An investigation of tissue-specific pharmacologies and inverse agonism at muscarinic acetylcholine receptors

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Abstract

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Evidence presented in this thesis supports the notion that certain muscarinic antagonists exhibit a greater functional affinity for the inhibition of M₃ muscarinic acetylcholine (mACh) receptor-mediated phosphoinositide turnover in guinea-pig urinary bladder than for M₁ receptor-mediated responses in the submandibular salivary gland of the same species. The present study suggests that the observed tissue-dependent pharmacologies cannot be explained in terms of multiple receptor subtypes. The muscarinic antagonists found to display such 'functional selectivity' in this investigation are used in the pharmacological management of overactive bladder, so it is possible that these properties contribute to the favourable side-effect profiles of some of these drugs. The potential molecular mechanisms underlying these observations are discussed.

To investigate the inverse agonist properties of these, and other, mACh receptor antagonists, a constitutively-active, mutant (CAM) M₂ mACh receptor was generated by a single amino acid change (N410Y) at the junction between the sixth transmembrane domain and the third extracellular loop of the M₂ receptor. The present study provides the first detailed characterisation of a CAM M₂ mACh receptor, demonstrating many of the hallmark features of a CAM receptor, including enhanced agonist-independent signalling, increased agonist binding affinity and functional potency and an increased partial agonist efficacy. All of the ligands assayed (previously classified as 'antagonists' at the M₂ receptor), similarly reduced the constitutive activity of the mutant receptor, indicating that they should be re-classified as inverse agonists at the M₂ mACh receptor. The CAM M₂ mACh receptor was also expressed at substantially lower levels than the wild-type receptor, but overnight incubation with atropine caused a significant up-regulation of cell surface receptor expression. However, not all inverse agonists were capable of up-regulating the CAM M₂ receptor. The implications of these findings for the clinical use of muscarinic antagonists and for mACh receptor signalling in general are discussed.
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Publications


Abbreviations

AC  adenylate cyclase
ACh  acetylcholine
cAMP  adenosine 3', 5'-cyclic monophosphate
CAM  constitutively-active mutant
CCh  carbamylcholine
CHO  Chinese hamster ovary
4-DAMP  4-diphenylacetoxy-N-methylpiperidine
DMSO  dimethyl sulphoxide
ECL  enhanced chemiluminescence
ETC  extended ternary complex model
GDP  guanosine 5'-diphosphate
GTP  guanosine 5'-triphosphate
GTPγS  guanosine 5'-O-(3-thiotriphosphate)
G-protein  heterotrimeric GTP-binding regulatory factor
HBS  HEPES-buffered saline
HEK  human embryonic kidney
Ins(1,4,5)P3  inositol 1,4,5-trisphosphate
KHB  Krebs-Henseleit buffer
mACh  muscarinic acetylcholine
MCh  methacholine
MT-7  muscarinic toxin 7
NMS  N-methyl scopolamine
OAB  overactive bladder
PKA  cyclic AMP-dependent protein kinase
PKC  protein kinase C
PLC  phospholipase C
PBS  phosphate-buffered saline
PTx  pertussis toxin
QNB  quinuclidinyl benzilate
SDS  sodium dodecyl sulphate
SMG  submandibular salivary gland
TCA  trichloroacetic acid
Chapter 1: Introduction

The aim of this introduction is to provide sufficient background information to support the discussion of the data presented in Chapters 3-6. Sections 1.1 and 1.2 provide a general introduction to the G-protein-coupled receptors (GPCRs) and in particular the expression, function, signalling and regulation of the muscarinic acetylcholine receptor family. Sections 1.3 and 1.4 summarise the roles of the muscarinic receptors in the regulation of smooth muscle and salivary gland function and discuss the use of muscarinic receptor antagonists in the treatment of overactive bladder. These sections will be of particular relevance to the data shown in Chapters 3 and 4. Finally, section 1.5 provides an introduction to the concepts of constitutive activity and inverse agonism at GPCRs, in particular the muscarinic receptors, and as such will provide a basis for the discussion of the data presented in Chapters 5 and 6.

1.1 The G-protein-coupled receptor family

G-protein-coupled receptors (GPCRs) are the largest family of integral membrane proteins involved in signal transduction. In vertebrates, they number more than 1000 (> 1 % of the genome) and are highly conserved throughout eukaryotic evolution. The nematode worm *Caenorhabditis elegans* genome encodes approximately 1100 GPCRs, constituting more than 5 % of its genome (Bargmann, 1998), while GPCRs have also been identified in plants (Plakidou-Dymock et al., 1998), protozoa (New & Wong, 1998) and yeast (Dohlman et al., 1991). GPCRs mediate physiological responses to a vast array of stimuli including peptide and non-peptide neurotransmitters, hormones, growth factors, lipids, ions, odorants, pheromones, photons and gustative molecules. Their fundamental importance to such a wide range of cellular responses is underlined by the fact that approx. 50 % of the current therapeutic agents on the market are targeted to GPCRs (Flower et al., 1999).

GPCRs are characterised by a signature seven α-helical transmembrane domains, forming a central core region, connected by 3 intracellular and 3 extracellular loops (Wess et al., 1997). Small ligands such as catecholamines bind in the cavity formed by transmembrane domains 3 and 6 and receptor activation is believed to alter the relative orientation of these transmembrane spans (Bockaert & Pin, 1999). The second and third intracellular loops of the receptor form the G-protein binding domain and since these links are directly connected to the third and sixth transmembrane domains,
it is proposed that the conformational change which occurs within the core domain is transduced into a rearrangement of the intracellular loops, uncovering previously masked G-protein-binding sites (Wess et al., 1997). Further discussion of the molecular mechanisms underlying receptor-G-protein interactions will be covered in sections 1.2.2 and 1.2.6.

1.1.1 Heterotrimeric G-proteins

The family of receptor-coupled G-proteins (guanine nucleotide binding proteins) possess a unique heterotrimeric structure, consisting of α, β, and γ subunits (Neer, 1995). The α-subunit contains a guanosine triphosphate (GTP)-binding domain (G domain), that is structurally similar to other members of the guanine nucleotide-binding protein superfamily, and an α-helical domain common to all α-subunits (Gudermann et al., 1996; Hamm, 1998). The β- and γ-subunits are tightly associated and form a non-dissociating functional unit. The α-subunit contains three flexible regions designated switches I, II and III, which form significant contacts with the βγ-dimer and change conformation in response to the guanine nucleotide binding status of the α-subunit (Morris & Malbon, 1999; Hamm, 1998).

Heterotrimeric G-proteins act as GPCR-activated molecular switches, transducing extracellular signals into intracellular responses by cycling between active and inactive states and between activated GPCRs and a subset of effectors specific for a given G-protein heterotrimer. This so-called ‘G-protein cycle’ is summarised in Figure 1.1. In the basal state, the guanosine diphosphate (GDP)-bound α-subunit and βγ-dimer are associated (1). When an activated receptor interacts with this GDP-bound heterotrimer, it induces GDP release (2), which is replaced by a molecule of GTP (3). This in turn induces conformational changes in the switch regions of the α-subunit, reducing the affinity of the α-subunit for the βγ-dimer, which leads to their dissociation (4). Go subunits in the GTP-bound state have 20-100 fold higher affinity for their effectors than the GDP-bound subunit, allowing specific regulation of classical effectors such as phospholipase C-β (PLC-β) or adenylate cyclase (AC). Similarly, βγ-subunits are free to interact with their own specific array of effector proteins (Guderman et al., 1996; Hamm, 1998; Morris & Malbon, 1999; Offermanns, 2003).
Figure 1.1 The G-protein cycle.
A - agonist
$R_i$ and $R_a$ - inactive and active receptors respectively
$\alpha$, $\beta$, $\gamma$ - heterotrimeric G-protein subunits
$E_1$ and $E_2$ - effectors coupled to $\alpha$-subunit and $\beta\gamma$-subunits respectively
Termination of signalling through G-proteins occurs via the inherent GTPase activity of the G-protein α-subunit, which catalyses the cleavage of GTP to GDP. This GTPase activity may be modified by a variety of accessory proteins including regulators of G-protein signalling (RGS proteins) and even several G-protein effector proteins such as PLC-β and this will be discussed in more detail later. With the α-subunit now GDP-bound, the cycle is completed by the re-association of α- and βγ-subunits, which acts as the inactivation mechanism for the βγ-dimer (Hamm, 1998; Offermanns, 2003).

In general, G-proteins are classified according to their α-subunits, though as the myriad regulatory roles of the βγ-subunit have become increasingly well understood, it is clear that this subunit may significantly contribute to the great diversity of G-protein signalling (Clapham & Neer, 1997). Indeed, the numerous permutations afforded by the combination of the three subunits may account for the wide variety of signalling profiles observed for a relatively small family of proteins. To date, 23 distinct α-subunits have been identified, encoded by 17 different genes (Numerberg et al., 1995). The α-subunits can be broadly divided into four groups: Goα, Gqα, Gsα and Gaiα. The roles and downstream effectors of these subunits will be given further consideration, in relation to the muscarinic acetylcholine (mACh) receptors, in section 1.2.2.

5 distinct β-subunits and 12 γ-subunits are currently known and while, with the exception of the βγ-subunit, the β-subunits share 80% identity, the γ-subunits are considerably more diverse (Guderman et al., 1997). βγ-dimers couple to a variety of intracellular effectors, including ion channels (Yamada et al., 1998; Zamponi & Snutch, 1998), PLC-β (Exton, 1996), adenylyl cyclase (Sunahara et al., 1996), phosphoinositide-3 kinase (PI3-K) (Vanhaesebroeck et al., 2001) and the mitogen-activated protein kinase (MAPK) cascades (Schwindinger & Robishaw, 2001). In some instances (e.g. PLC-β) βγ- and α-subunits may regulate effectors synergistically, but this is by no means a general property, with βγ-subunits often modulating effector function independently of their α-subunit partners (e.g. PI3-K) (Exton, 1996; Vanhaesebroeck et al., 2001).

Although most potential α/βγ pairings are capable of associating, there are exceptions (see Clapham & Neer, 1997). However, the specificity of effectors for different
combinations of β- and γ-subunits is generally poor, so the requirement for such a range of combinations is not fully apparent (Clapham & Neer, 1997). In addition to its direct effector regulatory roles, the βγ-subunit has been shown to enhance the binding of Gα to its cognate receptor and has profound effects on the conformational state of the α-subunit, resulting in an enhanced affinity for GDP (see Clapham & Neer, 1997).

1.1.2 Classification of GPCRs

GPCRs have traditionally been classified by criteria such as amino acid sequence, ligand binding and physiological features. The most frequently used system, the A-F system of Kolakowski (1994), was designed to encompass all GPCRs of both vertebrates and invertebrates. This suffers from the inherent problem that such diverse species possess radically different complements of GPCRs, not to mention the lack of sequence homology between more distantly related species (Fredriksson et al., 2003). More recently, as a result of the availability of the sequenced human genome (Venter et al., 2001) Fredriksson et al. (2003) proposed the GRAFS classification system, based upon 342 functional, non-olfactory human GPCR sequences. Five main families were identified and named as glutamate, rhodopsin, adhesion, secretin and frizzled/taste2 (Fredriksson et al., 2003). The rhodopsin family contains by far the most members (241 non-olfactory receptors) and is sub-divided into four groups: α, β, γ and δ. As suggested by their title, these GPCRs show the greatest homology to the proto-typical GPCR rhodopsin and generally correspond to the family A group of GPCRs in the A-F classification system. Within the α-group, the amine branch is one of five such clusters and includes the adrenergic, serotonin, dopamine, histamine and muscarinic receptors. The muscarinic receptor cluster displays the most sequence homology out of all of the amine clusters, sharing between 40-50 % identity (Fredriksson et al., 2003).

1.2 The muscarinic acetylcholine (mACH) receptors

1.2.1 Discovery and cloning

The physiological actions of acetylcholine (ACh) are mediated by the metabotropic muscarinic receptors and the ionotropic nicotinic receptors. The nicotinic receptors are mostly involved in fast neurotransmission and will not be discussed any further in this introduction. Early evidence of mACH receptor subtypes came from the work of Riker & Wescoe (1951), who found that gallamine was capable of antagonising
cardiac muscarinic receptors, but not those expressed in smooth muscle. Later, Barlow et al. (1976) reported significant differences in binding affinity for a number of muscarinic ligands, including 4-DAMP, between the atria and ileum of the guinea-pig. Hammer et al. (1980) then reported that pirenzepine was able to distinguish at least 3 sub-classes of muscarinic receptor, designating them M1 (in brain), cardiac M2 and glandular M3 (defined in salivary and lacrimal glands and later termed M3).

The cloning of complementary DNA for mACh receptor genes significantly advanced our understanding of the molecular nature of mACh receptor subtypes. Kubo et al. (1986a, b) first cloned the M1 and M2 receptors from porcine cortex and heart, respectively and later Bonner and co-workers cloned the M3, M4 and M5 receptors from rat and human (Bonner et al., 1987, 1988; Buckley et al., 1988). Each of the five genes encodes a seven transmembrane helix GPCR with significant homology with the rhodopsin-like family of receptors (Hulme et al., 1990). The five genes are intronless and there is a considerable degree of sequence homology between species (89-98% identity) (Wess, 1993). In addition, the five subtypes display at least 90% sequence homology with each other (Felder, 1995).

1.2.2 G-protein coupling and signal transduction

Despite their overall sequence homology, there is considerable divergence in the amino acid sequences of the third intracellular (i3) loops between the M1/M3/M5 and the M2/M4 receptors (Wess et al., 1997). This region is important in determining the G-protein coupling specificity of the receptors and may explain the distinct coupling characteristics of these two groups of mACh receptors (Wess, 1993). For instance, a four amino acid sequence (VTIL) at the interface of the i3 loop and sixth transmembrane domain of the M2 receptor has been identified as an essential component in the coupling of this subtype to its target Gia proteins (Liu et al., 1995).

In the case of the M3 mACh receptor, Blin et al. (1995) demonstrated that regions in the second intracellular (i2) loop and both N- and C-termini of the i3 loop accounted for the Gq11 coupling preference of the M3 receptor.

1.2.2.1 The Ga family of G-proteins

The Ga family consists of four members: Ga4, Ga11, Ga14 and Ga15/16. The α-subunits of Ga and Ga11 are closely related, displaying 88% sequence homology, but the other members exhibit less homology (e.g. Ga16 and Ga13 display only 55% sequence
Gq is expressed ubiquitously and G11 almost ubiquitously, while the expression profiles of G14 (spleen, kidney, testis and lung) and G15/16 (haematopoietic cells only) are considerably more restricted (Amatruda et al., 1991; Wilkie et al., 1991). The relative roles of the members of the Gq family are not yet clear, as receptors that activate them appear to activate Gq and G11 equally well (e.g. Offermanns et al., 1994a). Gq, G11 and G15/16 also seem to stimulate the different isoforms of PLC-β equally well, with a rank order of $\beta_1 \simeq \beta_3 > \beta_2$ (Rhee & Bae, 1997).

Evidence from mice lacking the genes for the individual members of the Gq family also indicates a certain amount of redundancy of function. Although mice lacking the gene for Gq exhibit ataxia and motor co-ordination deficits (Offermanns et al., 1997a, b) none of the other members of the Gq family yielded any obvious phenotypic changes when knocked out by homologous recombination (Offermanns et al., 1998; Davignon et al., 2000).

1.2.2.2 The phosphoinositide signalling pathway

The 'odd-numbered' mACh receptor subtypes (M1/M3/M5) are generally considered to predominantly couple through the PTx-insensitive Gq family of G-proteins (Peralta et al., 1987; Hulme, 1990). Activation of this family of G-proteins leads to the stimulation of the membrane-bound enzyme phospholipase C-β (PLC-β), which accelerates the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP2) into diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP3). These products act as second messengers, activating protein kinase C (PKC) and eliciting the release of Ca²⁺ from intracellular stores (see Figure 1.2 for an illustration of this pathway).

Figure 1.2 also depicts the major pathways by which inositol is recycled into the inositol phospholipid pool. The termination of IP3 signalling may occur via sequential dephosphorylation, with the terminal step being mediated by inositol monophosphatase (IMPase) (Atack et al., 1995). Alternatively, IP3 may first be specifically phosphorylated by inositol 1,4,5-trisphosphate 3-kinase to form inositol 1,3,4,5 tetrakisphosphate (Ins(1,3,4,5)P4). Ins(1,3,4,5)P4 has been suggested to perform a variety of signalling roles including a direct activation of extracellular Ca²⁺ influx (Luckhoff & Clapham, 1992), facilitation of IP3-mediated Ca²⁺ mobilisation (e.g. Smith, 1992) and protection of IP3 from hydrolysis by inositol phosphate 5-phosphatase (e.g. Hermosura et al., 2000). Whatever its physiological role(s), Ins(1,3,4,5)P4 is ultimately sequentially de-phosphorylated to inositol by a similar
Figure 1.2 The phosphoinositide cycle.
The hydrolysis of PtdIns(4,5)P₂ into DAG and Ins(1,4,5)P₃ and the recycling of inositol into the phospholipid pool, including the points at which Li⁺ modulates the cycle.
pathway to IP₃, allowing inositol to be re-incorporated into the phospholipid pool
(Atack et al., 1995).

As shown in Figure 1.2, both of these pathways of inositol recycling depend upon
the activity of IMPase. A crucial tool used in the study of the inositol phosphate pathway
is lithium, which is known to non-competitively inhibit both IMPase and inositol
polyphosphate 1-phosphatase in the millimolar range (Nahorski et al., 1991).
Inhibition of these enzymes leads to an accumulation of lower order inositol
phosphates upon PLC-linked receptor activation and as such may allow measurement
of radiolabelled inositol phosphate accumulation as an index of PLC activity (e.g.

1.2.2.3 Coupling of M₁, M₃ and M₅ mACh receptors to alternative signalling
pathways

In addition to their primary coupling partners, the Gₛ G-proteins, the 'odd-numbered'
mACh receptors have also been reported to couple to Gₛₒ-type proteins, as
demonstrated by a degree of PTx-sensitivity of mACh receptor-stimulated [³²P]-
GTPyS binding in CHO cells stably expressing M₁ or M₃ mACh receptors (Burford et
al., 1995a). Offermanns et al. (1994b) provided direct evidence for M₃ receptor-Gₛ
interactions by subtype-specific immunoprecipitation of [α-³²P]-GTP azidoanilide-
labelled Gₛ subunits. Later, Akam et al. (2001) provided similar evidence for both M₁
and M₃ receptors coupling to Gₛₒ proteins in CHO cells, by measuring muscarinic
agonist-stimulated [³⁵S]-GTPyS binding and immunoprecipitation of Gₛₒ proteins with
subtype-specific antibodies.

M₁, M₃ and M₅ receptor-mediated stimulation of adenylate cyclase has also been
reported in a variety of cell lines (Peralta et al., 1988; Felder et al., 1989; Jones et al.,
1991; Gurwitz et al., 1994; Burford et al., 1995b). Various different mechanisms have
been proposed to mediate this response, including Ca²⁺ (or Ca²⁺-calmodulin)-
mediated activation of adenylate cyclase isoforms (Felder et al., 1989) and direct
coupling to Gₛₒ (Jones et al., 1991). A direct Gₛ coupling to M₁ mACh receptors has
also been reported by Burford and Nahorski (1996), who found that a C-terminal-
directed anti-Gₛα serum significantly reduced carbachol-stimulated cyclic AMP
accumulation in CHO-m1 cells.
Many Gq/11-coupled GPCRs also signal via the G12 family of G-proteins (Offermanns, 2003). The G12 family consist of G12 and G13, which are widely expressed (Strathman & Simon, 1990) and have been demonstrated to couple to phospholipase D (PLD), c-Jun N-terminal kinase (JNK), the Na+/H+ exchanger and the Rho family of G-proteins (Buhl et al., 1995; Dhanasekaran & Dermott, 1996; Hooley et al., 1996; Plonlc et al., 1998). Indeed the M3 mACh receptor has been shown to signal to PLD via G12-type G-proteins and not via Gq, at least in HEK-293 cells (Rumenapp et al., 2001), and M1 and M3 receptors have been co-immunoprecipitated with G12 in PC3 and DU145 prostatic cell lines (Luthin et al., 1997). It is therefore clear that although considered primarily Gq/11-coupled GPCRs, the M1, M3 and M5 mACh receptors are also capable of signalling by several divergent G-protein pathways, though the challenge remains to determine the relative importance of these pathways in vivo.

1.2.2.4 The Gi0 family of G-proteins

The M2 and M4 mACh receptor subtypes are predominantly Gi0-coupled receptors, mediating pertussis toxin (PTx)-sensitive inhibition of adenylate cyclase and activation of inward-rectifier potassium conductances. G-proteins of the Gi0 family are generally expressed at higher levels than those of the other families, with Gi0, for instance, accounting for 1-2 % of total membrane protein in brain, where the two isoforms of this subtype (Gi0a and Gi0b) are localised (Hepler & Gilman, 1992). The most widely expressed members of this family are the G1 subtypes (G11, G12 and G13), while other isoforms perform specialist sensory functions and are localised accordingly e.g. transducin (G11 and G12) in retinal rod and cone cells, respectively (Morris & Malbon, 1999; Offermanns, 2003).

With the exception of G10 (expressed in platelets, adrenal gland and the CNS), all members of the Gi0 family contain a conserved cysteine residue in the C-terminus, that is the site of ADP-ribosylation catalysed by the exotoxin from Bordetella pertussis (i.e. pertussis toxin, or PTx) (Hanoune & Defer, 2001). The use of PTx has proven an important experimental tool for the dissection of numerous GPCR signal transduction pathways, by virtue of its specific and highly effective ‘uncoupling’ of Gi0 proteins from the receptor, which accounts for the block of GPCR-mediated adenylate cyclase inhibition by PTx observed in many systems (Rens-Domiano & Hamm, 1995).
1.2.2.5 The adenylate cyclase family

Molecular cloning has identified 9 membrane-bound and one soluble adenylate cyclase (AC) isoforms (Simonds, 1999). The distinct patterns of modulation of these diverse isoforms allow AC to integrate stimulatory and inhibitory signals from a broad range of signal-generating pathways. A number of excellent reviews discuss the diversity of adenylate cyclase (Sunahara et al., 1996; Simonds, 1999; Hanoune & Defer, 2001) and Table 1.1 summarises the regulation and expression of those isoforms cloned to date. ACs 1-8 are large integral membrane proteins with a cytoplasmic N-terminus, 2 sets of 6 transmembrane-spanning domains separated by large cytoplasmic domains which also comprise the two catalytic domains (C1 and C2) (Simonds, 1999). Activation by a variety of signals (see below) stimulates the hydrolysis of adenosine 5'-triphosphate (ATP) into adenosine 3',5'-cyclic monophosphate (cyclic AMP) and pyrophosphate. The regulation of AC activity is both complex and isoform-specific and is discussed briefly below.

All 9 membrane-bound isoforms of AC are unactivated by Gs and all but AC 9 are strongly stimulated by the plant diterpene forskolin (Premont et al., 1996), though these two activators bind at different sites. Forskolin binds within the catalytic core of AC, while Gs binds at the perimeter of the catalytic core, which may explain their synergistic activation of certain AC isoforms (Sunahara & Taussig, 2002). Gt and Go isoforms are capable of inhibiting the majority of AC isoforms, with the notable exception of AC 2 (Taussig et al., 1994), though not via direct competition with Go, as forskolin-stimulated AC activity is also inhibited. Instead, mutagenesis studies suggest that Go6 proteins bind to a site symmetrical to the Gs binding site (Dessauer et al., 1998).

G\textgamma\gamma subunits are also capable of activating (AC 2, 4 and 7) or inhibiting (AC 1, 5 and 6) AC, though this is usually only observed in the presence of co-stimulation by Gs, highlighting the potential for AC isoforms to integrate distinct signals into a convergent signal (Tang & Gilman, 1991; Federman et al., 1992; Yoshimura et al., 1996). The putative binding site for the \textgamma subunit has been mapped to amino acid residues 956 to 982 of AC 2, corresponding to the middle of the second catalytic domain (C2) (Chen et al., 1995, 1997). Interestingly, this sequence is only found in G\textgamma-regulated AC isoforms and a peptide corresponding to this putative binding
<table>
<thead>
<tr>
<th>AC isoform</th>
<th>Tissue distribution</th>
<th>Fsk</th>
<th>G-protein subunits</th>
<th>Ca(^{2+})</th>
<th>Protein kinases</th>
</tr>
</thead>
<tbody>
<tr>
<td>AC.1</td>
<td>Brain, adrenal medulla</td>
<td>+</td>
<td>+ve: (\alpha), -ve: (\beta), -ve: CaM, -ve: CaMKIV</td>
<td>+ve: PKC (weak)</td>
<td>-ve: CaMKIV</td>
</tr>
<tr>
<td>AC.2</td>
<td>Brain, lung, heart, skeletal muscle</td>
<td>+</td>
<td>+ve: (\alpha), -ve: (\beta)</td>
<td>-</td>
<td>+ve: PKC</td>
</tr>
<tr>
<td>AC.3</td>
<td>Brain, olfactory epithelium</td>
<td>+</td>
<td>+ve: (\alpha), -ve: (\alpha)</td>
<td>+ve: CaM, -ve: CaMKII</td>
<td>+ve: PKC (weak) -ve: CaMKII</td>
</tr>
<tr>
<td>AC.4</td>
<td>Brain, heart, kidney, lung, liver, uterus, BAT</td>
<td>+</td>
<td>+ve: (\alpha), -ve: (\beta)</td>
<td>-</td>
<td>-ve: PKC</td>
</tr>
<tr>
<td>AC.5</td>
<td>Brain, heart, kidney, lung, liver, uterus, BAT, adrenal</td>
<td>+</td>
<td>+ve: (\alpha), -ve: (\beta), (\alpha)</td>
<td>-ve (Ca(^{2+}))</td>
<td>-ve: PKA +ve: PKCa/(\gamma)</td>
</tr>
<tr>
<td>AC.6</td>
<td>Ubiquitous</td>
<td>+</td>
<td>+ve: (\alpha), -ve: (\beta), -ve: (\alpha), -ve: CaMKIV</td>
<td>-ve (Ca(^{2+}))</td>
<td>-ve: PKA, PKC</td>
</tr>
<tr>
<td>AC.7</td>
<td>Ubiquitous</td>
<td>+</td>
<td>+ve: (\alpha), -ve: (\beta)</td>
<td>-</td>
<td>+ve: PKC</td>
</tr>
<tr>
<td>AC.8</td>
<td>Brain, lung</td>
<td>+</td>
<td>+ve: (\alpha), -ve: (\alpha)</td>
<td>+ve: CaM</td>
<td>-</td>
</tr>
<tr>
<td>AC.9</td>
<td>Brain, skeletal muscle (weak)</td>
<td>+</td>
<td>+ve: (\alpha), -ve: (\alpha)</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Adapted from Sunahara et al. (1996); Simonds, (1999); Hanoune & Defer, (2001).

Fsk = Forskolin; CaM = Calmodulin; CaMKII = Calcium-calmodulin-dependent protein kinase II; PKC = Protein kinase C; PKA = cyclic AMP-dependent protein kinase.
region not only blocked $G_{i\gamma}$-mediated enhancement of $G_i$-stimulated AC 2 activity but also inhibited $G_{i\gamma}$-stimulated GIRK-1 potassium channel activity (Chen et al., 1995).

$Ca^{2+}$-calmodulin has also been reported to activate AC 1, AC 8 and possibly AC 3 (Krupinski et al., 1989; Choi et al., 1992; Cali et al., 1994), while $Ca^{2+}$ itself is able to inhibit AC 5 and AC 6 in the micromolar range (e.g. Yu et al., 1993). It is believed that capacitative $Ca^{2+}$ entry is mostly responsible for the inhibition of these isoforms, which are prevalent in the CNS and other excitable cells (see Table 1.1). This is one example of how the relative tissue distributions of AC isoforms are tightly regulated, providing another means by which the cell may modulate its integration of signalling inputs through adenylate cyclase.

A variety of protein kinases including PKC and PKA have also been reported to modulate the activity of various AC isoforms. For instance, PKA-mediated phosphorylation of AC 5 and AC 6 inhibits their responsiveness to $G_i$, acting in a negative feedback loop (Iwami et al., 1995). PKC may regulate ACs in an isoform-specific manner, with many isoforms being stimulated by PKC-mediated phosphorylation (see Sunahara & Taussig, 2002), while AC 6 is negatively regulated (Lai et al., 1997). However, further complexity is added by the observation that PKC phosphorylation may have differential effects, depending upon the stimulatory mechanism (i.e. $G_o$, $G_{i\gamma}$ etc) investigated (Zimmermann & Taussig, 1996).

1.2.2.6 $M_2/M_4$ mACh receptor modulation of adenylate cyclase

It is clear from the preceding discussion that in addition to the classical coupling of $G_{i\gamma}$-linked receptors, such as the $M_2$ and $M_4$ mACh receptors to the inhibition of adenylate cyclase, the liberation of $\beta_{i\gamma}$-subunits may lead to an activation of this enzyme. This depends to a large extent upon the AC isoforms expressed in the system. Thus, co-expression of mutationally active $G_o\alpha$ with AC 2 converted the response to activation of receptors that act through predominantly $G_{i\gamma}$-coupled receptors into a stimulation of cyclic AMP accumulation (Federman et al., 1992). This stimulatory response was sensitive to PTx and blocked by co-expression of the $\alpha$-subunit of transducin, which is known to bind $\beta_{i\gamma}$ subunits, implicating the latter in mediating the stimulation of AC 2 (Federman et al., 1992).
Similarly, Olianas and co-workers have shown that in the olfactory bulb, mACh receptors of the M_{4} subtype enhance basal and G_{\alpha}\text{-stimulated AC. activities in a G_{io}-sensitive manner, independent of intracellular Ca^{2+} (Olianas & Onali, 1992, 1996). Incubation with the \beta\gamma-subunit of transducin mimicked the effect of carbachol on olfactory bulb membranes, while \beta\gamma scavengers blocked the action of muscarinic stimulation on basal and G_{\alpha}\text{-stimulated AC. activity (Olianas et al., 1998).}

Direct activation of G_{\alpha} by the mACh receptors has also been reported. Dittman et al. (1994) found that the M_{4} receptor-mediated stimulation of the \beta\gamma-insensitive AC. 3 co-expressed in HEK-293 cells was not due to intracellular Ca^{2+} release or calmodulin-sensitive enzyme activation. It was, however, GTP-dependent, PTx-insensitive and could be mimicked by synthetic peptides derived from the G-protein-activating region of the M_{4} receptor, implicating a role for direct G_{\alpha} protein activation (Dittman et al., 1994). Okamoto & Nishimoto (1992) also found that synthetic peptides of the human M_{4} receptor were able to activate G_{\alpha}, albeit at higher concentrations than those required to activate G_{i}, supporting the notion that this receptor is capable of interacting with G_{\alpha} proteins.

Jones et al. (1991) reported a biphasic regulation of adenylate cyclase activity by the predominantly G_{io}-coupled M_{4} mACh receptor in CHO cells. PTx treatment also uncovered a more significant stimulation of cyclic AMP accumulation, suggesting that in the absence of G_{io} proteins capable of coupling to the M_{4} receptor, G_{i} coupling is enhanced. These data illustrate that the ‘switch’ between inhibition and stimulation of adenylate cyclase may depend upon a number of cellular factors, including receptor expression level, agonist concentration and the availability of specific G-protein isoforms.

Migeon & Nathanson (1994) found that JEG-3 cells transiently over-expressing mouse M_{4} and chick M_{4} receptors responded to carbachol with an enhanced cyclic AMP accumulation that was not reduced by PTx, excluding a significant role for \beta\gamma-subunit activation of AC. isoforms. At lower expression levels, M_{4} receptor activation caused a small inhibition of cyclic AMP accumulation at low agonist concentrations, with stimulation only occurring at higher agonist concentrations (Migeon & Nathanson, 1994). JEG-3 cells express G_{10}\alpha and G_{13}\alpha but not G_{12}\alpha (Montmayeur et al., 1993) and Migeon & Nathanson (1994) found that co-expression of G_{12}\alpha but not
the other two isoforms, enhanced the inhibitory effect of carbachol on AC activity. This suggests that the chick M₄ receptor requires G₂α for efficient inhibition of AC activity. In fact, Migeon et al. (1995) confirmed that both chick and human M₄ receptors preferentially coupled to the G₂α and G₆ isoforms over G₁α and G₉α, while the M₂ receptor was not selective.

In contrast, Akam et al. (2001) reported that methacholine stimulates marked increases in [³⁵S]-GTPγS binding to all three isoforms of Gᵢ in membranes prepared from CHO-m2 and CHO-m4 cells. No significant activation of Gₛ was observed in either case, contrasting with the data presented by Migeon et al. (1995) in JEG-3 cells. While this suggests that differences in G-protein availability/expression levels between the two cell lines may be crucial, differences in G-protein coupling may also be related to the choice of agonist. Akam et al. (2001) also found that the muscarinic partial agonist pilocarpine displayed greater efficacy for activation of G₁α₂α than G₁α₂α in both CHO-m2 and CHO-m4 membranes, suggesting that selective agonist-activated receptor-G-protein coupling may occur at the M₂ and M₄ receptors. In contrast, Uustare et al. (2004) examined a range of muscarinic agonists and found no significant differences in either potency or efficacy in their stimulation of [³⁵S]-GTPγS binding to Gᵢ or Gₛ proteins. However, the latter study was performed in S9 insect cells and therefore may be lacking in accessory proteins essential for the correct trafficking or localisation of signalling components.

Biphasic cyclic AMP responses have also been reported following stimulation of the M₂ mACh receptor, stably expressed in CHO cells (Michal et al., 2001). It was found that the occurrence and extent of stimulation of adenylate cyclase activity was dependent upon agonist efficacy, duration of incubation, receptor density, G₁₀ protein function and the extent to which agonists induce M₂ receptor internalisation (Michal et al., 2001). As would be expected, all agonists investigated inhibited AC activity more potently than they stimulated it, indicating the higher affinity of the M₂ receptor for G₁₀ proteins than Gₛ. In support of this, the stimulatory component was also more sensitive to a reduction in receptor expression level by oxyphenonium mustard treatment. However, following PTx pre-treatment, the stimulatory component was increased in both magnitude and potency, consistent with an increased availability of activated receptors to interact with Gₛ proteins (Michal et al., 2001).
It is therefore clear that under certain circumstances, both M₂ and M₄ mACh receptors are capable of signalling via G₄ proteins to activate adenylate cyclase. Such interactions are favoured in over-expressing receptor systems and at high levels of agonist stimulation (Jones et al., 1991; Michal et al., 2001). It is also likely that where the ratio of G₂:G₁₀ concentration is high, or where compartmentalisation of G-proteins and receptors enriches the local concentration of one or more components of the signal transduction pathway (Neubig, 1994), G₄ coupling will be more significant. The requirement for high levels of expression calls into question the physiological relevance of such responses observed mostly in recombinant cell systems. However, positive inotropic responses have been observed in response to muscarinic agonist stimulation (e.g., Imai & Ohta, 1988; Eglen et al., 1988a), but a link between these observations and M₂ receptor-mediated activation of G₄ remains to be investigated.

1.2.2.7 Alternative signalling pathways activated by M₂ and M₄ mACh receptors

The important roles of the βγ-subunits of heterotrimeric G-proteins in mediating signal transduction have been discussed previously, most notably in their activation of adenylate cyclase isoforms. In addition, Gβγ is known to activate isoforms 1-3 of PLC-β and this underlies the phosphoinositide responses of a number of predominantly G₁/₀-coupled receptors, including the M₂ and M₄ receptors (Ashkenazi et al., 1987; Peralta et al., 1988). However, unlike G₁/₀-mediated inhibition of adenylate cyclase activity, Gβγ-mediated PLC-β stimulation requires higher agonist concentrations and is usually associated with higher levels of receptor expression (Gudermann et al., 1996).

Another major effector of βγ-mediated signalling is the G-protein-activated K⁺ channel (GIRK), which is abundantly expressed not only in cardiac tissue, but also in the CNS and in endocrine cells (Yamada et al., 1998). Activation of the M₂ mACh receptor (in addition to a number of other GPCRs including 5-HT₁A, α₂-adrenoceptor and D₂ dopamine receptors) activates GIRK by the direct binding of Gβγ, liberated from heterotrimeric G-protein activation (Brown et al., 1991). Functional GIRK channels are composed of a combination of four subunits in heteromeric assemblies and are equipotently activated by a variety of βγ-dimers (Wickman et al., 1994; Lei et al., 2000). The βγ binding sites are located in the cytosolic N- and C-termini of the GIRK subunits (see Krapivinsky et al., 1998) and it has been proposed that the
binding of βγ-subunits to these regions is stabilised by PIP₂ binding and that this may underlie the synergistic effects of Gₚₚ and PIP₂ upon GIRK channel activation (Huang et al., 1998a).

In the heart, GIRK channel activation leads to a hyperpolarisation that is crucial to the bradycardic effect of parasympathetic cholinergic signalling. However, although both ACh and adenosine are able to activate GIRK currents via activation of M₂ and A₁ receptors respectively, noradrenaline does not activate GIRK channels, even though the β₁-adrenoceptor liberates βγ-subunits from Gₐ (see Yamada et al., 1998). However, as stated above, GIRKs exhibit little or no β or γ isoform specificity. Instead, the specificity seems to be conferred by the α-subunit with the preferred βγ-donors being G₂α and G₃α (Kozasa et al., 1996; Sowell et al., 1997). However, when overexpressed, PTx-insensitive G-proteins such as G₂ have been shown to activate GIRKs (Vorobiov et al., 2000), perhaps indicating that some form of co-localisation may be required to selectively target specific G-proteins to the GIRKs.

Peleg et al. (2002) demonstrated that G₂α directly gates GIRK channels in addition to providing βγ-subunits. When over-expressed in Xenopus oocytes, GIRK channels exhibit high basal activity (in contrast to their native basal activity) and weak activation in response to βγ-activation. However, co-expression of G₁α or G₃α reduced basal activity and allowed for more significant activation in response to Gβγ (i.e. a similar GIRK activity to that in native cells) (Peleg et al., 2002). This suggests that G₂α, either alone or as a part of a Gβγ heterotrimer, maintains the GIRK channel in a closed state in the absence of agonist activation. Both α- and βγ-subunits may therefore act in concert to regulate GIRK activity in the basal state and in response to agonist activation.

1.2.3 mACh receptor subtype function – evidence from knockout mice studies

Recent studies in genetically modified mice lacking one of the five mACh receptor genes have provided clarification of muscarinic receptor subtype distribution and, more importantly, function in both the periphery and the CNS (Wess, 2004). The evidence from 'knockout' mice studies and other studies relating to the function of each of the mACh receptor subtypes is summarised below. Once again, specific details relating to muscarinic receptor function in smooth muscle and salivary gland will be discussed later (see sections 1.3 and 1.4)
1.2.3.1 M<sub>1</sub> mACh receptor

Consistent with the significant expression of the M<sub>1</sub> receptor in the cortex and hippocampus, mice lacking the M<sub>1</sub> receptor failed to display muscarinic agonist-induced [³⁵S]-GTPγS binding in membranes from these brain regions (Porter et al., 2002), while phosphoinositide hydrolysis in cortex and hippocampus in response to muscarinic agonist in vivo was absent in M<sub>1</sub> knockout mice (Bymaster et al., 2002). Muscarinic agonist activation of the mitogen-activated protein kinase (MAPK) pathway in hippocampal slices and cortical cultures from M<sub>1</sub> receptor-deficient mice was also completely abolished (Hamilton & Nathanson, 2001; Berkeley et al., 2001).

In contrast to indications from earlier pharmacological experiments, M<sub>1</sub> receptors do not appear to be involved in hippocampus-dependent learning and memory tasks as these functions were unaffected in mice lacking the M<sub>1</sub> receptor (Miyakawa et al., 2001). However, behavioural studies have demonstrated that M<sub>1</sub> knockout mice displayed increased locomotor activity (Miyakawa et al., 2001) and Gerber et al. (2001) found this to be associated with increased striatal dopamine levels, consistent with the well-established involvement of mACh receptors in Parkinson's disease. Systemic administration of the muscarinic partial agonist pilocarpine causes epileptic seizures in wild-type mice, but in mice lacking the M<sub>1</sub> receptor, seizure activity in response to pilocarpine was completely abolished (Hamilton et al., 1997). This suggests that M<sub>1</sub> receptor activity may contribute to epileptic seizures in some cases.

Alzheimer's disease (AD) is associated with deficits in central cholinergic systems (Fisher et al., 1998). This has led to the proposal that M<sub>1</sub> receptor agonists and/or M<sub>2</sub> receptor antagonists may be suitable therapies for enhancing cholinergic signalling in AD patients (see Eglen et al., 1999). Numerous functionally M<sub>1</sub> selective agonists have been evaluated for the treatment of AD, but often with disappointing results, especially relating to efficacy. The clinical benefit of muscarinic therapies in AD therefore remains to be fully established.

Electrophysiological studies in M<sub>1</sub> receptor-deficient mice have highlighted the role of the M<sub>1</sub> receptor in mediating the muscarinic agonist-induced inhibition of the M current in sympathetic ganglia (Hamilton et al., 1997). Inhibition of this tonically-active voltage-dependent K<sup>+</sup> current leads to membrane depolarisation and an enhancement of neuronal excitability. However, mACh receptor-mediated
suppression of the M current in mouse hippocampal pyramidal cells remained unaltered in M1 knockout mice, indicating that other mACh receptor subtypes are capable of modulating the M current in the hippocampus at least (Rouse et al., 2000). Other electrophysiological deficits reported in the M1 knockout mice are the absence of the slow, voltage-independent muscarinic inhibition of N- and P/Q-type Ca\(^{2+}\) channels (Shapiro et al., 1999) and the loss of hippocampal muscarinic receptor-stimulated \(\gamma\)-frequency oscillations, believed to be involved in the performance of cognitive tasks (Fisahn et al., 2002).

1.2.3.2 M2 mACh receptor

Isolated atria from mice lacking the M2 receptor fail to display bradycardic responses to muscarinic agonist (Stengel et al., 2000), while preliminary in vivo observations suggest that vagally- or methacholine-induced bradycardia is also abolished in M2 receptor-deficient mice (see Wess, 2004). A crucial function of M2 receptors in the periphery is in the control of airway smooth muscle contraction and mucus secretion (see Coulson & Fryer, 2003). Although, as in many smooth muscle types, the M3 receptors directly mediate contraction, the release of ACh from the parasympathetic nerves (which control secretion and contraction) is predominantly under the inhibitory regulation of M2 receptors in the ganglia and at pre- and post-ganglionic nerve endings (Coulson & Fryer, 2003).

Evidence from studies with knockout mice indicates that although bronchoconstriction in response to muscarine is reduced by 60 % in M3 knockout mice, it is only in mice lacking both M2 and M3 receptors that constriction is entirely abolished (Struckmann et al., 2003). Both asthma and chronic obstructive pulmonary disease (COPD) are associated with increased parasympathetic activity and are characterised by increased basal tone and enhanced bronchoconstriction in response to a variety of irritants (see Coulson & Fryer, 2003). There is no evidence of alterations in postjunctional mACh receptors in either asthma or COPD (e.g. McGowan et al., 2002). Instead, reduced activity of inhibitory M2 autoreceptors is believed to underlie the hyperactivity of the parasympathetic innervation of the airways (e.g. Ten Berge et al., 1996).

Central functions of the M2 receptor suggested by studies with M2 receptor-deficient mice include thermo-regulation, corticosterone release and oxotremorine-induced
tremor (Wess, 2004). Gomeza et al. (1999) reported that M₂ knockout mice lacked oxotremorine-mediated tremor and akinesia, supporting earlier pharmacological data suggesting a role for striatal mACh receptors in mediating these muscarinic agonist-induced responses. Gomeza et al. (1999) also showed that mice lacking the M₂ receptor displayed reduced (but not completely abolished) hypothermic responses to systemic administration of oxotremorine, implicating roles for M₂ and non-M₂ mACh receptors in the regulation of body temperature. M₂ knockout mice also failed to respond to muscarinic agonist by releasing corticotrophin-releasing hormone (CRH), in the way that wild-type mice do (Hemrick-Luecke et al., 2002).

Shapiro et al. (1999) reported that mice lacking the M₂ receptor failed to display rapid, voltage-dependent mACh receptor-mediated inhibition of N- and P/Q-type Ca^{2+} channels in sympathetic ganglia. The role of the M₂ receptor in muscarinic agonist-induced analgesia was also demonstrated in M₂ knockout mice, where oxotremorine displayed a markedly reduced analgesic potency relative to wild-type (Gomeza et al., 1999). Various other effects relating to autoinhibitory pre-synaptic M₂ receptor populations have also been seen in M₂ receptor-deficient mice, including reduced carbachol-mediated inhibition of electrically-evoked [³¹H]-noradrenaline release from vas deferens, atria and urinary bladder (Trendelenburg et al., 2003) and altered kinetics of acetylcholine release the neuromuscular junction (Slutsky et al., 2003).

1.2.3.3 M₃ mACh receptor

Mice lacking the M₃ mACh receptor consumed significantly less food than their wild-type littermates and the adult males displayed a reduction in body weight of approximately 25 % (Yamada et al., 2001a). Further analysis indicated that M₃ receptor-deficient mice expressed significantly lower levels of the appetite-stimulating peptide melanin-concentrating hormone (MCH) (Yamada et al., 2001a). The authors proposed that M₃ receptor activity is necessary to maintain MCH expression, as muscarinic receptor stimulation enhances MCH expression (Bayer et al., 1999), and this is likely to underlie the hypophagia displayed by M₃ knockout mice. However, Matsui et al. (2000) found that feeding the mice a wet paste diet increased weight gain, suggesting that deficits in salivary flow could be responsible for reduced food intake. Since Yamada et al. (2001a) found that mice fed on either dry or wet food consumed less food and weighed less than wild-type animals, the precise relationship between M₃ receptor expression and feeding remains to be established.
Other phenotypes of M₃ receptor knockout mice include reduced hypothermic responses to oxotremorine (though effects were less pronounced than in M₂ knockout mice) (Bymaster et al., 2002) and dilated pupils (Matsui et al., 2000; Bymaster et al., 2002), consistent with the involvement of M₃ receptors in pupillary sphincter muscle tone.

1.2.3.4 M₄ mACh receptor

Many of the phenotypes associated with the absence of the M₄ mACh receptor relate to autoinhibitory roles of this receptor subtype (Wess, 2004). Zhang et al. (2002) reported that autoinhibition of acetylcholine release in the striatum is largely due to M₄ receptor activation, while that in the hippocampus and cerebral cortex is mediated by the M₂ receptor. Zhou et al. (2002) found that autoinhibition in the bladder was M₄ dependent, while that in the atria was mediated by a combination of M₄ and M₂ receptors.

Oxotremorine treatment failed to induce facilitation of dopamine release in striatal slice preparations from M₄ receptor-deficient mice (Zhang et al., 2002). It is known that the balance between muscarinic and dopaminergic signalling in the striatum is essential for the maintenance of locomotor co-ordination (Di Chiara et al., 1994), so imbalance between these two systems may underlie the enhanced locomotor activity observed in M₄ knockout mice (Gomez et al., 1999). Oxotremorine-mediated analgesia was completely abolished in M₂/M₄ double knockout mice, indicating that in addition to the major M₂ role (see above), the M₄ mACh receptor is also involved in muscarinic agonist-induced analgesia (Duttaroy et al., 2002).

1.2.3.5 M₅ mACh receptor

Dopamine release studies in vitro found that M₅ knockout mice displayed reduced striatal dopamine release relative to wild-type (Yamada et al., 2001b). However, work by Zhang et al. (2002) indicates that the M₄ receptor predominates in the muscarinic control of dopamine release in the striatum, so the physiological importance of the M₅-dependent pathway is not clear. The M₅ receptor has also been implicated in the control of dopamine release in the ventral tegmental area (VTA), a pathway predicted to contribute to the rewarding effects of drugs of abuse, in studies with mice lacking the M₅ receptor gene (Forster et al., 2002). Interestingly, the rewarding effects of morphine were substantially reduced in M₅ knockout mice, as were both the somatic
and affective elements of morphine withdrawal (Basile et al., 2002). These results suggest a potential role for the M₃ receptor in morphine addiction and point to a potential novel strategy for the treatment of drug addiction.

Mice lacking the M₃ receptor have also been used to investigate the potential role of this receptor subtype in the vasorelaxant effects of ACh (Yamada et al., 2001b). It was found that cerebral arteries and arterioles almost completely lost their vasorelaxant response to ACh in mice lacking the M₃ receptor, while extra-cerebral arteries maintained their sensitivity to ACh (Yamada et al., 2001b). It has been proposed that ACh-mediated vasodilatation might have a protective role during focal cerebral ischaemia and that it may also contribute to the pathophysiology of Alzheimer’s disease (see Wess, 2004).

Takeuchi et al. (2002) have reported that mice lacking the M₃ receptor drank twice as much water as wild-type mice and several studies with these mice have indicated roles for the M₃ receptor in salivation (Yeomans et al., 2001; Takeuchi et al., 2002; Bymaster et al., 2003). Further discussion of these observations is included in Section 1.4.

1.2.4 Pharmacology of the mACh receptors

1.2.4.1 Agonists

As described in the previous section, the mACh receptors provide numerous potential targets for therapeutic intervention. However, in order to successfully exploit this potential, ligands must ideally be selective for particular subtypes; otherwise, adverse effects due to actions on some of the numerous physiological functions regulated by muscarinic receptors may compromise the use of the therapy. However, in spite of decades of intense research, there are no mACh receptor agonists currently available with a high selectivity for an individual subtype.

McN-A-343 is perhaps the best example of a 'selective' muscarinic agonist, originally characterised as an M₁-selective ligand (Hammer & Giachetti, 1982). However, in recombinant systems it has displayed some selectivity for the M₄ receptor (Richards & Van Giersbergen, 1995) and has even been shown to be inactive at the M₁ receptor population mediating contraction of the canine saphenous vein (Watson et al., 1995). Agonists that display selectivity for a certain tissue response are therefore more accurately defined as 'functionally selective', since their discrimination between
subtypes is not based upon different affinities, but may instead relate to the prevailing receptor reserve, stimulus-response coupling efficiency, G-protein availability or the stabilisation of a specific receptor activation state (see Eglen & Watson, 1996; Heldman et al., 1996).

The failure to identify truly subtype-selective muscarinic agonists has not only restricted the therapeutic utility of drugs targeted to the muscarinic receptors, but has also limited their use in the classification of mACh receptor subtypes mediating a number of physiological responses. It is likely that a better understanding of the molecular processes involved in receptor activation, and in particular the propensity of agonists to selectively stabilise different active conformations of GPCRs, will be required to design more selective muscarinic agonists, capable of pinpointing the target response.

1.2.4.2 Antagonists

Although there are nearly 100 muscarinic antagonists currently available, none offer specificity for a single mACh receptor subtype and few even display greater than 10-fold selectivity for an individual subtype. This necessitates the use of a range of muscarinic antagonists when defining the receptor subtype expressed/functionally active in a given system (Eglen et al., 1996a). Table 1.2 describes some of the more selective ligands available and their estimated affinities for cloned mACh receptor subtypes, derived in radioligand binding and/or functional assays (adapted from Eglen et al., 1996a; Caulfield & Birdsall, 1998; Eglen & Nahorski, 2000).

MT-7 is clearly the most selective muscarinic antagonist identified to date, exhibiting sub-nanomolar affinity for the M1 receptor and at least micromolar affinity for all other subtypes (Adem & Karlsson, 1997; Bradley, 2000). MT-7 is one of a number of peptides with high affinity for the muscarinic receptors, isolated from the venom of the Eastern Green Mamba (Dendroaspis angusticeps). However, in common with many of the more selective muscarinic receptor antagonists, MT-7 exerts its antagonistic effect by binding to an allosteric site on the M1 mACh receptor, rather than binding at the orthosteric site in direct competition with the agonist (Olianas et al., 2000).
Table 1.2 Antagonist affinity constants (pKᵢ or pKₙ) for mammalian mACH receptors.

<table>
<thead>
<tr>
<th>Antagonist</th>
<th>mACH receptor subtype</th>
<th>Selectivity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M₁</td>
<td>M₂</td>
</tr>
<tr>
<td>Firenzepine</td>
<td>7.8-8.5</td>
<td>6.3-6.7</td>
</tr>
<tr>
<td>Methoctramine</td>
<td>7.1-7.8</td>
<td>7.8-8.3</td>
</tr>
<tr>
<td>4-DAMP</td>
<td>8.6-9.2</td>
<td>7.8-8.4</td>
</tr>
<tr>
<td>Darifenacin</td>
<td>7.5-7.8</td>
<td>7.0-7.4</td>
</tr>
<tr>
<td>p-F-HHSiD</td>
<td>7.4</td>
<td>6.7</td>
</tr>
<tr>
<td>Triptolamine</td>
<td>8.4-8.8</td>
<td>9.4-9.6</td>
</tr>
<tr>
<td>Oxybutynin</td>
<td>8.2</td>
<td>7.5</td>
</tr>
<tr>
<td>AF-DX 384</td>
<td>7.3-7.5</td>
<td>8.2-9.0</td>
</tr>
<tr>
<td>Secorverine</td>
<td>8.0</td>
<td>7.9</td>
</tr>
<tr>
<td>Tolveptidine</td>
<td>8.4</td>
<td>8.1</td>
</tr>
<tr>
<td>MT-3</td>
<td>6.7</td>
<td>5.9</td>
</tr>
<tr>
<td>MT-7</td>
<td>9.8</td>
<td>&lt;6.0</td>
</tr>
</tbody>
</table>

Adapted from Eglen et al. (1996a); Caulfield & Birdsall, (1998); Eglen & Nahorski, (2000).
1.2.4.3 Allosteric ligands

Clark & Mitchelson (1976) first recognised that the M₂ selective antagonist gallamine blocked the effects of ACh and carbachol in atria, but not by binding to the ACh binding site on the M₂ receptor. Instead, gallamine bound to an allosteric site on the receptor. It is now widely accepted that, like most GPCRs, the muscarinic receptors are subject to allosteric modulation (see Tucek & Proksa, 1995).

Binding of a modulator to the allosteric site(s) elicits a change in the conformation of the classical (orthosteric) binding site, such that its affinity for ligands binding to the orthosteric site is altered in some way. Most allosteric ligands exhibit negative co-operativity (reducing the affinity for orthosteric ligands), which can be difficult to distinguish from competitive antagonism (Christopoulos & Kenakin, 2002). For this reason, many of the more subtype-selective muscarinic receptor 'antagonists' identified have in fact turned out to be allosteric modulators e.g. methoctramine (Eglen et al., 1988b) and himbacine (Lee & El Fakahany, 1990).

One of the crucial characteristics of allosteric interactions is that the nature and extent of the effect is dependent upon the receptor subtype and the particular combination of allosteric and orthosteric ligand (Tucek & Proksa, 1995; Lazareno & Birdsall, 1996). For instance, the biphasic allosteric effects of alcuronium on the binding of [³H]-NMS at the M₂ receptor are not observed when [³H]-QNB is used as the radioligand (Tucek et al., 1990). Also, although low concentrations of gallamine slow the dissociation of [³H]-NMS from all five mACh receptor subtypes, there is a wide range in potency (M₂ > M₄ > M₁ > M₃ > M₀) (Ellis et al., 1991).

The difference in susceptibility to allosteric modulation between the M₂ receptor and the M₃ and M₄ subtypes is common to the majority of allosteric interactions reported for a number of different ligands and was used by Ellis and colleagues to investigate the nature of the allosteric binding site on the M₂ receptor (Ellis & Seidenberg, 2000). The use of chimeric M₂/M₃ and M₂/M₅ receptor constructs identified a 31 amino acid stretch corresponding to portions of the third extracellular loop and the sixth transmembrane domain of the M₂ receptor as being essential for the potency of gallamine at the M₂ subtype (Ellis & Seidenberg, 2000). However, the use of other allosteric modulators in the same system illustrated that other epitopes were involved in mediating allosteric effects, with a single threonine residue (T423) in the M₂
receptor crucial in the definition of subtype selectivity of a number of allosteric modulators (Ellis & Seidenberg, 2000). Earlier studies identified a unique EDGE sequence in the second extracellular loop of the M<sub>2</sub> receptor as being crucial in mediating the effects of gallamine upon [³H]-NMS binding (Leppik et al., 1994). In general, therefore, it seems that the allosteric binding site(s) may be located close to the orthosteric site, but more extracellularly. The importance of unique residues, such as in the EDGE epitope, to the effects of certain modulators on the M<sub>2</sub> receptor may explain not only why the M<sub>2</sub> receptor is more readily modulated by allosteric ligands, but also the basis for the pronounced M<sub>2</sub>-selectivity of certain allosteric 'antagonists' (such as methoctramine) i.e. in binding to less conserved regions than the orthosteric binding site, it is possible to achieve a greater degree of subtype selectivity.

Finally, the presence of multiple allosteric sites on the mACh receptors is becoming increasingly well accepted (Birdsall et al., 2001). Early evidence for the biphasic effects of certain allosteric modulators (e.g. tubocurarine, gallamine) on the dissociation of [³H]-NMS from the M<sub>2</sub> receptor is not consistent with a single allosteric binding site (e.g. Ellis et al., 1991). More recent studies have identified the protein kinase inhibitor KT5720 as a high affinity allosteric modulator of the M<sub>1</sub> receptor, which allosterically enhances ACh or [³H]-NMS binding (i.e. is positively co-operative) without influencing gallamine binding, even though the latter agent is able to bind simultaneously with KT5720 and an orthosteric ligand (Lazareno et al., 2000). This is consistent with the presence of at least 2 distinct allosteric binding sites, as has also been suggested for the α<sub>1</sub>-adrenoceptor (Leppik et al., 2000).

1.2.5 mACh receptor structure

As a member of the seven transmembrane receptor superfamily and in particular as a prototypical family A or 'rhodopsin-like' receptor, the mACh receptors display a significant sequence homology with rhodopsin. The availability of a high resolution X-ray crystallographic structure of rhodopsin (Palczewski et al., 2000) has therefore provided a firm basis for the prediction of the structure of the muscarinic receptors. Lu and colleagues have used the rhodopsin structure as a template to build a model for the structure of the M<sub>1</sub> ground state (Lu et al., 2002). Coupled with earlier scanning and point mutagenesis studies on the M<sub>1</sub> (and other) mACh receptor, the residues contributing to the orthosteric agonist/antagonist binding site have been mapped, in
addition to determining the intramolecular interactions required to hold the receptor in the inactive and active conformations (Lu et al., 2002; Hulme et al., 2003).

Hulme and colleagues have mapped the residues contributing to the binding site for the agonist, acetylcholine, and the antagonist, N-methyl scopolamine (NMS), demonstrating that the quaternary ammonium head-groups of these ligands interact with an aromatic cage, comprising the side-chains of amino acid residues in transmembrane domains (TMs) 3, 6 and 7 (Lu et al., 2002; Hulme et al., 2003). Differences in the residues involved in the binding of agonist and antagonist include those towards the top of TM5 and TM6, mutation of which reduces ACh, but not NMS affinity, and contacts deep within the membrane in TMs 3, 5 and 6. The latter group of residues are likely to interact with the phenyl ring of NMS, shared by atropine and its analogues, reinforcing the hydrophobic latch between TMs 3, 5 and 6. This may act to stabilise the receptor in the ground state and perhaps provide a structural basis for the inverse agonist activity of atropine and NMS (Lu et al., 2002; see section 1.5).

Lu et al. also identified some of the potential conformational changes that might occur upon agonist activation of the M1 receptor. The efficacy of ACh is highly dependent upon both the negative charge of the highly conserved aspartate residue within TM3 (believed to electrostatically interact with the cationic head-group of ACh) and the phenyl rings of the tyrosine residues within the aromatic cage (see Lu et al., 2002). It is proposed that a constriction of the aromatic cage around the quaternary ammonium head-group of ACh might precipitate the movements of TM6 and TM7 relative to TM3 and contribute to the activation of the receptor. Restriction of the outward movement of TM6 relative to TM3 (by the engineering of Zn$^{2+}$ bridges between them) was found to inhibit the activation of rhodopsin, β2-adrenoceptor and parathyroid hormone receptor, confirming the importance of this movement in GPCR activation (Sheikh et al., 1999). The greater mobility of TM7 upon receptor activation has been demonstrated in a number of ways, including the state-dependent disulphide cross-linking of rhodopsin (Yu et al., 1999).

The residues involved in mediating the activation of G-proteins have been mapped to the cytoplasmic ends of TMs 3, 5, 6 and 7, the H8 helix and the second and third intracellular loops (see Lu et al., 2002). Agonist binding opens a pocket between the TM helices, into which the C-terminal sequence of the G-protein α-subunit can

23
project (Bourne, 1997). Kostenis and colleagues have identified several residues within the second and third intracellular loops and TM6, which are important in mediating the interaction of the muscarinic receptors with their cognate G-proteins (Kostenis et al., 1997a; 1997b). In addition to these interactions with the C-terminus of the α-subunit, stabilising interactions between the Gβγ-subunit and the C-terminus of the receptor (in particular the H8 helix) have been reported for rhodopsin (Phillips & Cerione, 1992), while evidence for a βγ-subunit binding domain in the third intracellular loop of the muscarinic receptors has been provided (Wu et al., 2000).

Other notable structural features of the mACh receptor family include two conserved cysteine residues in the first and third extracellular loops, between which a disulphide bond is formed (Kurtenbach et al., 1990). Another cysteine residue in the C-terminus of the M2 receptor (C457) provides a site for palmitoylation, which may enhance signalling efficiency (Hayashi & Haga, 1997). All muscarinic receptor subtypes contain at least 1 potential glycosylation site within their N-terminus, but the functional relevance of this modification is not yet clear. Enzymatic deglycosylation of muscarinic receptors has been shown to have no influence upon agonist or antagonist binding in reconstituted lipid vesicles (Ohara et al., 1990) and glycosylation-deficient M2 receptor mutants demonstrated that glycosylation was not required for cell surface localisation, ligand binding, functional coupling to the inhibition of adenylate cyclase or protection against rapid degradation (Van Koppen & Nathanson, 1990).

1.2.6 Compartmen탈isation of mACh receptor signalling

As our understanding of the complexity and diversity of signal transduction advances, the requirement for mechanisms conferring specificity within signalling pathways becomes even more apparent. Until quite recently the notion that the myriad components of signal transduction cascades are freely mobile within the plasma membrane was an established one. However, accumulating evidence suggests that the specificity of molecular interactions within the cell is not merely prescribed by the three-dimensional structures of the individual components. Observations that different receptors coupled to the same G-protein in a single cell type can induce different cellular responses, has challenged the classical view of GPCR signal transduction as a linear pathway (Steinberg & Brunton, 2001).
It is becoming increasingly well accepted that GPCRs and downstream signalling proteins can assemble to generate signalling complexes, based around scaffolding proteins, which concentrate the components of a signal transduction pathway in a discrete region of the cell (Steinberg & Brunton, 2001). A prototypical scaffolding protein is AKAP150, a member of the A-kinase anchoring protein (AKAP) family (Colledge & Scott, 1999). AKAP150 has been reported to associate with the β2-adrenoceptor, facilitating cyclic AMP-dependent phosphorylation of the receptor by anchoring PKA in close proximity to the receptor (Fraser et al., 2000).

In relation to the muscarinic receptors, an interesting example is that of the caveolin family of scaffolding proteins. This family comprises three isoforms, namely caveolin-1, -2 and -3, and form the main structural components of caveolae (Liu et al., 2002). Caveolae are vesicular invaginations of the plasma membrane, for which numerous cellular functions have been proposed, including vesicular transport, cholesterol homeostasis and the compartmentalisation and regulation of signal transduction pathways (see Razani et al., 2002). They are highly enriched in cholesterol and have been identified in abundance in smooth and striated muscle cells, fibroblasts, adipocytes, endothelial cells and type I pneumocytes, but are apparently absent from lymphocytes and CNS neurons (Okamoto et al., 1998). An interesting property of these specialised lipid rafts is that they appear to selectively accumulate a wide variety of signalling molecules, including GPCRs, G-proteins, src kinases and second messenger-regulated kinases (see Razani et al., 2002). This led to the proposal that caveolae may be involved in the regulation of signal transduction.

Caveolins 1 and 2 are almost ubiquitously expressed, while caveolin-3 is restricted to cardiac, skeletal and smooth muscle (Razani et al., 2002). Li et al. (1995) provided the first evidence that these proteins might directly interact with caveolae-localised signalling proteins, when they reported that a 20 amino acid peptide derived from caveolin-1 potently inhibited the activity of heterotrimeric G-proteins in GTP hydrolysis assays. This region has since been characterised to mediate the binding of caveolin-1 to a wide range of signalling proteins and as such has been termed the "caveolin scaffolding domain" (Couet et al., 1997). All of the proteins found to interact directly with caveolin do so via a conserved caveolin-binding domain, corresponding to either ΦXΦXXXΦ or ΦXXXXΦXXΦ (where X = any amino acid and Φ = Phe, Trp or Tyr) (Couet et al., 1997).
However, the role of caveolae in GPCR signalling is not merely to provide a static framework for the assembly of signalling networks. It has been demonstrated that although β_2_-adrenoceptors are highly enriched in cardiomyocyte caveolae at rest, they may transit out of caveolae upon receptor activation (Ostrom et al., 2001). This is thought to target the β_2_-adrenoceptors to clathrin-coated pits following the termination of signalling through G_s and can be blocked by co-expression of the C-terminus of GRK-2, which prevents activation of endogenous GRK-2 (Ostrom et al., 2001). In contrast to the β_2_-adrenoceptor, neither β_1_-adrenoceptors nor M_2_ mACh receptors are enriched in caveolae, but following activation by muscarinic agonist, the M_2_ receptor translocates into caveolae, as demonstrated by an increased association with caveolin (Feron et al., 1997; Rybin et al., 2000). The differential localisation of receptors in different membrane compartments has many functional implications, commensurate with the relative concentrations of G-protein and effector proteins in the local environment. For instance, the coincident exclusion of β_1_-adrenoceptor and M_2_ mACh receptor species from caveolae may explain the ability of the latter to inhibit adenylate cyclase activation by the β_1_ but not the β_2_ subtype (Aprigliano et al., 1997).

Rybin et al. (2000) also showed that the cardiac adenylate cyclase isoforms (5 and 6) are enriched in the caveolae of cardiomyocytes. However, this does not appear to increase the efficiency of β-adrenoceptor activation of adenylate cyclase, as might be expected. Instead, the caveolar environment appears to have an inhibitory influence upon adenylate cyclase activity, since the cyclodextrin-mediated disassembly of caveolae leads to an enhanced cyclic AMP accumulation in response to forskolin treatment or β-adrenoceptor activation (Rybin et al., 2000). Indeed, caveolae have been found to exert inhibitory effects on the vast majority of signalling intermediates found to be enriched in caveolae and/or found to interact with caveolins (see Razani et al., 2002). In particular, most tyrosine- and serine/threonine-kinases possess caveolin-binding motifs within their active catalytic domains (Couet et al., 1997) and are therefore potently inhibited by caveolin binding.

Caveolin binding is also known to inhibit heterotrimeric G-protein activation either by preferentially binding to the GDP-bound α-subunit (caveolin-1) or by accelerating GTP hydrolysis via an intrinsic GTPase-activating activity (caveolin-2) (Okamoto et al., 1998). It has been proposed that this may be implicated in the heterologous desensitisation subsequent to M_2_ and M_3_ mACh receptor activation in rabbit ileal
smooth muscle cells (Murthy & Makhlouf, 2000). M₂ mACh receptors have themselves been reported to bind to caveolin-3 upon receptor activation in both cardiac myocytes (Feron et al., 1997) and in rabbit ileum (Murthy & Makhlouf, 2000). In the latter study, this was not considered a pre-requisite for heterologous desensitisation, as the M₃ mACh receptor was not found to co-precipitate with caveolin-3 (either before or after agonist activation), yet was still capable of inducing desensitisation (Murthy & Makhlouf, 2000). However, inspection of the sequences of the cloned human muscarinic receptors indicates that the consensus caveolin-binding domain identified in the M₂ receptor (WWWXXWXXY; see Couet et al., 1997) is conserved in all five mACh receptor subtypes. Given that both G₆ (e.g. Lisanti et al., 1994) and PIP₂ are also enriched in caveolae (as much as 50 % of the total cellular PIP₂ of A431 cells was present in caveolae; Pike & Casey, 1996) it is possible that they might form scaffolds for PLC signalling pathways, perhaps downstream of M₁/M₃/M₅ receptor activation.

A precedent for PLC-linked signalling in caveolae involves bradykinin-mediated PLC activation in A431 cells (Pike & Casey, 1996; Pike & Miller, 1998). Treatment of A431 cells with bradykinin (or EGF) selectively stimulated PIP₂ hydrolysis in caveolae, without any significant turnover of non-caveolar PIP₂ (Pike & Casey, 1996). Removal of cholesterol from caveolae by cyclodextrin treatment disrupted caveolae and profoundly reduced the bradykinin- (and EGF)-stimulated PIP₂ hydrolysis and de-localised G₆ and PIP₂ from caveolae into the plasma membrane (Pike & Miller, 1998). It has since been proposed that both B₁ and B₂ bradykinin receptors translocate into caveolae upon agonist activation (see Ostrom, 2002; Sabourin et al., 2002) and that here, the receptors are able to efficiently couple to G₁₁-medaited PLC activation and Ca²⁺-mobilisation, as well as activation of cPLA₂. These data therefore suggest that the localisation of signalling proteins and lipids to caveolae may in some instances be required for efficient PIP₂ turnover and that caveolae may in fact be the primary sites for hormone-stimulated PIP₂ hydrolysis (Pike & Casey, 1996).

1.2.7 mACh receptor desensitisation

1.2.7.1 The role of phosphorylation in mACh receptor desensitisation

The prolonged exposure of most GPCRs to agonist results in an attenuation of receptor responsiveness, known as desensitisation. As is the case with many GPCRs,
agonist-induced desensitisation of the mACh receptors is usually associated with receptor phosphorylation (Kwatra & Hosey, 1986). The mACh receptors are phosphorylated on serine and threonine residues within the third intracellular loop and C-terminal tail by second messenger-regulated protein kinases (PKA and PKC), G-protein-coupled receptor kinases (GRKs) and casein kinase 1α (see Van Koppen & Kaiser, 2003). Second messenger-regulated protein kinases possess the unique ability to phosphorylate either active or inactive receptors, enabling them to initiate “heterologous” desensitisation, whereby inactive receptors may be desensitised by the activation of distinct receptors. In contrast, the GRKs are only recruited to activated GPCRs and therefore mediate “homologous” desensitisation in response to agonist activation.

1.2.7.2 PKC- and casein kinase 1α (CK1α)-mediated phosphorylation of the mACh receptors

Although both M1 and M3 mACh receptors have been demonstrated to be substrates for PKC-mediated phosphorylation, agonist-mediated phosphorylation of these receptors has been shown to be independent of PKC (Tobin et al., 1993; Haga et al., 1996). In agreement with this, although phorbol ester treatment significantly reduced M3 receptor-mediated phosphoinositide and Ca2+ responses in SH-SY5Y cells, inhibition of PKC was without effect upon agonist-mediated responses (Willars et al., 1996). These data suggest that although the M1 and M3 mACh receptors are capable of acting as substrates for PKC, physiological activation of the receptors fails to elicit a sufficient activation of PKC (or activation of the necessary PKC isoform(s)) to phosphorylate the receptor, while phorbol ester-mediated phosphorylation is likely to result from un-physiological levels of PKC activation (or activation of alternative PKC isoforms). This is supported by the observation that the phorbol ester PDBu induced a faster and possibly greater translocation of PKCα than M3 receptor activation in SH-SY5Y cells (Willars et al., 1996).

Casein kinase 1α (CK1α) has also been implicated in muscarinic M3 receptor phosphorylation, as it has been demonstrated to bind to and phosphorylate the third intracellular loop of the M3 receptor in an agonist- and βγ-subunit-dependent manner (Budd et al., 2000, 2001). CK1α-mediated phosphorylation does not appear to desensitise the M3 receptor, but may instead act to facilitate M3 receptor-mediated
activation of the extracellular signal-regulated kinases 1 and 2 (ERK 1/2) (Budd et al., 2001). Although the functional role(s) of CK1α phosphorylation is not yet fully understood, the presence of CK1α phosphorylation motifs in a number of different GPCRs suggests a more widespread physiological role (Tobin, 2002).

1.2.7.3 GRKs 2 and 3 in mACh receptor desensitisation

The GRK family comprises seven members, including GRKs 1 and 7, which are specifically expressed in the retina, and the more widely distributed non-visual GRKs 2-6. The most extensively studied of these kinases are GRKs 2 and 3 (formerly known as β-adrenergic receptor kinases 1 and 2, or β-ARK 1 and 2), which are ubiquitously expressed and have been implicated in the phosphorylation and desensitisation of a number of GPCRs, including the muscarinic receptors (see Ferguson, 2001; Van Koppen & Kaiser, 2003; Willets et al., 2003). In unstimulated cells, GRKs 2 and 3 are found in the cytosol and translocate to the plasma membrane upon agonist activation of their target receptors. This is mediated in part by their association with the βγ-subunit of the activated heterotrimeric G-protein (Pitcher et al., 1992), but also by the binding of plasma membrane PIP$_2$ to the pleckstrin homology domain of the kinases (Pitcher et al., 1995).

Haga et al. (1996) provided strong evidence for GRK2-mediated phosphorylation of the M$_1$ mACh receptor on residues in its third intracellular loop. Similarly, Wu et al. (2000) found that the M$_3$ receptor is phosphorylated on specific residues with the third intracellular loop, in close proximity to a putative Gβγ binding site proposed to mediate the synergistic activation of GRK2 by Gβγ and activated receptor. This is in agreement with the earlier work of DebBurman et al. (1995) who reported that reconstituted membranes of S9 cells expressing the M$_3$ receptor recruited GRKs 2 and 3 to phosphorylate the receptor in an agonist-dependent manner. The physiological relevance of this is highlighted by the observation that mice lacking the gene for GRK3 display enhanced airway responsiveness to methacholine, suggesting that GRK3 may contribute to the in vivo regulation of M$_3$ and/or M$_2$ receptor function in airway smooth muscle (Walker et al., 1999).

Despite its distinct G-protein coupling characteristics, the M$_2$ mACh receptor also appears to be phosphorylated in an agonist-dependent manner by GRK2 on specific residues within its third intracellular loop (Haga et al., 1994; Nakata et al., 1994;
Kameyama et al., 1994; Pals-Rylaarsdam et al., 1995). The use of either deletion (Pals-Rylaarsdam et al., 1995) or substitution (Pals-Rylaarsdam & Hosey, 1997) strategies within the third intracellular loop highlight the importance of phosphorylation of serine and threonine residues in this region for M2 receptor desensitisation. Also, the expression of dominant-negative GRK2 mutants significantly reduced agonist-induced phosphorylation and desensitisation of the M2 receptor (Tsuga et al., 1994; Pals-Rylaarsdam et al., 1995), indicating that in recombinant cell systems at least, GRK2 is capable of mediating agonist-induced desensitisation of the M2 receptor.

1.2.7.4 GRKs 4, 5 and 6 in mACl receptor desensitisation

The roles of the less intensively studied members of the non-visual GRK family, GRKs 4-6, in GPCR regulation are becoming increasingly well appreciated. Interestingly, genetically modified mice lacking GRK5 displayed enhanced responsiveness to the muscarinic agonist oxotremorine in a variety of classic muscarinic responses, including hypothermia, tremor, salivation and hypoactivity (Gainetdinov et al., 1999). Oxotremorine-induced desensitisation of [35S]-GTPyS binding in membranes isolated from the brain stem and striatum of the GRK5 knockout mice was completely abolished (Gainetdinov et al., 1999). Taken together, these data indicate that GRK5 contributes to the regulation of responses mediated by both central and peripheral muscarinic receptors in vivo, and supports earlier evidence that GRK5 was capable of phosphorylating the M2 mACh receptor (Tsuga et al., 1998). It is also interesting to note that GRK5 knockout mice displayed identical responses to serotonergic and dopaminergic agonists as wild-type mice, indicating that GRK5 appears to display some selectivity for the regulation of the muscarinic receptors (Gainetdinov et al., 1999).

GRK6 was first implicated in the regulation of the M3 muscarinic receptor when it was observed that overexpression of either GRK3 or GRK6 enhanced agonist-dependent phosphorylation of the endogenously expressed M3 receptor in SH-SY5Y cells (Willets et al., 2001). Endogenous GRK6 was subsequently shown to contribute to M3 receptor phosphorylation and desensitisation in SH-SY5Y cells (Willets et al., 2002). This was based upon the observation that the expression of a dominant-negative, kinase-dead GRK6 led to a 50% reduction in both phosphorylation and receptor-Gq/11 uncoupling (Willets et al., 2002). This contrasts with the earlier work of
DebBurman et al. (1995), who found that neither GRK5 nor GRK6 were capable of significantly phosphorylating M3 receptors expressed in S9 cell membranes. M1, M2 and M3 receptors have also been reported to be poor substrates for GRKs 5 and 6 in vitro (Kunapuli et al., 1994; Loudon & Benovic, 1997). However, given the weight of evidence from the GRK5 knockout mice and the use of the neuroblastoma SH-SY5Y cell line, it would seem that the in vivo regulation of GRKs 5 and 6 may be considerably more complex than can be appreciated in vitro, or in a recombinant system. Indeed factors such as the palmitoylation status of GRK6 (Stoffel et al., 1998) and PKC-mediated phosphorylation of GRK5 (Chuang et al., 1996) have been shown to profoundly influence the activity of these kinases and further investigation is required to fully understand their regulation and function.

1.2.7.5 Phosphorylation-independent desensitisation

GRKs 2 and 3 have also been found to regulate GPCRs in a phosphorylation-independent manner, most likely via high affinity binding of their RGS-like domain to Gq11α, thus preventing association with PLC-β (see Willets et al., 2003). Expression of the N-terminal RGS-like domain alone is sufficient to selectively inhibit the activation of PLC resulting from stimulation of a number of Gq11-coupled GPCRs expressed in recombinant cell systems (Sallese et al., 2000). Willets et al. (2004) recently demonstrated phosphorylation-independent desensitisation of muscarinic M1 receptor signalling in cultured hippocampal neurons. Expression of either wild-type or catalytically-inactive GRK2 almost completely abolished agonist-induced IP3 production, suggesting that even in the absence of kinase activity, GRK2 is capable of desensitising M1 receptor-mediated signalling (Willets et al., 2004). Perroy et al. (2003) recently utilised short interfering RNA (siRNA) to show that GRK4 was capable of regulating the GABAB receptor independently of phosphorylation, indicating that other members of the GRK4 family of kinases might also suppress receptor-G-protein coupling independently of phosphorylation.

1.2.7.6 The role of RGS proteins in GPCR desensitisation

The mechanisms of desensitisation discussed in the previous sections relate to the regulation of the receptor subsequent to the activation of the downstream signalling cascades. However, G-protein inactivation provides another means by which the kinetics of signal transduction may be modulated. The hydrolysis of GTP to GDP on
the G-protein α-subunit terminates signalling through this subunit and although this is mediated by the intrinsic GTPase activity of the α-subunit itself, it is known that the rate of this reaction may be increased by up to 2 orders of magnitude by a variety of GTPase-activating proteins (GAPs) (see Wilkie & Ross, 2000). Many of these GAPs belong to the regulators of G-protein signalling (RGS) family of proteins, of which more than 30 have been identified (De Vries et al., 2000; Wilkie & Ross, 2000). One surprising finding from the extensive study of this family of proteins is that the majority of RGS proteins display little selectivity for particular Gα subunits (Hepler, 2003). Indeed, in vitro most RGS proteins act as GAPs for members of the Gj family and a subset of those are also capable of inactivating Gq (Hepler, 1999; De Vries et al., 2000).

In contrast to the apparent lack of selectivity for G-protein α-subunits, many RGS proteins do display receptor-dependent selectivity for the inactivation of functional responses. For instance, Wang et al. (2002) reported that in rat aortic smooth muscle, endogenous RGS3 negatively regulates M3 receptor-mediated signalling, while RGS5 inhibits angiotensin AT1α receptor-activated signal transduction. Since the MAP kinase response assayed in response to stimulation of each receptor is likely to proceed through a Gq11-dependent pathway (based upon the insensitivity of each MAP kinase response to PTx), the apparent selectivity of RGS3 and RGS5 cannot be explained by G-protein selectivity, but instead indicates that selectivity is conferred by the GPCR itself (Wang et al., 2002). There is now considerable evidence that GPCRs play a pivotal role in determining RGS protein recruitment to the signalling pathway (Hepler, 2003). Other examples involving the muscarinic receptors include RGS4 selectivity for the inhibition of mACH receptor- versus CCK receptor-mediated Ca2+-mobilisation in pancreatic acinar cells (Zeng et al., 1998) and RGS8, which selectively suppressed M1, but not M3 receptor-mediated Ca2+-activated Cl− currents in Xenopus oocytes (Saitoh et al., 2002).

Roy et al. (2003) recently reported that RGS2 and RGS4 are selectively recruited to the plasma membrane by either G-proteins or by GPCRs that activate those G-proteins. Selective recruitment of RGS4 by the M2 mACH receptor and G12α was mirrored by the greater potency of RGS4 (versus RGS2) for promoting steady-state Gαi GTPase activity (Roy et al., 2003). The use of G-protein mutants with low affinity for RGS proteins demonstrated that recruitment initiated by G-proteins required direct
RGS protein binding and this occurred irrespective of the activation state of the receptor or G-protein (Roy et al., 2003). Given the receptor/G-protein selectivity and relative stability of the interactions between these signalling elements and the RGS proteins, the emerging evidence indicates that RGS proteins are selectively recruited to plasma membrane-associated multiprotein complexes to modulate the activity of the associated Go subunit (Hepler, 2003). Given that RGS proteins are also known to attenuate Go-mediated signal generation by a functional inhibition of Go-effector coupling (so-called 'effector antagonism'; e.g. Hepler et al., 1997), it is clear that the localisation of the different components of the signalling cascade might promote selective inactivation of G-protein signalling at more than one point in the pathway.

Further evidence for the contribution of both muscarinic receptors and their interacting G-proteins in the recruitment of specific RGS proteins came from the observation that the spontaneous association of RGS4 with anionic phospholipid vesicles was stabilised by the presence of either M1 receptor/Go or M2 receptor/Gi proteins (Tu et al., 2001). Bernstein et al. (2004) recently provided definitive evidence that RGS proteins are capable of selectively binding directly to mACh receptor subtypes. For instance, RGS2 but not RGS16 binds to the third intracellular loop of the M1 mACh receptor and, importantly, when co-expressed in CHO cells RGS2 (but not RGS16) co-localises with the M1 receptor (Bernstein et al., 2004). In agreement with the work of Zeng and colleagues, who found that the N-terminal domain of RGS4 conferred receptor-selective inhibition of Go signalling, Bernstein et al. reported that the N-terminus of RGS2 was both necessary and sufficient for the RGS-receptor i3 loop interaction. They also demonstrated that the third intracellular loops of all five subtypes of the mACh receptor family displayed unique binding profiles for a range of RGS proteins (Bernstein et al., 2004), suggesting that these interactions might explain the ability of GPCRs to selectively recruit RGS proteins and provide a structural basis for the assembly of these proteins into membrane-associated protein scaffolds, as has been proposed (Sierra et al., 2000).

1.2.8 Internalisation of mACh receptors

1.2.8.1 A role for internalisation in desensitisation?

In common with the majority of GPCRs, prolonged exposure to agonist not only leads to the uncoupling of receptor and G-protein, but also causes the receptor to undergo
the process of internalisation (Koenig & Edwardson, 1997); however, the functional role of this process has been the subject of debate for a number of years. Initially, it was proposed that receptor internalisation might be the process by which GPCRs undergo a rapid desensitisation in response to agonist activation (e.g. Cheung et al., 1989). However, it was observed that in the case of the muscarinic receptors (and many other GPCRs), the rate at which desensitisation occurred was significantly greater than the loss of receptors from the cell surface (Tobin et al., 1992), indicating that internalisation was not a pre-requisite for desensitisation. Furthermore, Pals-Rylaarsdam et al. (1995) found that while a dominant-negative GRK2 mutant reduced agonist-dependent phosphorylation and desensitisation of the M2 mACh receptor, agonist-induced internalisation was unaffected, supporting the notion that desensitisation and internalisation are distinct processes. Similarly, Willets et al. (2002) found that a dominant-negative GRK6 mutant significantly inhibited phosphorylation and desensitisation of the M3 receptor, while receptor internalisation was unaltered.

1.2.8.2 The link between phosphorylation and internalisation

It is now understood that desensitisation and internalisation are distinct, but interrelated functions as for most GPCRs, agonist-induced phosphorylation facilitates internalisation as well as the uncoupling of receptors from their cognate G-proteins. Putative phosphorylation sites in the third intracellular loop of the M1, M2 and M3 mACh receptors have been identified in mutagenesis studies as being crucial for agonist-induced internalisation (Moro et al., 1993; Pals-Rylaarsdam & Hosey, 1997). The role of GRKs in muscarinic receptor internalisation was first demonstrated by the over-expression of GRK2 in COS-7 cells co-expressing the M2 mACh receptor (Tsuga et al., 1994). Whereas wild-type GRK2 enhanced agonist-induced internalisation of the M2 receptor, co-expression of a dominant-negative GRK2 mutant (K220W) had the opposite effect (Tsuga et al., 1994). However, the contribution of GRK subtypes to mACh receptor internalisation appears to be dependent not only on the receptor subtype, but also on the cell background (see Van Koppen & Kaiser, 2003). For instance, the K220W GRK2 mutant had no effect on the M2 receptor when expressed in HEK-293 or BHK-21 cells (Tsuga et al., 1994; Pals-Rylaarsdam et al., 1995), nor did it affect the internalisation of the M3 receptor expressed in COS-7 cells (Tsuga et al., 1998). This probably reflects the differential
expression patterns of the GRK isoforms, with cells expressing lower endogenous levels of GRK2 likely to be more sensitive to the expression of the dominant-negative GRK2 mutant.

In many cases phosphorylation is not an absolute requirement for internalisation, but instead acts to stabilise a receptor conformation that promotes the interaction with accessory proteins involved in the endocytic process. GRK-phosphorylation of muscarinic receptors increases their affinity for β-arrestin proteins, which, in addition to uncoupling the receptor from its associated heterotrimeric G-protein, targets the receptor for internalisation via clathrin-coated pits (Goodman et al., 1996; Vogler et al., 1999). Expression of dominant-negative β-arrestin mutants that are either unable to bind to clathrin or phosphorylated muscarinic receptors, significantly reduced agonist-mediated internalisation of M₁, M₃ and M₄ receptors in HEK-293 cells (Vogler et al., 1999; Claing et al., 2000). Over-expression of a dominant-negative clathrin mutant also inhibited the internalisation of the M₁, M₃ and M₄ receptors (Vogler et al., 1999), highlighting the importance of β-arrestin and clathrin in the internalisation of these mACh receptor subtypes.

In addition to the β-arrestins, the interaction of mACh receptors with other accessory proteins may also be important for mediating internalisation. The βγ-subunit binding domain identified in the M₃ receptor, for instance, has been implicated in internalisation, since mutant receptor lacking this binding site exhibit attenuated internalisation, despite ligand-binding and functional responses being unaffected (Wu et al., 2000). However, it still remains a possibility that the compromised internalisation of these receptors is due to a disruption in the GRK-mediated phosphorylation of the M₃ receptor. Further downstream, additional β-arrestin binding proteins such as AP-2 (Laporte et al., 1999) might be necessary for the efficient targeting of GPCRs to clathrin-coated pits, while the phosphorylation status of β-arrestin proteins is known to regulate their ability to bind clathrin and initiate internalisation, underscoring the multiple potential sites for regulation of GPCR internalisation (see Ferguson, 2001 for review).

1.2.8.3 The unique nature of M₂ mACh receptor internalisation

In contrast to the other mACh receptor subtypes investigated, the M₂ receptor appears to internalise in an arrestin- and clathrin-independent manner (Schlador & Nathanson,
The mechanism by which $M_3$ receptors are internalised is not yet fully understood, but it has been reported to display a unique sensitivity to mutants of dynamin (Werbonat et al., 2000). It has been suggested that the $M_3$ receptor may internalise via caveolae but this may be dependent upon the cell system investigated (Dessy et al., 2000; Roseberry & Hosey, 2001). Delaney et al. (2002) have subsequently reported that in HeLa cells, $M_3$ receptor internalisation is dependent upon the small G-protein ARF6. The same study found that once internalised, $M_3$ receptors quickly localise to endosomal compartments expressing clathrin-coated pit markers, suggesting a convergence of clathrin-dependent and -independent pathways downstream of internalisation (Delaney et al., 2002).

1.2.8.4 Is activation required to facilitate internalisation?

The relationship between GPCR activation and internalisation has been called into question by the observation that the binding of antagonists to certain GPCRs is capable of enhancing the rate of receptor endocytosis (Roettger et al., 1997; Bhowmick et al., 1998). Also, etorphine, is capable of stimulating phosphorylation and internalisation of the $\mu$-opioid receptor in HEK-293 cells, while morphine only elicits similar responses when GRK2 is over-expressed (Zhang et al., 1998). These observations have been taken as evidence that different ligands are capable of stabilising distinct receptor conformations that are able to discriminate G-protein activation from GRK recruitment/phosphorylation (see Ferguson, 2001).

However, Koenig & Edwardson (1996) demonstrated the importance of cell background in the intracellular trafficking of the muscarinic receptors when they provided quantitative evidence that the apparent loss of mACh receptors from the cell surface in response to agonist was dependent upon both receptor subtype and the cell type in which they are expressed. This is because the rate of recycling of receptors is unaffected by the activation state of the receptor, but is intrinsic to the cell type (Szekeres et al., 1998a). It is the rate of endocytosis (the removal of receptors from the plasma membrane) that is increased by agonist activation and the extent to which it is enhanced is proportional to the intrinsic activity of the agonist (Szekeres et al., 1998a). Therefore, in a cell system where the rate of recycling is low and the maximal capacity for endocytosis is high, even agonists displaying as little as 3 % of the intrinsic activity of a full agonist can cause an apparent loss of up to 50 % of the surface $M_3$ receptor number (Szekeres et al., 1998a). Given that such a weak partial
agonism would probably be undetected in most standard assays of functional activity, it is possible that 'antagonists' capable of internalising GPCRs are in fact weak partial agonists and that a low level of recycling and an efficiently coupled receptor system might uncover this agonism (Szekeres et al., 1998a). Differences in agonist efficacy are also likely to underlie the ability of one agonist to induce GRK-mediated phosphorylation and internalisation, where others activate the receptor in the absence of these adaptive responses (e.g. Zhang et al., 1998). The nature of the cell background is likely to have further influences in this respect, particularly in the relative expression levels of GRK isoforms.

1.2.8.5 Is mACh receptor internalisation and recycling necessary for resensitisation?

The role of receptor internalisation and recycling in receptor desensitisation and resensitisation has also been found to depend upon the cell background (see Van Koppen & Kaiser, 2003 for review). The classical view is that upon agonist activation, GPCRs are internalised via a GRK- and β-arrestin-dependent mechanism. Receptors associated with acidic, endocytic compartments are then de-phosphorylated by a GPCR phosphatase. This resensitises the receptors and following their recycling to the plasma membrane they are ready for another round of receptor activation (Pitcher et al., 1995).

However, in the case of the muscarinic receptors, Tobin et al. (1992) observed that resensitisation of the M3 receptor occurs rapidly at the plasma membrane, while internalisation of the M4 receptor has actually been found to delay receptor resensitisation (Bogatkevitch et al., 1996). In contrast, Szekeres et al. (1998a) found that inhibition of endocytosis and recycling of the (mostly) M3 mACh receptor population expressed in SH-SYS5Y cells decreased resensitisation, suggesting a role for receptor recycling in the recovery from desensitisation. Szekeres et al. (1998b) highlighted the role of receptor reserve in the process of resensitisation, demonstrating that removal of the 50 % receptor reserve for Ca\textsuperscript{2+} mobilisation in SH-SYS5Y cells by irreversible receptor alkylation decreased the rate of resensitisation. The authors proposed that receptor reserve might facilitate rapid resensitisation of functional responses and it is therefore conceivable that inevitable differences in reserve between different cell types might in part explain the disparity in the reported contribution of receptor internalisation and recycling in receptor resensitisation.
1.3 Urinary bladder (detrusor) smooth muscle contraction

1.3.1 Mechanisms of detrusor smooth muscle contraction

The autonomic innervation of the urinary bladder consists of the sympathetic (adrenergic) fibres of the hypogastric nerve and the parasympathetic fibres in the pelvic nerve. Both sympathetic and parasympathetic nervous systems act in concert to effect the two phases of micturition; the filling phase and the emptying phase (for review, see De Groat & Yoshimura (2001); Chess-Williams (2002)). Smooth muscle in the bladder neck region expresses a predominant α1-adrenoceptor population, through which sympathetic activity (which predominates during the filling phase) maintains the tone in this region to constrict the bladder outlet. Also during the filling stage, cholinergic signalling is suppressed while sympathetic stimulation of β-adrenoceptors in the bladder dome and body cause relaxation, allowing bladder capacity to increase.

During bladder emptying, sympathetic activity is inhibited and parasympathetic drive is increased. This results in relaxation of the bladder outlet region through reduced adrenergic-mediated contraction in the bladder neck and acetylcholine (ACh)-induced nitric oxide (NO) release in the urethra (Nagahama et al., 1998). In humans, ACh is the major parasympathetic excitatory transmitter, though co-released ATP plays a bigger role in some other species. Coupled with relaxation of the bladder neck and urethra, ACh-mediated contraction of bladder dome and body allows bladder emptying to occur with little increase in intravesical pressure.

Activation of the cholinergic system is therefore the major pathway by which micturition is initiated in humans (Andersson, 1993). Two mACh receptor subtypes (M2 and M3) have been identified in bladder smooth muscle by a variety of techniques (see Eglen et al., 1996a). Radioligand binding studies, for example in guinea pig (Nilvebrant & Sparf, 1983) and human (Nilvebrant et al., 1985), identified a high density of muscarinic receptors. Northern blot analysis in human demonstrated the presence of mRNA for the M2 and M3 receptors only (Yamaguchi et al., 1996). Quantitative immunoprecipitation with receptor-specific antibodies confirmed this finding at the level of protein expression in rat, human, rabbit and guinea pig bladder membranes (Wang et al., 1995). Wang et al. (1995) demonstrated a 75%
predominance of the $M_2$ subtype in all species except rat, where the $M_2$ subtype comprised 90% of the total muscarinic receptor population.

However, pharmacological characterisation of the muscarinic receptors mediating contraction of detrusor muscle agree on the predominant involvement of the minority $M_3$ receptor population in rat (Wang et al., 1995; Longhurst et al., 1995), rabbit (Wang et al., 1995; Choppin et al., 1998), mouse (Choppin & Eglen, 2001a), guinea pig (Wang et al., 1995; Ikeda et al., 1999), pig (Yamanishi et al., 2000; Sellers et al., 2000), dog (Choppin & Eglen, 2001b) and human (Chess-Williams et al., 2001; Fetscher et al., 2002). $M_3$ receptor stimulation has been demonstrated to stimulate phosphoinositide hydrolysis in the detrusor smooth muscle of a number of species, including guinea pig (Noronha-Blob et al., 1989), rat (Mimata et al., 1995) and human (Andersson et al., 1991; Harriss et al., 1995) and this pathway is believed to be involved in inducing contraction of smooth muscle.

In common with all smooth muscle types, the crucial event in the initiation of bladder detrusor contraction is the elevation of intracellular Ca$^{2+}$. Ca$^{2+}$ binds calmodulin and this complex activates myosin light chain kinase (MLCK). Phosphorylation of myosin light chain (MLC) by MLCK allows the myosin ATPase to be activated by actin, leading to contraction of the smooth muscle (see Horowitz et al., 1996 for review). Modulation of the intracellular concentration of Ca$^{2+}$ is therefore an obvious way in which smooth muscle contraction may be regulated. However, both MLCK and MLC phosphatase (MLCP), which mediates the dephosphorylation of MLC and therefore plays an equally pivotal role in regulating the amplitude of force production, are targets for a variety of signal transduction cascades (Pfitzer, 2001). The modulation of smooth muscle tone therefore involves the integration of a complex network of signalling pathways, as illustrated in Figure 1.3.

Considerable evidence exists for a significant role of influx of extracellular Ca$^{2+}$ in response to muscarinic receptor activation in bladder (Batra et al., 1987; Fovaeus et al., 1987; Jezior et al., 2001). Recent studies by Michel's group have demonstrated a key role for L-type VOCCs in rat and human urinary bladder contraction to carbachol (Fleischman et al., 2004; Schneider et al., 2004a, b). A thorough discussion of the integration of intracellular and extracellular Ca$^{2+}$ pools in the regulation of smooth muscle contraction is beyond the scope of this Introduction, but a summary of the proposed sequence of ionic currents activated in smooth muscle in response to
Figure 1.3 Signal transduction pathways involved in the initiation of smooth muscle contraction by the M₃ mACH receptor. Relaxatory pathways are indicated in red.
A muscarinic agonist is outlined in Figure 1.4 (see section 1.3.4 for details of the role of the M₂ mACh receptor in smooth muscle contraction).

1.3.2 Mechanisms of detrusor smooth muscle relaxation

The mechanisms by which sympathetic stimulation mediates smooth muscle relaxation are closely linked to the pathways through which contractile force is generated. A thorough discussion of smooth muscle relaxation is beyond the scope of this introduction but the proposed interaction between contractile and relaxant pathways are highlighted in Figure 1.3. For extensive reviews of this area, see Kotlikoff & Kamm, 1996; Carvajal et al., 2000; Woodrum & Brophy, 2001.

1.3.3 Pre-junctional mACh receptors

In the urinary bladder, pre-junctional mACh receptors are located on both parasympathetic and sympathetic nerve endings. The bladder is somewhat unique in that it contains both inhibitory and facilitatory pre-junctional receptors, while other tissues such as heart, ileum and trachea express only inhibitory receptors on pre-junctional nerve endings (Somogyi & De Groat, 1999). Facilitatory muscarinic receptors have been identified as being of the M₁ subtype in a variety of species including rat (Somogyi et al., 1994) and rabbit (Tobin & Sjogren, 1995). Pre-junctional inhibitory receptors appear to be species-dependent, being identified as M₂ receptors in rat (Braverman et al., 1998) and rabbit (Tobin & Sjogren, 1995) and M₄ receptors in guinea-pig (Alberts, 1995), mouse (Zhou et al., 2002) and human (D'Agostino et al., 2000).

The relative influence of these two seemingly antagonistic systems appears to depend upon the frequency, pattern and duration of neuronal stimulation (Somogyi & De Groat, 1999). Intermittent field stimulation, consisting of short trains separated by 5 second intervals, failed to activate facilitatory mechanisms in rat bladder, while continuous stimulation caused a significant increase in ACh release (Somogyi & De Groat, 1999). This facilitation was sensitive to atropine and pirenzepine and the lower range of the continuous firing protocols used mimics physiological firing rates observed in bladder (Somogyi & De Groat, 1999). It would therefore seem that facilitatory pre-junctional M₁ receptors predominate during neuronal stimulation of bladder contraction, augmenting ACh release and thereby acting to potentiate bladder contraction during micturition. In contrast, while the bladder is relaxed during the
Figure 1.4 The interactions between M₂ and M₃ mACh receptors and ion channels in the regulation of smooth muscle contraction. A simplified proposed sequence of ionic conductances upon muscarinic receptor activation is indicated by the numbers 1 to 4.
filling phase and parasympathetic drive is low, inhibitory pre-junctional M₂/M₄ receptors are likely to predominate, acting to further dampen the neuronal input to the detrusor smooth muscle (Somogyi et al., 1994).

Direct PKC activation by phorbol esters leads to a facilitation of ACh release from nerves serving the rat bladder (Somogyi & De Groat, 1999). Also, PKC inhibition attenuated continuous stimulation-facilitated ACh release, but had no effect on non-facilitated release, implicating PKC in mediating the M₁ receptor-stimulated ACh release (Somogyi & De Groat, 1999). Somogyi et al. (1994) demonstrated that facilitated ACh release in the bladder was dependent upon the extracellular Ca²⁺ concentration. It is known that under non-facilitatory conditions, N- and to a lesser extent P/Q-type Ca²⁺ channels mediate a Ca²⁺ influx that contributes to transmitter release from the nerve terminals of the urinary bladder (Waterman, 1996). When facilitation is induced by continuous stimulation, a nifedipine-sensitive Ca²⁺ entry occurs, which is essential for mediating facilitated ACh release (Somogyi et al., 1997). This has led to the proposal that M₁ receptors mediate facilitated ACh release via activation of PKC, phosphorylation and activation of L-type VOCCs and subsequent Ca²⁺ influx, which facilitates neurotransmitter release (Somogyi & De Groat, 1999).

1.3.4 The role of the M₂ mACh receptor in bladder smooth muscle contraction

1.3.4.1 Pharmacological evidence

Despite its predominant expression, the physiological role of the M₂ muscarinic receptor in a variety of smooth muscle types (Eglen et al., 1996a) remains enigmatic. As described earlier, M₃ receptor-mediated activation of PLC and subsequent Ca²⁺ release from intracellular stores has been strongly implicated in mediating smooth muscle contraction and the muscarinic receptor-mediated inositol phosphate accumulation and contractile responses in most smooth muscle cell types have been demonstrated to be largely insensitive to pertussis toxin (PTx) treatment (e.g. Thomas & Ehlert, 1994). However, Candell et al. (1990) first proposed that activation of the M₂ receptor population of smooth muscle might act to oppose the relaxant effects of cAMP. Ehlert and co-workers subsequently designed experiments to investigate this further in guinea-pig ileum (Thomas et al., 1993). They attempted to isolate the M₂ receptor population by selectively inactivating muscarinic M₃ receptors in ileal
smooth muscle cells with 4-DAMP mustard treatment. Following extensive washing, the tissue is exposed to a contractile agent such as histamine and a relaxant such as isoprenaline, which together produce no net contractile effect. It was found that under these conditions, muscarinic agonist (Oxo-M) treatment elicited a potent re-contraction that was antagonised by AF-DX 116 in a manner consistent with an M₂-mediated response (Thomas et al., 1993). In agreement with this, it was found that the muscarinic agonist-mediated re-contraction was sensitive to PTx, indicating that inactivation of the M₃ receptor population uncovers an M₂/Gᵢₒ-mediated inhibition of cyclic AMP-dependent relaxation (Ehlert & Thomas, 1995). It has also been demonstrated that even without prior inactivation of the M₃ receptor population, in the presence of relaxants such as isoprenaline or forskolin, contraction of the guinea-pig ileum to Oxo-M is moderately sensitive to PTx (Thomas & Ehlert, 1994), as are contractions in response to P2X receptor-stimulated release of endogenous ACh in the same tissue (Sawyer et al., 2000).

Use of these techniques has identified similar M₂-mediated re-contractions in guinea-pig colon (Sawyer & Ehlert, 1998, 1999), bovine and guinea-pig trachea (Ostrom & Ehlert, 1998, 1999), rat oesophagus (Eglen et al., 1996b) and rat bladder (Hegde et al., 1997), in addition to the original work in guinea-pig ileum. However, subtle differences, perhaps indicative of tissue- or species-dependent variation, have been observed in some cases. For instance, although the re-contraction response to Oxo-M following 4-DAMP mustard treatment in the guinea-pig colon was sensitive to PTx treatment, the M₂-selective antagonist AF-DX 116 only weakly antagonised the residual contraction, yielding a functional Kᵦ estimate intermediate between those expected at M₂ and M₃ receptors, respectively (Sawyer & Ehlert, 1998). This suggested an interaction between M₂ receptors and the residual M₃ population, which were still able to elicit a phosphoinositide response despite approx. 96 % being inactivated by alkylation (Sawyer & Ehlert, 1998). The authors proposed that a model in which the M₃ receptor signals through two parallel pathways could explain the apparent discrepancies in their data. The first pathway (A) is a simple M₃-mediated contraction, while the second component (B) involves the coincident activation of two "silent" pathways (one through the M₂ receptor along with an alternative M₃ pathway), together generating a contraction (Sawyer & Ehlert, 1999). In this model, the M₂ receptor is unable to elicit contraction by itself (consistent with the lack of
effect of PTx and the M₂-like pharmacological profile of contraction in the absence of relaxing agents) and contraction through pathway B is conditional upon M₃ receptor activation. Pathway A will normally predominate, perhaps because of the large receptor reserve for direct contraction to potent muscarinic agonists like Oxo-M, mediating a maximal contraction at concentrations too low to evoke a response through component B. However, following inactivation of the majority of M₃ receptors, the sensitivity of pathway A may be sufficiently reduced such that contraction now occurs primarily via an M₂-dependent, yet M₂-potentiated (and therefore PTx-sensitive) mechanism (i.e. pathway B). According to the mathematical model employed in the authors' analysis, competitive antagonism of contraction will resemble that predicted for the less sensitive receptor mechanism (now pathway A). The "M₂-like" profile found for AF-DX 116 may therefore be resolved with an M₂-dependent (i.e. PTx-sensitive) mechanism of contraction. While this model appears to fit the authors' data adequately, it still leaves the exact nature of the M₂/M₃ interaction somewhat unclear.

An M₂-mediated re-contraction has also been observed in rat urinary bladder smooth muscle (Hegde et al., 1997). Selective inactivation of the M₃ receptor population with 4-DAMP mustard was again employed to uncover a muscarinic agonist (in this case (+)-cis-dioxolane)-mediated re-contraction in the presence of KCl (pre-contraction) and the β-adrenoceptor agonist isoprenaline (relaxant). Coupled with the lack of effect of (+)-cis-dioxolane in tissues relaxed with the potassium channel activator pinacidil, these observations are consistent with a cAMP-dependent reversal of isoprenaline-mediated relaxation (Hegde et al., 1997). However, pharmacological analysis of the muscarinic receptor involved in this response proved inconclusive as both the M₂-selective compound methoctramine and the M₃-selective darifenacin antagonized the re-contracitive responses with affinities intermediate between those expected for these ligands at M₂ and M₃ receptors, respectively (Hegde et al., 1997). This is reminiscent of the findings of Sawyer and Ehler (1998), using AF-DX 116 in the guinea pig colon, and could be interpreted as evidence of a contractile mechanism dependent upon both M₂ and M₃ receptor activation.

Hegde and co-workers also examined volume-induced bladder contractions (VIBC) in vivo (Hegde et al., 1997). These represent physiological responses to bladder wall distension, in this case induced by rapid filling with saline. Potency estimates for a
range of antagonists tested at inhibiting this response were found to correspond best with binding affinity estimates at the human recombinant muscarinic M₂ receptor (Hegde et al., 1997). Surprisingly, the weakest correlation was seen with the M₃ receptor, raising questions regarding the physiological relevance of M₃ mediated contractions in the bladder. However, it should be noted that a correlation of in vivo functional Kᵦ estimates with radioligand binding affinity estimates derived at human receptors recombinantly expressed in CHO cells may not provide the most accurate of comparisons (Gillberg et al., 1998). Also, the M₃-selective agent darifenacin was moderately potent at inhibiting VIBC at doses that did not influence heart rate, indicating at least partial involvement of the M₃ receptor (Hegde et al., 1997). Pretreatment with the β-adrenoceptor antagonist propranolol significantly decreased the potency of methoctramine, but not darifenacin, indicating the involvement of two independent muscarinic receptor-driven pathways in the generation of VIBC. Overall, it is not possible to explain the data in this study without invoking a role for the M₂ receptor, providing an important in vivo correlate of the in vitro work supporting the involvement of the M₂ receptor in smooth muscle contraction.

1.3.4.2 Evidence from mouse ‘knockout’ models

The study of mice lacking the genes for individual subtypes of mACh receptors (so-called ‘knockout’ mice) has provided further insight into the roles of these subtypes in smooth muscle contraction as well as numerous other cellular processes (Wess, 2004). Their availability has allowed the study of the effects of stimulating the M₂ and M₃ receptor subtypes in isolation in various smooth muscle types, something not previously possible with the pharmacological tools available. Muscarinic M₃ receptor knockout mice display severely distended bladders in the male population, but interestingly a less acute distension in the female population (Matsui et al., 2000). Nonetheless, both sexes displayed maximal contractile responses to carbachol of only 5% of wild-type (Matsui et al., 2000), highlighting the major contribution of the M₃ subtype to micturition. Pharmacological characterisation of the residual carbachol-mediated contraction in mice lacking the gene for the M₂ receptor, strongly suggests that it is mediated via the M₂ population (Matsui et al., 2000), based upon an increased Kᵦ value for methoctramine and a low Kᵦ for pirenzepine.

This M₂-mediated contraction, albeit a modest one, appears to contradict the model proposed by Ehlert, in which the M₂ receptor is only able to elicit contraction in the
presence of $M_3$ receptor stimulation (see above; Ehlert, 2003). It is possible that the absence of $M_3$ receptor expression has induced a compensatory increase in the capability of the $M_2$ receptor to mediate contraction. Alternatively, the $M_2$ receptor may have a slightly more prominent role in smooth muscle contraction in the mouse than in guinea-pig and rat, where the majority of pharmacological studies have previously focussed (e.g. Eglen et al., 1996b; Hegde et al., 1997; Sawyer & Ehlert, 1998, 1999). Evidence from $M_3$ knockout mice suggests that even within the same species, striking differences in the relative contractile roles of $M_2$ and $M_3$ receptors can be observed between different smooth muscle types (Stengel et al., 2002). It was found that although carbachol-mediated contraction of urinary bladder smooth muscle from $M_3$ knockout mice was reduced to less than 10% of wild-type, smooth muscle from trachea and stomach fundus of knockout mice demonstrated approx. 50% of wild-type contractions to carbachol (Stengel et al., 2002). This would suggest that, at least in mice, the $M_2$ receptor plays a more critical role in mediating cholinergic contractions in trachea and stomach fundus than in urinary bladder.

A similar study using mice lacking the $M_2$ receptor gene demonstrated that a two-fold higher concentration of carbachol was required to initiate contraction of urinary bladder, trachea and stomach fundus smooth muscle of knockout mice, compared to their wild-type littermates (Stengel et al., 2000). However, although less potent, carbachol was still capable of eliciting maximal contractile responses in smooth muscle lacking functional $M_2$ receptors, suggesting that $M_2$ receptor activation may act to increase the sensitivity or potency of the $M_3$-mediated response to carbachol (Stengel et al., 2000), in line with the proposed $M_2$ role in gastrointestinal smooth muscle suggested by Ehlert. Clearly the tissue-dependent differences in $M_2$-mediated contraction observed in $M_3$ knockout mice (Stengel et al., 2002) are not seen in mice lacking the $M_2$ receptor gene, perhaps because the significant contraction observed in some smooth muscle types of $M_3$ knockout mice is actually due to a compensatory up-regulation of $M_2$ receptor-mediated contraction.

In mice lacking both $M_2$ and $M_3$ mACh receptor genes, severe urinary bladder distension was observed only in males, in agreement with the phenotype observed in $M_3$ knockout mice (Stengel et al., 2000; Matsui et al., 2002). Cholinergic contractions in these ‘double-knockout’ mice were completely abolished, supporting the notion that ACh-mediated contraction of detrusor smooth muscle is mediated by a
combination of $M_2$ and $M_3$ mACh receptor subtypes (Matsui et al., 2002). An interesting observation in mice lacking both $M_2$ and $M_3$ receptor genes is that despite a complete lack of cholinergic contraction, bladder and intestines were mostly healthy (with the exception of the bladder distension observed in males). It therefore seems that a certain degree of functional redundancy exists in ileum (as reported previously; Furness, 2000) and bladder with alternative neurotransmitter pathways (most likely non-adrenergic non-cholinergic (NANC) transmitters such as ATP; see Ralevic & Burnstock, 1998) able to compensate for a loss of cholinergic signalling. This may not be the case in other smooth muscle types (such as trachea and stomach fundus), perhaps explaining the greater reliance upon an enhanced $M_2$ receptor-mediated contraction in these tissues from mice lacking the $M_3$ receptor.

1.3.4.3 Evidence from mACh receptor-mediated desensitisation to contractile stimuli

Another technique utilised to investigate the roles of mACh receptor subtypes in smooth muscle contraction has been to examine heterologous desensitisation to high levels of muscarinic stimulation. This is based upon the assumption that the muscarinic receptors that mediate contraction will also mediate the desensitisation of the response to other contractile agents. Eglen et al. (1992) first reported that the $M_3$ selective antagonist $p$-FHHSiD was able to block carbachol-mediated desensitisation of guinea-pig ileum contraction. In contrast, $M_1$ and $M_2$ selective antagonists had no effect upon desensitisation, indicating a major role for the $M_3$ receptor in desensitisation and by inference, contraction (Eglen et al., 1992).

However, more recent work in the same tissue by Ehlert’s group indicates that both $M_2$ and $M_3$ mACh receptors are involved in mediating both heterologous and homologous desensitisation (Ehlert et al., 2001; Shehnaz et al., 2001). Pertussis toxin pre-treatment was able to significantly reduce ACh-mediated desensitisation of contraction to both histamine (Shehnaz et al., 2001) and oxotremorine-M (Ehlert et al., 2001). This might appear surprising, as PTx has no effect upon contraction (in the absence of relaxant agents), but the authors noted the higher agonist concentration used in desensitisation experiments (30 μM ACh), indicating that perhaps $G_i$-mediated contraction occurs in response to high agonist concentrations (Ehlert et al., 2001). However, this calls into question the physiological significance of the $M_2$ role in contraction/desensitisation as maximal contractile responses are achieved with
much lower agonist concentrations, via direct contraction downstream of \( M_3 \) receptor activation (Sawyer & Ehlert, 1999).

Shehnaz et al. (2001) also found that 4-DAMP mustard treatment significantly reduced ACh-mediated heterologous desensitisation, indicating that both \( M_2 \) and \( M_3 \) receptors may be involved in the desensitisation of histamine-induced contractions in guinea-pig ileum. It was also found that desensitisation had no effect on the phosphoinositide turnover in response to subsequent challenge with histamine or oxotremorine-M, suggesting that desensitisation occurs somewhere downstream of PLC activity (Ehlert et al., 2001; Shehnaz et al., 2001). A recent study by the same group utilised the availability of \( M_2 \) and \( M_3 \) knockout mice, finding that ACh-induced heterologous desensitisation was abolished in the ilea of mice lacking either \( M_2 \) or \( M_3 \) mACh receptors (Griffin et al., 2004). Pharmacological inhibition of desensitisation in wild-type guinea-pig ileum yielded a profile most consistent with an \( M_3 \) receptor-mediated response. Mathematical modelling of a response mediated via the coincident activation of two receptor subtypes indicated that the competitive antagonism of this response should display a pharmacological profile similar to that of the least sensitive pathway (Griffin et al., 2004). These data in guinea-pig ileum therefore support the evidence from knockout mice indicating that both \( M_2 \) and \( M_3 \) receptors contribute to ACh-induced desensitisation in ileal smooth muscle and suggest that the \( M_2 \)-linked pathway is less sensitive than that coupled to \( M_3 \) receptor activation. Overall, this would suggest that the \( M_2 \)-linked pathway might only be recruited at high levels of muscarinic stimulation (such as used to induce desensitisation) and that under physiological conditions, the \( M_2 \) receptor may have only a minor regulatory role in smooth muscle contraction.

1.3.4.4 Mechanisms of \( M_2 \) receptor-mediated smooth muscle contraction

1.3.4.4.1 Reversal of relaxant-induced cyclic AMP accumulation

A variety of intracellular pathways have been suggested to mediate the \( M_2 \) receptor-mediated contraction of smooth muscle. As discussed earlier, in the presence of agents that cause relaxation by increasing cytosolic cyclic AMP concentrations, a high potency reversal of this relaxation is mediated by the \( M_2 \) receptor. Further evidence for this mechanism was provided by the observation that in mice lacking the \( M_2 \) receptor gene, the relaxant effects of both forskolin and isoprenaline in ileum, trachea
and urinary bladder were greater than those observed in the same tissues from wild-type mice (Matsui et al., 2003). This suggests a significant physiological role for M₂ receptor-mediated reversal of β-adrenoceptor-induced cyclic AMP accumulation in the smooth muscle contractile responses to muscarinic agonist in these tissues.

While ATP is known to act via P₂ purinoceptors to cause a direct contraction of smooth muscle (Ralevic & Burnstock, 1998), adenosine, a purine nucleoside produced by the metabolism of ATP, causes detrusor relaxation via A₁ receptors (Nicholls et al., 1992). Accordingly, Giglio et al. (2001) observed a biphasic response to ATP in the rat bladder, characterized by a transient contraction, followed by a sustained relaxation that was sensitive to the adenosine receptor antagonist 8-para-sulfophenyltheophylline (Giglio et al., 2001). This suggests that the relaxatory phase is at least in part due to the breakdown of ATP to adenosine. The adenosine analog, 2-chloro-adenosine, alone had no significant effects on carbachol pre-contracted tissue, but in the presence of the M₂-selective antagonist methoctramine, caused substantial relaxations of the bladder strips (Giglio et al., 2001). This suggests that the M₂ receptor might be involved in the reversal of adenosine-mediated relaxation in detrusor, most likely by attenuating adenosine (A₂B receptor)-mediated elevation of intracellular cAMP (Giglio et al., 2001).

**1.3.4.1.2 Modulation of ion channels**

In addition to the attenuation of cAMP-induced relaxation, which may occur as a result of M₂ receptor activation, the M₂ muscarinic receptor is known to modulate various ion channels present in the plasma membranes of smooth muscle cells. Inoue & Isenberg (1990) first reported that muscarinic receptor stimulation in smooth muscle activated a non-selective cation current (I_{cat}), which they reported to be sensitive to pertussis toxin. Subsequent work also identified that the current was Ca²⁺-dependent (Lee et al., 1993). It has been proposed that muscarinic stimulation of this inward cation current in smooth muscle triggers the activation of L-type VOCCs, leading to depolarisation and subsequent contraction. Its modulation and activation may therefore be critical in regulating the contraction of many smooth muscle types, including detrusor, though few studies have characterised the I_{cat} current in the bladder.
Bolton & Zholos (1997) investigated the pharmacology of Icat activation and found that, while Mz-selective antagonists competitively antagonised it, a non-competitive reduction in the maximal current evoked by carbachol was seen with M3-selective antagonists, with affinities consistent with an M3-mediated action. The authors proposed that the current was gated by the Mz receptor, but also modulated by an M3 receptor-dependent mechanism (Bolton & Zholos, 1997). The role of the M3 receptor in the activation of Icat was confirmed by the use of antibodies directed to specific G protein α-subunits in a number of studies. Wang et al. (1997) found that antibodies directed against Gα and all 3 isoforms of Gi blocked muscarinic activation of Icat in equine tracheal smooth muscle cells. In contrast, studies in ileal (Yan et al., 2003) and gastric (Kim et al., 1998) smooth muscle have identified Gq activation as the primary pathway by which Icat is stimulated in response to muscarinic agonist. Yan et al. (2003) also demonstrated that βγ subunits were not involved in Icat activation. Similar experiments using antibodies directed to Gq/11 proteins provided less consistent results. Although Lee et al. (2003) found that Gq/11 antibodies blocked Icat generation in mouse gastric myocytes, they were without effect in equine trachea (Wang et al., 1997) and guinea-pig ileum (Yan et al., 2003).

Wang et al. (1997) highlighted the additional role of the M3 receptor in the activation of Icat, reporting that M3 receptor antagonism in equine trachea prevented Icat activation and depletion of intracellular Ca2+ stores had the same effect. Okamoto et al. (2002) found a correlation between the relative potencies of a range of muscarinic agonists in inducing Ca2+ release via M3 receptor activation and in activating Icat in guinea-pig ileum. This supports the notion that M3 receptor activation potentiates M2 receptor-stimulated gating of Icat, but since Icat was recorded under conditions where intracellular Ca2+ was clamped at 100 nM, the involvement of alterations in Ca2+ levels in mediating this potentiation would appear unlikely (Okamoto et al., 2002). Surprisingly, no correlation was found between the rank orders of potency of agonists in stimulating Icat activation and in inhibiting cAMP accumulation, despite both responses being considered to occur as a result of M2 receptor activation (Okamoto et al., 2002). Given the evidence that Gβ mediate the activation of Icat, the authors suggest that different G proteins mediate the two effects, with cyclic AMP inhibition largely occurring as a result of the activation of Gi isoforms.
Rhee et al. (2000) found that although PTx completely abolished I_{cat} generation in gastric antrum smooth muscle cells, 4-DAMP-mediated inhibition of the current elicited by a single concentration of carbachol was characteristic of a competitive inhibition of an M_{2}-mediated response. This differs from the work of Bolton & Zholos, who found that M_{3} receptor antagonism caused a non-competitive reduction in the maximal response with no shift in carbachol potency (Bolton & Zholos, 1997).

While the authors cite differences in intracellular Ca^{2+} buffering as the cause of these discrepancies, a consensus regarding the relative contributions of the M_{2} and M_{3} mACh receptors in I_{cat} generation remains elusive.

A recent study in ileal smooth muscle cells suggests that even when intracellular Ca^{2+} is strongly buffered, the PLC inhibitor U73122 is still capable of inhibiting muscarinic receptor-mediated activation of I_{cat} (Zholos et al., 2004). Both carbachol- and GTPγS-stimulated I_{cat} were similarly blocked by U73122 and also by a low concentration of ρ-FHHSID (Zholos et al., 2004). Overall, these data indicate that M_{3} receptor-mediated activation of PLC stimulates I_{cat} in a Ca^{2+}-independent manner. Zholos et al. (2004) also ruled out the involvement of the cyclic AMP pathway, Ca^{2+} store depletion, DAG and Ins(1,4,5)P_{3} production, phospholipase A_{2} and PC-PLC activation in the generation of I_{cat}. It is possible that depletion of phosphatidylinositol 4,5-bisphosphate (PIP_{2}) might be involved in activating I_{cat}, in a similar manner to the role of PIP_{2} in the activation of Ik_{ACh} (Huang et al., 1998a), but no direct evidence for this is available. Wang et al. (1999) reported that stimulation of the M_{2} receptor in equine tracheal myocytes activates I_{cat} via PI3-Kγ and atypical PKC isoform(s).

Zholos et al. (2004) did not investigate this pathway, so it is tempting to speculate that this may be the mechanism by which the M_{2} receptor activates I_{cat}.

Bladder smooth muscle of both guinea-pig and human has been demonstrated to express iberiotoxin (IbTX)-sensitive Ca^{2+}-dependent K^{+} channels (BK_{Ca}) and activation of these channels causes relaxation of bladder smooth muscle (Trivedi et al., 1995). Heppner et al. (1997) demonstrated that IbTX treatment increased the electrical excitability of the guinea-pig bladder (prolonging action potential duration and increasing the rate of action potential firing). Muscarinic receptor stimulation in a variety of other smooth muscle types has been shown to activate BK_{Ca} channels (e.g. Wade & Sims, 1993; Carl et al., 1995), most likely mediated by M_{3} receptor-stimulated Ca^{2+} release. In this way, BK_{Ca} channel opening (and subsequent outflow
of K⁺ acts as an inhibitory feedback mechanism to counteract the depolarising effects of Ca²⁺-mobilising receptor activation.

Shishido et al. (1996) found that M₂ receptor blockade had an inhibitory effect upon carbachol-induced contraction of the rat bladder. Moreover, when Ca²⁺-activated K⁺ channels were also inhibited (by charybdotoxin) under conditions of M₂ receptor antagonism, carbachol responses were restored to control levels (Shishido et al., 1996). Subsequent work by the same group demonstrated that muscarinic receptor activation in rat bladder smooth muscle induced a transient activation of BKCa (measured as an IbTX-sensitive outward current), which decayed back to control levels upon prolonged (> 10 s) application of carbachol (Nakamura et al., 2002). Pertussis toxin treatment or antagonism of the M₂ mACh receptor led to a persistent outward current upon carbachol stimulation, indicating that the M₂ receptor inhibits BKCa via a Gipor-dependent pathway (Nakamura et al., 2002). Similar PTx-sensitive muscarinic receptor-mediated inhibition of BKCa has been reported in other smooth muscle types, including trachea (Kotlikoff et al., 1992) and intestine (Cole & Sanders, 1989).

Figure 1.4 illustrates the proposed sequence of ionic conductances developing upon muscarinic receptor activation and their role in inducing smooth muscle tone. M₃ receptor mediated Ca²⁺ release from intracellular stores activates BKCa, which will act to hyperpolarise the cell through the outward flow of K⁺ ions. However, inhibition of the channel via an M₂ receptor-dependent pathway will counteract the Ca²⁺-dependent activation of BKCa, perhaps acting to amplify/prolong the contractile response to muscarinic agonist. BKCa channels have been investigated as potential therapeutic targets in the treatment of lower urinary tract disorders (Imai et al., 2001) and their importance in the control of micturition is underlined by studies using mice lacking different subunits of the BKCa channel (Petkov et al., 2001; Meredith et al., 2004). The latter study found that mice lacking the gene for the pore-forming subunit of BKCa exhibited enhanced spontaneous and nerve-evoked bladder contractions, leading to increased urinary frequency (Meredith et al., 2004).

There is some evidence that the M₂ receptor may also exert a direct influence on the influx of Ca²⁺ through L-type VOCCs in smooth muscle cells. For instance, Jin et al. (2002) reported that the M₂ receptor in rabbit colonic smooth muscle cells was able to activate the L-type VOCC via G₁ and c-Src. In agreement with this, Callaghan et al.
(2004) found that ACh enhanced Ca^{2+} current in rabbit portal vein myocytes through activation of the M_{2} receptor, Goβ_{Y}, PI3-K, a novel PKC isoform and c-Src. This is a similar pathway to that reported by Wang et al. (1999) for the activation of I_{cat} in equine trachea. However, the physiological relevance of this pathway in smooth muscle contraction is not clear.

1.3.4.4.3 M_{2}/M_{3} receptor cross-talk in smooth muscle contraction

The pathways through which both the M_{2} and M_{3} mACh receptors may influence smooth muscle contraction are varied and by no means independent. Accordingly, there are numerous examples in the literature of cross-talk between signal transduction pathways initiated by such G_{0/12} and G_{q/11}-linked receptors (for review, see Selbie & Hill, 1998; Werry et al., 2003). Although the M_{2} receptor is capable of weakly stimulating PLC activity, this is only generally observed when the receptor is expressed at high levels in recombinant cell systems (Peralta et al., 1988; Offermanns et al., 1994) and in general, activation of Go-coupled GPCRs in vivo is insufficient to stimulate PLC activity (Selbie & Hill, 1998). In contrast, the interplay between G_{0/12} and G_{q/11}-linked pathways is often characterised by a Go/(mediated potentiation of G_{q/11}-stimulated PLC activity, increases in intracellular Ca^{2+} levels, PKC activity or arachidonic acid production (Selbie & Hill, 1998). It is widely thought that this cross-talk is mediated via Go/(derived βγ subunits (Zhu & Birnbaumer, 1996; Quitterer & Lohse, 1999), which are capable of stimulating PLC-β activity (Katz et al., 1992; Blank et al., 1992). Chan et al. (2000) highlighted the potential for synergy between G_{0/12} and G_{q/11} stimulation in the activation of PLC-β activity, demonstrating that δ- and κ-opioid receptors, expressed in COS-7 cells, could only activate PLC-β when co-expressed with G_{q16}. The partial sensitivity of this response to PTx and to βγ scavenging illustrated that enhancement of G_{q/11}-dependent signals by G_{0/12}-linked receptors requires PLC-β activation and occurs via the βγ subunit (Chan et al., 2000).

It has also been reported that short-term agonist pre-treatment of HEK 293 cells stably expressing either M_{2} or M_{3} mACh receptors can induce a long-lasting heterologous potentiation of receptor-stimulated inositol phosphate formation (Schmidt et al., 1996, 1998). In each case the potentiation is fully PTx-sensitive but is also dependent upon PKC activity (Schmidt et al., 1996, 1998), indicating that both G_{0/12} and PKC are required for potentiation to occur. In CHO cells co-expressing recombinant human M_{2}
and M₂ receptors, PTx treatment was found to decrease the potency with which methacholine stimulated Ins(1,4,5)P₃ formation by approx. 3 fold (Homigold et al., 2003).

This suggests that at least in a recombinant cell system, M₂ receptor activation is capable of potentiating M₃ receptor-mediated signalling through PLC. There are also examples of the effects of Gαi/Gq/11 cross-talk in modulating native cellular processes, including smooth muscle contraction (e.g. Camps et al., 1992). It is therefore possible that co-stimulation of M₂ receptors (as occurs when ACh stimulates smooth muscle contraction) may potentiate the M₃ receptor-mediated inositol phosphate signal and subsequent Ca²⁺ release and muscle contraction. However, as discussed earlier, inositol phosphate and contractile responses in gastrointestinal smooth muscle are generally insensitive to PTx in the absence of prevailing relaxant activity (Thomas & Ehlert, 1994), so the potential for the M₂ receptor to augment M₃ receptor-mediated PLC activity in vivo remains to be proven.

M₂ and M₃ receptor-activated pathways may also converge at points downstream of PLC activation. For instance, the M₂ receptor has been shown to couple both positively (White et al., 2003) and negatively (Higashida et al., 1997) to cyclic ADP-ribose (cADPR) activity in porcine airway smooth muscle and NG108-15 neuroblastoma cells respectively. cADPR was originally identified by its ability to mobilise Ca²⁺ from intracellular stores in sea urchin eggs (Lee et al., 1989). Galione et al. (1991) showed that cADPR releases Ca²⁺ from a ryanodine-sensitive pool and it has since been demonstrated to mediate agonist-induced Ca²⁺ signalling in a variety of tissues, including smooth muscle (e.g. Kuenmerle & Makhlouf, 1995; Prakash et al., 1998). It is therefore possible that M₂ receptor activation may play a general role in augmenting cholinergic-mediated intracellular calcium signalling in smooth muscle, via release from ryanodine-sensitive stores.

1.3.5 Pathological alterations in cholinergic signalling in detrusor smooth muscle

Much of the work relating to the muscarinic regulation of smooth muscle contraction has been performed in apparently healthy tissue. However, where therapeutic intervention might be required to normalise smooth muscle activity (such as in disorders of the lower urinary tract), signal transduction pathways within the tissue
may be altered/compromised. For this reason, some researchers have attempted to generate animal models of lower urinary tract disorders (Braverman et al., 1999).

For instance, bilateral ablation of the major pelvic ganglion of the rat (denervation) results in a rat unable to void (Braverman et al., 1999). The bladders develop hypertrophy and are 'supersensitive' to exogenously applied carbachol (Braverman et al., 1998). Denervation is also associated with a 50 % increase in total mACh receptor expression in detrusor, which is attributable to a 60 % increase in M2 receptor expression, with no change in M3 receptor expression (Braverman et al., 1998). This is in general agreement with an earlier study that reported a 37 % increase in [3H]-QNB binding sites in denervated rat bladder relative to control (Gunasena et al., 1995). However, Nilvebrant et al. (1986) found that although total mACh receptor density decreased upon urinary diversion, bladders were still supersensitive to muscarinic agonist. These data would suggest that although mACh receptor expression may be regulated by bladder function or nervous input, the supersensitivity to cholinergic stimulation is not solely due to increased receptor density. Braverman and co-workers suggest that in the denervated bladder, the absence of nervous input removes the tonic desensitisation of mACh receptor signalling, causing the appearance of an enhanced sensitivity to muscarinic agonist (Braverman et al., 1998).

Denervation was also shown to alter the muscarinic receptor subtype profile of carbachol-induced bladder contraction (Braverman et al., 2002). The suitability of denervation as a model for detrusor instability is supported by the observation that a reduced density of innervation is often associated with bladder dysfunction (e.g. Mills et al., 2000). Consistent with numerous previous studies, Braverman et al. (2002) found that in normal bladders, the pharmacological profile of contraction was consistent with an M3-mediated response. However, denervated bladder contractions were antagonised with low affinity by both M3 and M2 selective ligands, indicating that two or more pathways may be interacting to elicit contraction (Braverman et al., 2002). Accordingly, synergistic effects were seen with a combination of methoctramine and p-FHHSiD in the inhibition of carbachol-induced contraction in denervated, but not normal bladders (Braverman et al., 2002). Interestingly, pretreatment of normal bladders with thapsigargin to deplete intracellular Ca2+ stores mimicked the effect of denervation (Braverman et al., 2002). The authors suggest that the M3-dependent contraction that normally predominates, acts through the release of
intracellular Ca^{2+} and that compromising this pathway uncovers a less sensitive M_2-dependent contraction. This is clearly reminiscent of the model proposed by Sawyer & Ehlerlert to explain their observations in 4-DAMP mustard-treated colonic smooth muscle and may indicate that rather than denervation inducing an alteration in the mACh receptor coupling to contraction, it may be simply uncovering a pre-existing, redundant M_2-dependent pathway.

Following their observations in denervated rat bladder, Braverman's group investigated the bladders of seven humans who had suffered spinal cord injuries and displayed neurogenic bladder dysfunction, compared with a control group of bladders from organ donors (Pontari et al., 2004). Pharmacological analysis of the mACh receptor subtypes mediating contraction provided variable and conflicting results in both groups. Experiments using the more selective agents methoctramine and darifenacin indicated that the organ donor bladders contracted via an M_3-dependent mechanism (Pontari et al., 2004). Data from the spinal cord injured group indicated an M_2-like profile in 4/7 cases, but either M_3 or intermediate between M_2/M_3 affinities were derived in the other 3 bladders. Although this suggests a shift towards an M_2 receptor-mediated contraction in neurogenically dysfunctional bladders, in agreement with the earlier work in rat (e.g. Braverman et al., 2002), the variability in the data and the relatively small number of samples used necessitates caution in interpreting the results. It should also be noted that all of the patients suffering bladder dysfunction had been taking some form of anti-muscarinic medication for varying periods of time prior to their death (Pontari et al., 2004). The long-term effects of such muscarinic antagonists upon mACh receptor expression/coupling are not apparent and it would have been interesting to compare the relative expression levels of the mACh receptor subtypes in these patients, as this may have been a factor in the apparent inter-individual variability of contractile profiles.

Bladder outlet obstruction is a common condition in older men and is most frequently observed as a result of benign prostatic hyperplasia (BPH). Secondary damage to the bladder can occur, including hypertrophy and reduced innervation, leading to secondary symptoms of unstable bladder activity, such as frequency, urgency and urge incontinence. Bladder outflow obstruction in a variety of animal species leads to similar characteristic symptoms, so this model is commonly employed to study bladder instability. Krichevsky et al. (1999), using this model in rat, showed that the
pharmacological profile of bladder contraction to carbachol was still consistent with an M₃-mediated response. No alteration in sensitivity to carbachol was observed either, although potassium-induced contractions were reduced, suggesting that the detrusor was less susceptible to depolarisation (Krichevsky et al., 1999).

It is well established that lower mammals exhibit a significant purinergic component of neuronally-mediated bladder contraction, while this is generally found to account for less than 5% of contraction in healthy human bladder (De Groat & Yoshimura, 2001). However, it has been reported that the purinergic component increases in animal models of detrusor instability (Calvert et al., 2001) and in cases of idiopathic instability and instability secondary to bladder outlet obstruction in humans (Bayliss et al., 1999). Fry et al. (2002) reported that ATP was significantly more potent in inducing contraction of detrusor strips taken from unstable bladders compared with stable controls. Moreover, the non-hydrolysable ATP analog α,β-methylene ATP displayed an even greater potency in eliciting contraction, suggesting a deficit in the ecto-ATPase activity in unstable bladders may underlie the greater sensitivity to ATP (Fry et al., 2002). Accordingly, it was found that endogenous ATPase activity in unstable bladders was only approx. 50% of that observed in stable bladders (Fry et al., 2002). Alterations in alternative signalling pathways such as this may therefore influence the relative importance of muscarinic receptor subtypes in eliciting contraction. In light of these apparent alterations in ATPase activity, it would also be interesting to know if the acetylcholine-esterase (AChE) activity is altered in disease states.

Alterations in the expression/function of proteins involved in the signalling cascades initiated by mACh receptor activation in smooth muscle might also have implications for the relative involvement of receptor subtypes in initiating contraction. For instance, it has been reported that Gαo expression levels increase with age in the rat urinary bladder (Derweesh et al., 2000). Previous studies have shown that detrusor relaxation in response to forskolin and isoprenaline decreases in older rats (Wheeler et al., 1990). Derweesh et al. (2000) showed that PTx and PKA inhibitors were able to reverse the age-dependent decrease in isoprenaline-mediated cyclic AMP accumulation, suggesting that increased Gαo levels may enhance the inhibitory coupling of β-adrenoceptors to adenylate cyclase. The selective increase in Gα₁γ expression in older rat left ventricle produced a significant increase in the potency of
mACh receptor-mediated inhibition of β-adrenoceptor-stimulated cyclic AMP accumulation (Kilts et al., 2002), so it might be expected that the enhanced Goαi expression in older rats will be associated with an increase in M2 receptor-mediated reversal of relaxation. Age-dependent changes, such as these, highlight the importance of the age of animal models, particularly in the study of age-related disorders, such as those affecting the lower urinary tract.

The potential role of cyclic ADP ribose (cADPR) in muscarinic agonist (in particular M2 receptor)-mediated increases in intracellular Ca²⁺ in smooth muscle has been discussed earlier. It is interesting to note that in a model of airway smooth muscle hyper-responsiveness, the sensitivity of ACh-mediated calcium signalling to the cADPR antagonist 8-bromo-cADPR was significantly increased, indicating that in this model disease state, a shift in Ca²⁺ mobilising pathway is observed (Deshpande et al., 2003). Although the muscarinic receptor subtype mediating the enhanced cADPR signalling was not investigated in this study, it is possible that the involvement of the M2 subtype in contraction (via cADPR) may be increased in airway smooth muscle hyper-responsiveness. Whether similar changes may occur in other hyper-responsive smooth muscle types (such as in lower urinary tract disorders) remains to be investigated.

Finally, it is not only the mACh receptors of the detrusor smooth muscle that may be altered by pathologies of the lower urinary tract. Somogyi and co-workers have identified that the muscarinic facilitation of ACh release from parasympathetic nerve terminals of the urinary bladder in spinal cord transected (SCT) rats, mediated by pre-junctional mACh receptors, exhibits a leftward shift in the frequency-response curve, such that facilitation occurs at lower frequencies of stimulation (Somogyi et al., 1998). Later work by the same group identified that whereas in normal rats, pirenzepine inhibits the facilitation of ACh release at concentrations selective for M1 mACh receptors, the same response in SCT rats is blocked by low concentrations of 4-DAMP, but not by pirenzepine (Somogyi et al., 2003), suggesting an alteration in the mACh receptor subtype mediating facilitation from M1 (in normal rats) to M3 in SCT rat bladder. The functional significance of this change is not clear, but it highlights another example of the plasticity of the mACh receptor population in the bladder.
1.3.6 Pharmacological management of overactive bladder (OAB)

The term 'overactive bladder' has been defined as “a medical condition referring to
the symptoms of frequency and urgency, with or without urge incontinence, when
appearing in the absence of local pathologic or metabolic factors that would account
for these symptoms” (Abrams & Wein, 2000). The total number of OAB sufferers
worldwide has been estimated to be between 50 and 100 million. The prevalence is
likely to be underestimated as many studies have only considered patients with
incontinence, while it is thought that only approximately half of OAB patients
actually experience incontinence. In a recent survey of 6 European countries, it was
found that the overall prevalence of OAB was around 17% in men and women over
40 years of age (Milsom et al., 2001). It was also reported that incidence of OAB
consistently increased with advancing age (Milsom et al., 2001).

OAB does not identify any specific causes or pathologies and in fact encompasses
many previously described disorders of the lower urinary tract, including unstable
bladder, neurogenic bladder and neuropathic bladder (Mostwin, 2002). Damage to
CNS pathways involved in micturition by spinal cord injury was one of the first
causes of OAB to be identified. Damage to suprapontine inhibitory pathways in
Alzheimer’s disease or stroke have since been implicated in causing bladder
overactivity (Griffiths et al., 1994), while animal models of Parkinson’s disease have
demonstrated the potential role of compromised dopaminergic and glutamatergic
pathways in OAB (Mostwin, 2002).

OAB can also occur as a result of increased afferent activity, perhaps via sensitisation
ofafferent nerve fibres in the bladder (Mostwin, 2002). Bladder outlet obstruction,
secondary to prostatic enlargement, bladder neck dysfunction, uncoordinated
sphincter activity etc, have been shown to increase nerve growth factor production
and induce neuronal enlargement (Steers et al., 1988). Also, in experimental animal
models, bladder outlet obstruction is associated with OAB-like symptoms and
alterations in bladder smooth muscle consistent with denervation (Mostwin, 2002).
Brading (1997) suggested a myogenic basis for OAB, where perhaps an increase in
intercellular signalling between detrusor cells leads to a state of spontaneous over-
activity. However, it is likely that the bladder would still require the co-ordinated
nervous input from the CNS to initiate a voiding contraction and concomitant urethral
relaxation.
Although the aetiology remains uncertain and is likely to involve both myogenic and neurogenic components, mACh receptor antagonists inhibit contractile responses to ACh in overactive bladder, as well as reducing pressure during filling (Moreland et al., 2004). The efficacy of muscarinic antagonists in alleviating the symptoms of OAB is generally good, but their use is limited by their often severe side-effect profiles, which include blurred vision, constipation, drowsiness, tachycardia and dry mouth. The challenge in recent years has therefore been to identify agents that display greater bladder selectivity. However, it is clear from the complex nature of the muscarinic receptor-mediated control of micturition (as discussed above) and the widespread expression of mACh receptors, that subtype selectivity alone may not be sufficient to ensure adequate selectivity for bladder contractions. Present knowledge about the major current anti-muscarinic OAB therapies oxybutynin and tolterodine, together with the recently FDA-approved darifenacin, is reviewed in the following sections.

1.3.6.1 Oxybutynin

Oxybutynin is a potent, competitive mACh receptor antagonist that has been commonly prescribed for the treatment of OAB for over 20 years. It displays some degree of selectivity for $M_1$ and $M_3$ mACh receptors over other muscarinic subtypes (Noronha-Blob & Kachur, 1991) with up to 10-fold selectivity for $M_3$ versus $M_2$ receptor subtypes reported (Nilvebrant et al., 1997a). Oxybutynin has high affinity for the muscarinic receptor population of the human urinary bladder and potently antagonises carbachol-induced contractions (Nilvebrant et al., 1985; Waldeck et al., 1997). In addition to its anti-muscarinic activity, oxybutynin acts as a direct smooth muscle relaxant and local anaesthetic (e.g. Kachur et al., 1988). This is likely to be linked to its actions as a Ca$^{2+}$ channel blocker (Wada et al., 1995), though oxybutynin is 500 times weaker as a smooth muscle relaxant than as an antimuscarinic agent (Kachur et al., 1988). It is therefore generally considered that oxybutynin exerts its clinical effects through its action as a muscarinic antagonist, since therapeutically relevant plasma concentrations are within the nanomolar range (Chapple, 2000).

Clinically, oxybutynin undergoes extensive first-pass metabolism, producing several metabolites, the major one being $N$-desethyl oxybutynin, which has similar
pharmacological properties to that of the parent compound (Waldeck et al., 1997). Following oral administration, levels of this metabolite may be six-fold higher than oxybutynin and might therefore be responsible for producing adverse effects. Attempts to improve the tolerability of oxybutynin have therefore centred upon reducing its first-pass metabolism, including intravesical instillation and extended-release oral formulations (for review, see Chappie, 2000).

Although clinically effective, severe dry mouth (xerostomia) has been reported in at least 50 % of patients taking oxybutynin (Yarker et al., 1995). This is a major factor leading to up to 27 % of patients discontinuing treatment. It has been reported that as little as 18 % of patients taking immediate release oxybutynin remain on long-term therapy for 6 months or more (Kelleher et al., 1997). Although the adverse effect profile of oxybutynin has severely limited its tolerability, the once-daily, extended-release formulation shows encouraging signs of improvement. Gleason et al. (1999) reported an 83-90 % reduction in incontinence episodes in patients taking this drug relative to placebo and significantly reduced incidence of dry mouth has been reported with extended-release (25 %) versus immediate release (46 %) oxybutynin (Anderson et al., 1999).

1.3.6.2 Tolterodine

Tolterodine is a competitive non-subtype selective mACh receptor antagonist (Nilvebrant et al., 1997a). In functional studies in vitro tolterodine and oxybutynin are similarly potent in the inhibition of carbachol-induced contractions of both guinea-pig (Nilvebrant et al., 1997a) and human (Yono et al., 1999). Similar in vivo data in the anaesthetised cat demonstrated that both oxybutynin and tolterodine are capable of inhibiting ACh-induced bladder contractions with high affinity (Nilvebrant et al., 1997a). Unlike oxybutynin, tolterodine does not appear to block Ca\(^{2+}\) channels (Yono et al., 1999) and so it would appear to act as a 'pure' muscarinic antagonist.

The major active metabolite of tolterodine (PNU-200577 or DD01) exhibits a similar pharmacological profile to the parent compound (Nilvebrant et al., 1997b). It is therefore likely that this plays some role in the therapeutic and/or adverse effects observed upon tolterodine administration. Despite the apparent lack of subtype selectivity, moderate doses of tolterodine demonstrated no significant cardiovascular, CNS, respiratory or renal side-effects in animal models (Wefer et al., 2001). It was
only upon administration of higher doses that classical anti-muscarinic side-effects (e.g. dry mouth, tachycardia, reduced GI motility) were observed. Indeed, in clinical trials tolterodine was equally as efficacious as oxybutynin but displayed a superior side-effect profile (Abrams et al., 1998; Malone-Lee et al., 2001). Overall, adverse effects were less frequently observed in tolterodine-treated patients (69 % versus 81 %) and fewer patients treated with tolterodine (37 %) reported dry mouth than amongst the patients taking oxybutynin (61 %) (Malone-Lee et al., 2001).

1.3.6.3 Darifenacin
Darifenacin is an M₃ selective mACh receptor antagonist, which has recently received FDA approval for use in the treatment of OAB. Since the M₃ receptor subtype was identified as the major subtype responsible for mediating ACh-induced detrusor contractions (e.g. Chess-Williams et al., 2001), it was proposed that compounds selective for this subtype might be effective in relieving the symptoms of OAB, without the side-effects caused by antagonism at the other mACh receptor subtypes (Wallis & Napier, 1999). This led to the development of darifenacin, which displays sub-nanomolar affinity for the M₃ mACh receptor and significant selectivity for the M₃ versus M₂ subtypes. Wallis & Napier (1999) reported this selectivity to be approximately 60 fold, while Gillberg et al. (1998) found darifenacin to display 47 fold higher affinity for the M₃ subtype than for the M₂ receptor.

This selectivity has also been demonstrated in functional in vitro studies, where darifenacin potently inhibited muscarinic agonist-induced contractions of guinea-pig bladder (pA₂ = 8.66) with selectivity over M₂ receptor-mediated contractions of guinea-pig atria (pA₂ = 7.48) and M₁ receptor-mediated contractions of rabbit vas deferens (pA₂ = 7.90) (Wallis & Napier, 1999). In similar experiments, atropine, oxybutynin and tolterodine all failed to display significant selectivity for the M₃ receptor-mediated contraction of urinary bladder (Wallis & Napier, 1999). Darifenacin was also found to be extremely potent (pA₂ = 9.34) for the inhibition of carbachol-mediated contraction of human detrusor smooth muscle in in vitro experiments (Miyamae et al., 2003). In the same study, it was demonstrated that while oxybutynin significantly inhibited KCl- and CaCl₂-induced contractions, darifenacin was without effect on these contractions, indicating that unlike oxybutynin, darifenacin does not appear to block Ca²⁺ channels (Miyamae et al., 2003). It has also been reported that the major active metabolite of darifenacin is 9-fold less potent than
the parent compound and is unlikely to have a significant role in the therapeutic actions of darifenacin (Kerbusch et al., 2004).

However, in several *in vitro* pharmacological studies, darifenacin has displayed unsurmountable antagonism of agonist-induced functional responses. Darifenacin has unsurmountably antagonised muscarinic agonist-induced bladder contractions in rat (Hegde et al., 1997; Hirose et al., 2002; Schneider et al., 2004a), pig (Yamanishi et al., 2000), human (Fetscher et al., 2002; Schneider et al., 2004b) and also in rat submaxillary gland (Meloy et al., 2001). However, in other studies in rabbit (Choppin et al., 1998) and human (Miysaae et al., 2003) urinary bladder, darifenacin has behaved competitively and surmountably over the concentration range tested. Furthermore, Clarke et al. (2003b) recently demonstrated that darifenacin is a competitive, reversible antagonist at the human M₃ mACh receptor recombinantly expressed in CHO cells. The basis of the unsurmountable antagonism observed in certain studies remains to be resolved, but may relate to insufficient equilibration or other methodological considerations.

*In vivo* studies in the anaesthetised dog also support the M₃ selectivity of darifenacin. Darifenacin did not increase heart rate at concentrations sufficient to completely inhibit pelvic nerve-stimulated bladder contractions, while some effects were seen at higher doses of the non-selective tolterodine (Gupta et al., 2002). The *in vivo* M₃/M₁ selectivity of darifenacin was also confirmed in a separate study in anaesthetised dogs, where high doses of darifenacin failed to influence the M₁ receptor-mediated gastric acid secretion (Alabaster, 1997). The M₃ selective nature of darifenacin is supported by clinical data, which has found that the incidence of cardiac and CNS adverse effects experienced by patients taking darifenacin is not significantly greater than in placebo-treated patients (Haab et al., 2004). Darifenacin significantly improved bladder capacity, micturition frequency, frequency of urgency, severity of urgency and number of incontinence episodes and no patients withdrew as a result of dry mouth (Haab et al., 2004). However, in an earlier study using a small number of patients, a single dose (10 mg) of darifenacin significantly improved urodynamic parameters, but also caused significant impairment of salivary flow (Rosario et al., 1999). However, based upon the limited clinical data currently available, it would seem that darifenacin certainly displays a superior side-effect profile to oxybutynin and further investigation of its long-term tolerability in the clinic is eagerly awaited.
1.3.6.4 Tissue-dependent functional selectivity of muscarinic antagonists

Although it is clear that an \(M_3\) mACh receptor subtype-selective antagonist might exhibit a favourable side-effect profile in relation to \(M_1\) and \(M_2\) receptor-mediated effects in particular, it would be expected that such an agent would severely compromise other physiological effects mediated by the \(M_3\) receptor (such as salivary secretion and gastrointestinal motility). Indeed, the most commonly indicated side-effects (and often those which lead to patient discontinuation of treatment) in antimuscarinic therapy are \(M_3\) receptor-mediated. It was therefore of obvious therapeutic interest when certain muscarinic antagonists were observed to inhibit urinary bladder contractions at concentrations that did not influence salivary secretion. Comparison of the inhibition of carbachol-stimulated \(^{86}\text{Rb}\) efflux from submandibular salivary glands with the inhibition of ACh-mediated bladder contractions in guinea-pig indicated that darifenacin (6-fold) and tolterodine (2-fold) both displayed higher affinity for the inhibition of bladder contractions than for the inhibition of salivary gland responses (Newgreen & Naylor, 1996). In vivo, selectivity was even more pronounced, as darifenacin displayed a 10-fold selectivity for inhibition of pelvic nerve-stimulated bladder contractions versus salivary secretion in anaesthetized dog (Gupta et al., 2002). In the same study tolterodine was 4-5 fold selective but notably, atropine was non-selective (Gupta et al., 2002).

In rat, darifenacin was also found to dose-dependently inhibit micturition pressure and increase micturition volume and interval at doses lower than those required to inhibit salivary secretion (see Wallis & Napier, 1999). Further in vivo support for the functional bladder selectivity of tolterodine comes from experiments performed in the anaesthetized cat, where tolterodine was approx. 2.5-fold selective for the inhibition of ACh-induced bladder contractions versus electrically-evoked salivation (Nilvebrant et al., 1997a). Gillberg et al. (1998) also found that tolterodine and the \(M_2\)-selective antagonist AQ-RA 741 were 2.5-3.3 and 1.4-2.7 fold selective for bladder in anaesthetized cat. However, in the latter study, it was reported that darifenacin displayed slightly higher affinity for the inhibition of salivary responses than for inhibition of bladder contractions (Gillberg et al., 1998).

Although there is convincing evidence in some studies of a genuine tissue-dependent functional selectivity exhibited by certain muscarinic antagonists, in some cases the evidence is contradictory. This is perhaps best exemplified by oxybutynin, which
displayed approx. 4-fold bladder selectivity in the anaesthetized dog (Gupta et al., 2002), but was reported to exhibit the opposite profile in anaesthetized cat (approx. 2-fold selective for salivation; Nilvebrant et al., 1997a). In guinea-pig in vitro studies, Newgreen & Naylor (1996) reported that oxybutynin, like atropine, was non-selective between bladder and salivary glands. Ikeda et al. (2002) found that oxybutynin was essentially non-selective between Ca\(^{2+}\) responses in guinea-pig urinary bladder and mouse submandibular salivary gland. However, considerable inter-species variation has been reported for the functional affinities of muscarinic antagonists (Wust et al., 2002), so comparisons between tissues from different animals may not be valid. These differences may also explain the apparently conflicting evidence relating to the selectivity profiles of certain antagonists when investigated using different animal models.

However, Kobayashi et al. (2004) found that in the same species (Cynomolgus monkeys), oxybutynin, tolterodine and darifenacin were all slightly selective for the inhibition of Ca\(^{2+}\) mobilization in submandibular salivary glands relative to urinary bladder. In contrast, the same group reported that in rats, tolterodine, oxybutynin, darifenacin and atropine were all approximately 2-fold bladder selective when measuring carbachol-induced Ca\(^{2+}\) mobilization, but only tolterodine was selective for bladder when carbachol-mediated contraction was compared with salivary secretion (Ohtake et al., 2004). However, where these “selectivities” result from only marginal differences in functional affinity and where all of the compounds investigated display a degree of selectivity for one tissue preparation (e.g. Kobayashi et al., 2004), caution ought to be exercised in the interpretation of the data. It is possible that some intrinsic difference in the tissue preparation may result in a ‘frame-shifting’ of affinities between preparations, which could be incorrectly interpreted as a tissue-dependent pharmacological phenomenon. The studies where a reference compound, such as atropine, displays no selectivity between tissues (e.g. Newgreen & Naylor, 1996; Gupta et al., 2002) perhaps provide the most convincing evidence of tissue-specific pharmacology.

Several other muscarinic antagonists have been found to display differences in affinity between smooth muscle preparations. Dicyclomine, an early treatment for urinary urge incontinence, was found to be more active against muscarinic M\(_3\) receptors in rabbit and human urinary bladder than in ileum (Downie et al., 1977). The muscarinic
M₃-selective antagonist pFHHSiD exhibited significantly lower pA₂ values in tracheal smooth muscle than in ileal smooth muscle of the same species (Eglen et al., 1990), while the affinity of zanifenacin (another M₃-selective agent) at tracheal M₃ receptors was lower than at either ileal or oesophageal M₃ receptors (Watson et al., 1995). More recently, the M₃ selective antagonist solifenacin has been shown to selectively inhibit carbachol-induced Ca²⁺ mobilization in the bladder of both Cynomolgus monkeys (Kobayashi et al., 2004) and rat (Ohtake et al., 2004), relative to salivary gland cells of the same species.

Despite the preponderance of M₃ selective antagonists that also display functional tissue selectivity, it is not possible to reconcile such tissue-dependent selectivity with selectivity for a particular subtype, as both atropine and tolterodine are non subtype-selective (Nilvebrant et al., 1997a; Caulfield & Birdsall, 1998), yet display differing inter-tissue pharmacological profiles within the same species (e.g. Gupta et al., 2002). Further investigation is required to determine the nature of this selectivity and to examine the importance of factor, such as species and functional output in defining functional affinities of both subtype selective and non-selective mACh receptor antagonists in native tissues.

1.4 mACh receptor-mediated regulation of salivary secretion

The mucosal surfaces of the oral cavity contain many diverse salivary glands. However, the main focus of research into the control of secretion in mammals has been on the 3 types of ‘major’ salivary glands: sublingual, parotid and submandibular. These glands contain one or both of two types of secretory acinar cell types: serous and mucous cells. Serous cells are actively involved in the synthesis of proteinaceous material and predominate in serous glands such as the parotid gland (Pinkstaff, 1980). Mucous cells predominate in mucous glands such as the sublingual gland, where they synthesize and release large quantities of the high molecular weight glycoproteins, mucins, which act to protect mucosal epithelia. Other salivary glands, such as the submandibular glands, contain a mixed population of serous and mucous cells (Pinkstaff, 1980). While all salivary glands are under the control of the autonomic system (and hence receive both cholinergic and sympathetic innervation), the predominant innervation of mucous glands such as the sublingual gland is
parasympathetic (Baum, 1993). The cholinergic control of both fluid and mucin secretion is therefore of primary importance.

1.4.1 The mACh receptor subtype expression profiles of the major salivary glands

Numerous studies have attempted to characterise the muscarinic receptors expressed in the different major salivary glands by a variety of methods. Early radioligand binding studies in guinea pig parotid gland indicated the presence of a homogeneous population of muscarinic receptors, with binding affinities consistent with what we would now classify as M3 receptors (Nilvebrant & Sparf, 1983). The use of ‘subtype-specific’ antisera to immunoprecipitate muscarinic receptors from mouse parotid gland however suggested that 15% of the total mACh receptor population was M1, the remaining 85% being of the M3 subtype (Watson et al., 1996). Binding experiments with the subtype-selective antagonists methoctramine and pirenzepine supported this, but found that the ‘M1’ population had atypical characteristics, more closely resembling an M2 receptor (Watson et al., 1996). It is possible that this was due to contamination of the preparation by nervous tissue. In support of this, Dai et al. (1991) identified the presence of mRNA for the M3, but not the M2 receptor, in rat parotid gland by Northern blot analysis. They also used subtype-specific antisera to immunoprecipitate muscarinic receptors and found that 93% of precipitated receptors were of the M3 subtype (Dai et al., 1991).

Barras et al. (1999) examined the ability of a range of antagonists to inhibit inositol phosphate accumulation in rabbit parotid gland and found that affinity estimates most closely matched those expected at the M3 mACh receptor (Barras et al., 1999). Bockman and co-workers identified the presence of mRNA for M3, M4 and M5 receptor subtypes in a rat parotid gland-derived cell line, using RT-PCR (Bockman et al., 2001). However, both binding and functional assays using a range of selective antagonists, generated affinity estimates most closely correlating with those expected at the M3 receptor, indicating that the M4 and M5 subtypes were not expressed at significant levels and/or were not functional (Bockman et al., 2001). Tobin et al. (2002) also found that in vivo, pirenzepine displayed a low inhibitory potency for salivary secretion in rat parotid glands. Overwhelming evidence therefore points to a near-homogeneous population of M3 receptors mediating the cholinergic control of secretion in parotid salivary glands.
In the case of the sublingual gland, the majority of evidence points to a mixed population of muscarinic receptors. This might be expected as M₁ receptors are preferentially expressed on mucous secretory cells, while serous cells express M₃ receptors in abundance (Buckley & Burnstock, 1986). Since the sublingual gland comprises both of these cell types (Pinkstaff, 1980), it would seem likely that both M₁ and M₃ subtypes (at least) might be involved in the cholinergic regulation of secretion.

Early radioligand binding studies suggested a heterogeneous receptor population in the rat sublingual gland (Martos et al., 1987). Watson & Culp (1994) detected mRNA for both M₁ and M₃ receptors and binding studies on rat sublingual glands suggested a mixed population of M₁ and M₃ receptors (Watson & Culp, 1994). Immunoprecipitation of solubilized receptors with subtype-specific antisera also found significant proportions of both M₁ and M₃ receptors, in whole glands and in isolated acini, indicating that the M₁ component is not merely due to contamination by neuronal tissue (Watson & Culp, 1994). The same group later demonstrated that both M₁ and M₃ receptors were involved in the regulation of secretion from rat sublingual glands (Culp et al., 1996). M₃ receptors alone were insufficient to induce a maximal muscarinic response, as the M₁-selective antagonist m₁-toxin inhibited maximal secretion by only 40% (Culp et al., 1996). More recently, Tobin et al. (2002) reported that both 4-DAMP and pirenzepine had inhibitory effects on salivary secretion in rat sublingual gland, supporting the notion that this gland expresses both M₁ and M₃ mACh receptors.

The situation in the submandibular gland appears to be slightly more controversial and may be species-dependent. The radioligand binding work of Martos et al. (1987) found a homogeneous population of muscarinic M₃ receptors in the rat submandibular gland (Martos et al., 1987) and functional studies in the same tissue were consistent with an M₃-regulated phosphoinositide turnover (Laniyonu et al., 1990). More recent radioligand binding studies, utilizing compounds with a greater degree of selectivity for the M₃ subtype (e.g. zamifenacin, darifenacin), also suggest that binding affinities in the rat submandibular gland strongly correlate with those at human cloned M₃ receptors (Moriya et al., 1999). However, in in vivo experiments, Tobin et al. (2002) demonstrated that the M₃ receptor is largely responsible for salivary secretion in the rat, but that other muscarinic receptors may also be involved. Specifically, a role for
an M₁ receptor population was identified in mediating indirect effects upon protein secretion, via nitric oxide release (Tobin et al., 2002).

In rabbit submandibular gland, quantitative immunoprecipitation assays indicated that M₁ and M₃ receptors were expressed in approximately equal proportions, in addition to a small amount of M₂ receptor, believed to be of neuronal origin (Dorje et al., 1991). Also working in the rabbit submandibular gland, Tobin (1995) demonstrated that in vivo carbachol-induced salivary secretion was potently antagonized by both pirenzepine and 4-DAMP, implicating roles for both M₁ and M₃ receptor subtypes in this tissue response. However, it is interesting to note that pirenzepine was considerably less potent in vitro, where the regulation of potassium release was consistent with the predominant involvement of the M₂ receptor (Tobin, 1995).

Competition radioligand binding studies in mouse submandibular gland demonstrate a predominant M₃ receptor population (80-86%) and a minor M₁ population (14-20%) (Watson et al., 1996). This is supported by recent data utilising the availability of mice lacking the genes for the M₁ and/or M₃ mACh receptors (Nakamura et al., 2004). In mice lacking the M₃ receptor, carbachol-induced Ca²⁺ responses in submandibular gland acinar cells were reduced by approx. 85%, but were completely abolished in cells from mice lacking both M₁ and M₃ mACh receptors (Nakamura et al., 2004). In support of these in vitro observations, pilocarpine-induced salivation was found to be completely abolished in mice lacking both M₁ and M₃ mACh receptors, while the presence of either receptor subtype alone was sufficient to produce a lesser response to pilocarpine (Gautam et al., 2004). This suggests a major role for the M₃ receptor and a minor role for the M₁ receptor in mediating cholinergic signalling in the submandibular glands of the mouse, in striking agreement with the binding studies of Watson et al. (1996).

In vivo experiments performed in the same study demonstrated that the M₁ receptor-mediated salivation response in M₃ knockout mice was less sensitive to pilocarpine stimulation (i.e. a higher dose of pilocarpine was required to induce similar salivation) (Nakamura et al., 2004). The authors propose that the lower dose of pilocarpine more accurately reflects the physiological level of stimulation, indicating that M₃ receptor-mediated salivation might predominate under physiological conditions (Nakamura et al., 2004). Interestingly, it was noted from the in vitro Ca²⁺ signalling experiments that the M₁ responses were not observed in all M₃ knockout cells, suggesting a
heterogeneous expression of functional $M_1$ receptors, in contrast to the apparently ubiquitous $M_3$ receptor expression in wild-type mice (Nakamura et al., 2004). However, the functional significance of this remains unclear.

Controversy regarding "non-$M_3$" mACh receptors in the submandibular glands is not limited to the potential involvement of the $M_1$ subtype. There is some evidence for the presence of the $M_5$ muscarinic receptor from radioligand binding studies (Flynn et al., 1997) and functional microphysiometry studies in rat submandibular gland (Meloy et al., 2001). The latter study used a range of muscarinic antagonists to generate an affinity profile that correlated best with human cloned $M_3$ and $M_5$ receptors (Meloy et al., 2001). The lack of ligands genuinely selective between these two receptor subtypes prevented a definitive conclusion being drawn regarding the subtype(s) present in this tissue. This highlights the need for caution when interpreting such data, even when using a large number of antagonists.

Mice lacking the $M_5$ receptor gene showed moderately reduced salivation in response to pilocarpine only at longer times (15-60 min) after injection (Yeomans et al., 2001; Takeuchi et al., 2002). In contrast, Bymaster et al. (2003) reported that salivation measured at 15 min post-injection was not altered in $M_5$ knockout mice, seemingly implicating the $M_3$ receptor in slow responses to muscarinic agonist. However, Nakamura et al. (2004) recently reported that salivary secretion in mice lacking both $M_1$ and $M_5$ receptors was not significantly different to wild-type over a 30 min timescale. Submandibular gland acinar cells from $M_1/M_5$ knockout mice also produced similar Ca$^{2+}$ responses to cells from wild-type animals, so the role of non-$M_5$ mACh receptors in the submandibular gland remains controversial.

Overall, data from studies utilizing mACh receptor subtype knockout mice indicate that the $M_3$ receptor is not involved in salivary secretion (Gomez et al., 1999; Bymaster et al., 2003), but potential roles for all 4 other subtypes have been proposed. Bymaster et al. (2003) found that mice lacking either $M_1$ or $M_4$ receptors exhibited approx. 30% lower salivary secretion in response to pilocarpine. Mice lacking the $M_3$ subtype displayed a much more significant reduction in saliva production, in support of the findings of Matsui et al. (2000), who reported a complete loss of pilocarpine response in $M_3$ knockout mice. In contrast, however, Yamada et al. (2001a) reported significant pilocarpine-induced salivation in mice lacking the $M_3$ receptor, implicating non-$M_3$ subtypes in this response.
Most of the work discussed above, however, agrees on the crucial role of the M₃ receptor in the cholinergic modulation of salivation. Species-dependent differences and variations in experimental techniques and functional readouts may account for many of the apparently contradictory observations in the literature. It is clear that a more complete understanding of the complex regulation of salivary secretion will be required to successfully develop anti-muscarinic therapies with minimal salivary gland-based side-effects.

1.5 Constitutive activity and inverse agonism at GPCRs

Classical receptor theory proposed that agonist binding to a single quiescent receptor state induces a conformational change in the GPCR to its functionally active state. It was believed that only ligands possessing both affinity and efficacy (i.e. agonists) were capable of eliciting this conformational change, while antagonist ligands (which display affinity but not efficacy at the receptor) bound to the receptor, preventing agonists from binding but not directly influencing the receptor ‘state’. However, the observation that GPCRs are able to activate G-proteins in the absence of agonist stimulation (so-called ‘constitutive’ activity) challenged this view (Costa & Herz, 1989). Moreover, Costa & Herz (1989) provided the first example of what we now know as ‘inverse agonism’. They demonstrated that an elevated basal GTPase activity in NG108-15 cells was reduced by the δ-opioid receptor ‘antagonist’ ICI 174864 in a concentration-dependent manner and more importantly, that this effect was blocked by another opioid receptor antagonist MR2266 (Costa & Herz, 1989). The latter observation ruled out the possibility that the observed constitutive activity was due to trace levels of opioid agonist in the system and instead demonstrated that a sub-set of ‘antagonist’ ligands might be capable of actively inhibiting the spontaneous G-protein activation mediated by GPCRs.

In the following sections, the emerging evidence for constitutive activity and inverse agonism as general principles of GPCR behaviour will be discussed with reference to the more seminal work of the last 15 years in this area. Particular attention will be given to evidence relating to the ability of the mACh receptor family of GPCRs to exhibit constitutive activity in both native and recombinant systems. Current
knowledge of the prevalence and potential therapeutic benefits of inverse agonists at
the muscarinic receptors and others will also be focussed upon.

1.5.1 Stimulus-biased systems as a means of investigating constitutive activity
and inverse agonism in GPCRs

1.5.1.1 The use of constitutively-active mutant (CAM) receptors

Initial scepticism of the phenomena of constitutive activity and inverse agonism was
largely based upon the requirement for specialised cellular conditions (i.e. a
constitutively-active receptor system), in order to observe inverse agonism. Advances
in our understanding of the various ways in which to generate such biased systems
have facilitated the study of constitutive activity (and as a result, inverse agonism).
One of the most successful means of increasing the propensity of GPCRs to
spontaneously activate G-proteins has been the use of constitutively-active mutant
(CAM) receptors (for review, see Seifert & Wenzel-Seifert, 2002; Cotecchia et al.,
2003). The first example of this came from the work of Cotecchia et al. (1990), who
found that the substitution of 3 amino acids in the C-terminus of the third intracellular
loop of the α1-adrenoceptor generated a mutant receptor that, when expressed in
COS-7 cells, exhibited a basal inositol phosphate accumulation of more than double
that of the wild-type receptor. Single mutations of either of two of these amino acids
(Lys290 and Ala293) also generated moderately constitutively-active receptor
mutants (Cotecchia et al., 1990). Indeed, a more thorough investigation at one of these
residues (Ala293), provided the extraordinary finding that substitution of this residue
by any other amino acid, significantly increased the level of constitutive activity (but
to varying degrees) when expressed in COS-7 cells (Kjelsberg et al., 1992).

Following on from this work, Lefkowitz’s group developed a CAM β2-adrenoceptor
by replacing a C-terminal portion of the third intracellular loop with the
corresponding region of the α1B-adrenoceptor (Samama et al., 1993). This generated a
mutant receptor which was constitutively coupled to the stimulation of adenylate
cyclase and which also displayed what are now considered characteristic features of
the majority of GPCR CAMs: enhanced binding affinity for agonists (with the
increase proportional to the intrinsic activity of the agonist); increased potency of
agonists for functional responses; and enhanced intrinsic activity of partial agonists
(Samama et al., 1993). As a result of this work, Lefkowitz and colleagues proposed an
extension to the ternary complex model (TCM), as the classical form of the TCM did not adequately fit their data with the β2-adrenoceptor CAM (Samama et al., 1993). In this refined model, the equilibrium (defined by the equilibrium constant, L) between the inactive receptor (R or R̅) and the active state (R* or Rₐ) is incorporated into the TCM (see Figure 1.5). The model assumes that only the Rₐ form is able to bind the G-protein, forming the ARₐG ternary complex. The extent to which the ligand A affects the equilibrium between R and Rₐ is denoted by the constant α, while its ability to stabilise the ternary complex ARₐG is determined by the constant γ; the molecular efficacy of the ligand A is therefore defined by the product of these two constants, αγ. The extended ternary complex (ETC) model has subsequently become widely accepted as a good approximation (albeit an over-simplification) of the molecular processes involved in receptor activation (Kenakin, 2002).

Having identified that Gₛ- and Gₒ/₁-coupled receptors could be mutated to generate constitutively-active receptors, Cotecchia and colleagues mutated a single residue in the C-terminal portion of the third intracellular loop of the Gₒ/₁-linked α₂A-adrenoceptor to create mutant receptors, which constitutively inhibited D₁A receptor-mediated cyclic AMP accumulation in HEK-293 cells (Ren et al., 1993). It is therefore apparent that GPCRs coupling to all of the major classes of G-protein are capable of exhibiting constitutive activity. This is supported by the observation that around 50 % of GPCRs for which constitutive activity has been reported couple to Gₒ/₁ proteins and approximately 25 % couple to Gₛ and Gₒ/₁, respectively (see Seifert & Wenzel-Seifert, 2002). However, this reflects the overall coupling profile of GPCRs to the three major classes of G-protein, so there does not appear to be any difference in the propensity for receptors coupled to different classes of G-protein to exhibit constitutive activity (Seifert & Wenzel-Seifert, 2002).

1.5.1.2 Over-expression of wild-type receptors

Since the initial characterisation of constitutive activity and inverse agonism in the δ-opioid system in NG108-15 cells (Costa & Herz, 1989) it has become increasingly evident that wild-type receptors can display constitutive activity when expressed either in recombinant cells or in non-engineered 'physiological' tissues/cells. High GPCR expression levels facilitate the detection of constitutive activity, as even for receptors with a low intrinsic tendency to adopt the active conformation(s), the
Figure 1.5 The Extended Ternary Complex (ETC) model

The receptor exists in inactive (Ri or R) and active (Ra or R*) conformations in an equilibrium defined by the constant L. Ra can spontaneously interact with G-protein (G) to produce a constitutively active species (RaG).

- $K_a$: equilibrium constant for receptor and ligand
- $K_g$: equilibrium constant for receptor and G-protein
- $L$: allostereic constant defining the proportion of receptor in active versus inactive states
- $\alpha$: factor defining the effect of ligand binding upon the equilibrium between Ri and Ra
- $\gamma$: factor defining the effect of ligand binding upon the affinity of Ra for G
The absolute number of active receptors will increase in proportion to the expression level (Kenakin, 2004). Many GPCRs therefore exhibit significant constitutive activity when expressed at levels between 10 and 40 pmol/mg of membrane protein in insect cells (e.g. Chidiac et al., 1994; Pozvek et al., 1997; Ross et al., 1999; Brys et al., 2000).

However, some receptors exhibit significant basal activity even when expressed at relatively low levels, closer to those found in native systems. For instance, even when expressed at levels of 1 pmol/mg of membrane protein and below, the rat H₃ histamine receptor was found to exhibit pronounced activation of adenylate cyclase in the absence of agonist stimulation in CHO cells (Smit et al., 1996). The H₂ receptor-selective antagonists cimetidine and ranitidine both acted as inverse agonists in concentration-dependently reducing the constitutive cyclic AMP accumulation in cells expressing H₂ receptors, while burimamide had no effect on basal adenylate cyclase activity (Smit et al., 1996). This study was one of the first to demonstrate constitutive activity and inverse agonism at near-physiological levels of receptor expression, but the failure to observe significant constitutive activity of numerous other GPCRs at similar expression levels is indicative of the varying propensity for receptors to spontaneously assume their active state(s).

Indeed, even closely related GPCRs often display different levels of constitutive activity when expressed at similar levels. For instance, the β₂-adrenoceptor exhibited spontaneous activity when expressed in cardiac myocytes from β₁β₂-knockout mice, while expression of the β₁-adrenoceptor (even at higher levels) failed to elicit constitutive activity (Zhou et al., 2000). Interestingly, when either the β₁ or the β₂ adrenoceptors were fused to the long isoform of Gαs, fixing the stoichiometry of receptor and G-protein, similar levels of constitutive activity were observed (Wenzel-Seifert et al., 2002). This might suggest that the availability of G-protein is limiting the extent of constitutive activity, rather than the inherent properties of the receptors themselves. It has been proposed that the differential localisation of the β₁ and β₂ adrenoceptors in cardiomyocytes (in caveolae; see section 1.2.6) might therefore modulate the basal activity of these receptor systems by regulating their access to G-protein effector populations (see Milligan, 2003).

The over-expression (by 40-100 fold) of wild-type β₂-adrenoceptors in genetically modified mice demonstrated that constitutive activity could be induced in vivo (Bond...
et al., 1995). Baseline left atrial tension was increased 3-fold in mice over-expressing the β2-adrenoceptor relative to wild-type, while cardiac contractility in vivo was significantly greater in the transgenic mice (Bond et al., 1995). At the biochemical level, basal cyclic AMP accumulation was increased in β2-adrenoceptor over-expressing cardiac membranes (Milano et al., 1994; Bond et al., 1995). All of these readouts of constitutive activity were sensitive to the inverse agonist ICI-118,551 and this inhibitory effect was competitively antagonised by the apparently neutral antagonist alprenolol (Bond et al., 1995). Surprisingly, over-expression of a β2-adrenoceptor CAM in mice hearts failed to induce a similar phenotype, perhaps because of the relatively low expression level (approx. 3 fold above endogenous β-adrenoceptor levels) (Samama et al., 1997). However, chronic exposure to either inverse agonist or neutral antagonist (but not agonist) caused a significant up-regulation of CAM β2-adrenoceptor (by approx. 50-fold). This led to similar increases in adenylate cyclase activity and enhanced contractility of cardiac tissue both in vitro and in vivo as seen in wild-type over-expressing mice (Samama et al., 1997). The effect of antagonist/inverse agonist ligands in up-regulating the CAM β2-adrenoceptor is likely to be due to a stabilisation of what has been demonstrated in vitro to be a relatively unstable receptor protein (Gether et al., 1997), while ligand-induced up-regulation of the CAM β2-adrenoceptor had previously been demonstrated in NG108-15 cells (MacEwan & Milligan, 1996). The de-stabilising effects of constitutively activating mutations and the stabilisation and resulting up-regulation of GPCRs by inverse agonist ligands has been observed with a number of different CAMs and will be discussed in greater detail in section 5.3 (see also Milligan & Bond, 1997).

1.5.1.3 G-protein over-expression

Another approach that has been successfully used to enhance the basal activity of wild-type GPCRs is to over-express their cognate G-proteins. Theoretically, this too should shift the equilibrium in favour of the formation of the active (Rg) state, assuming that G-proteins display a higher affinity for Rg than R. This was first demonstrated experimentally for the muscarinic receptors by Burstein and colleagues in 1995, when they observed an elevated constitutive activation of the M3 receptor expressed in NIH-3T3 cells upon transient co-expression of the Gαq subunit (Burstein et al., 1995). This also indicates that, as proposed for the β-adrenoceptors in cardiac
myocytes, the availability of G-proteins might provide a means by which the
constitutive activity of a system can be regulated. Further characterisation of the
effects of Gq over-expression upon muscarinic receptor pharmacology revealed that
each of the Gq-coupled mACh receptors (M₁, M₂ and M₃) behaved similarly
(Burstein et al., 1997). Gq over-expression induced constitutive activity (representing
approx. 20-30 % of the maximal response to agonist), enhanced the potency of all
agonists tested and increased the efficacy of partial agonists. All antagonists tested
(including atropine, pirenzipine, QNB and NMS) were also capable of completely
reversing the constitutive activity with potencies consistent with their affinity for
inhibiting agonist-mediated responses (Burstein et al., 1997). Since it has been shown
that G-protein expression levels may be regulated in response to muscarinic receptor
activation (Mitchell et al., 1993), it is possible that constitutive receptor activity may
be regulated at this level in vivo.

Other factors relating to the cellular background in which a receptor is expressed
might influence the extent to which that receptor is active in the absence of agonist
stimulation, as recently reviewed by Milligan (2003). For instance, the constitutive
activity of mGluR1a and mGluR5 receptors is modulated by specific interactions
between their C-terminal tails and the scaffolding protein Homer (Ango et al., 2001).
When expressed endogenously in cerebellar granule cells, the receptors are quiescent
in the absence of agonist, while in HEK-293 cells, pronounced basal inositol
phosphate turnover is observed (Ango et al., 2001). Treatment of cerebellar granule
cells with Homer 3 antisense oligonucleotides led to an mGluR inverse agonist-
sensitive increase in basal inositol phosphate generation, suggesting that Homer 3
negatively regulates the basal activity of the mGluR 1a and 5 receptors in neuronal
tissue (Ango et al., 2001).

1.5.2 Constitutive activity and inverse agonism in non-engineered systems

Many of the early examples of constitutive activity in endogenous GPCR populations
involve the muscarinic receptors and as such will be discussed later (see section
1.5.3). However, inverse agonists have revealed constitutive activity of α₂D-
adrenoceptors endogenously expressed in RIN5AH cells (Tian et al., 1994) and of β-
adrenoceptors in turkey erythrocytes (Gotze & Jakobs, 1994) and guinea-pig and
human cardiomyocytes (Mewes et al., 1993). Leeb-Lundberg et al. (1994) also
demonstrated that treatment of rat myometrial cells expressing B₂ bradykinin receptors with bradykinin receptor ‘antagonists’ decreased basal inositol phosphate accumulation, suggesting that B₂ receptors are spontaneously active in the myometrium.

One of the most convincing examples of constitutive activity in vivo is provided by the work of Arrang and colleagues, who investigated the spontaneous activity of the histamine H₃ autoreceptor in rat brain (Morisset et al., 2000; Rouleau et al., 2002). Initially, H₃ receptor constitutive activity was identified in recombinant CHO cells expressing as little as 300 fmol/mg of membrane protein and both inverse agonists (e.g. thioperamide) and a neutral antagonist (proxyfan) were identified in this system (Morisset et al., 2000). Subsequent experiments in rat brain demonstrated constitutive H₃ receptor activity by measuring either autoreceptor-mediated [³H]-histamine release or basal [³⁵S]-GTPγS binding to rat brain membranes and in both assays proxyfan reversed both agonist and inverse agonist-mediated effects (Morisset et al., 2000). Rouleau et al. (2002) later showed that both human and rat H₃ receptors display significant constitutive activity at physiological expression levels (of less than 500 fmol/mg protein), while inverse agonists at H₁ and H₂ receptors failed to reduce basal [³⁵S]-GTPγS binding in rat brain membranes. This provides a further indication that even within the same tissue, different receptors/receptor subtypes might display distinct profiles of constitutive activity.

1.5.3 Examples of constitutive activity and inverse agonism at the mACh receptors

1.5.3.1 Wild-type receptors

Although Costa & Herz provided the first definitive demonstration of inverse agonism at a GPCR in 1989, there were numerous earlier indications in the literature that ‘antagonists’ were capable of inducing opposite effects to agonists. Perhaps the earliest of these observations came from work on the cardiac mACh receptor population. Giles & Noble (1976) reported that while ACh significantly reduced the slow, inward cation current in bullfrog atria, atropine had the opposite effect in increasing the current. Also, in rabbit atrial cells, Soejima & Noma (1984) demonstrated that both atropine and scopolamine were capable of reducing the basal activity of ACh-activated K⁺ channels.
Other, less direct indications of inverse agonism at cardiac muscarinic receptors came from radioligand binding data in membranes. For instance, Berrie et al. (1979) showed that GTP enhanced the binding of $[^3H]$-NMS binding to rat heart membranes and later, Burgisser et al. (1982) reported similar findings using $[^3H]$-QNB and the non-hydrolysable GTP analogue Gpp(NH)p on frog heart membranes. It is well established that guanine nucleotides uncouple G-protein and receptor, thus stabilising the receptor in its inactive (R$i$) state. The low affinity of agonists for the R$i$ state is evident in radioligand binding studies, for instance, where the curve for the displacement of radiolabelled antagonist by agonist is shifted in the presence of GTP (e.g. Lohse et al., 1984). If, however, an inverse agonist displays higher affinity for R$i$ than R$a$, it might be expected that its affinity for the receptor population might be enhanced in the presence of GTP. The early observations of increased binding of the ‘antagonists’ $[^3H]$-NMS and $[^3H]$-QNB to cardiac muscarinic receptors in the presence of GTP are therefore consistent with such a model (Berrie et al., 1979; Burgisser et al., 1982).

However, the problem with many of these earlier studies is that the potential for endogenous ACh present in the system to be activating the muscarinic receptors in the ‘basal’ state was not addressed. Hilf & Jakobs, in 1992, were the first to attempt to address this concern. They demonstrated that atropine (and a variety of other muscarinic ‘antagonists’, including pirenzepine, HHSiD and AF-DX 384) concentration-dependently reduced the basal $[^35S]$-GTP$\gamma$S binding in porcine atrial membranes (Hilf & Jakobs, 1992). They also used acetylcholine esterase to degrade any endogenous ACh that was present and found that atropine was still capable of inhibiting $[^35S]$-GTP$\gamma$S binding with a similar potency and to the same extent as in non-acetylcholine esterase treated membranes, providing convincing evidence that atropine (and the other ligands tested) acts as an inverse agonist in porcine atrial membranes (Hilf & Jakobs, 1992).

Hanf et al. (1993) provided further electrophysiological evidence of the inverse agonist activity of atropine on cardiac mACh receptors. They investigated $I_{Ca}$ in frog ventricular cells and found that, following prior stimulation of $I_{Ca}$ by isoprenaline, ACh significantly reduced the current (Hanf et al., 1993). In contrast, atropine concentration-dependently enhanced $I_{Ca}$ either in the presence or absence of ACh. In rat myocytes, the prior stimulation of $I_{Ca}$ was not required as basal activity was high.
enough to support both inhibition by ACh and stimulation by atropine (Hanf et al., 1993).

One of the most thorough early investigations of constitutive activity and inverse agonism at the muscarinic receptors was that of Jakubik et al. (1995), who studied both the natively-expressed M₂ receptor subtype (in rat cardiomyocytes) and the M₁-M₄ receptor subtypes recombinantly expressed in CHO cells. They found that for all four subtypes expressed in CHO cells and for the M₂ receptor in rat cardiomyocytes, treatment with either atropine, NMS and in some cases QNB and AF-DX 116 caused a concentration-dependent decrease in the production of inositol phosphates (for M₁ and M₃ receptors) or an increase in the synthesis of cyclic AMP (for M₂ and M₄ receptors) with EC₅₀ values close to that expected for the binding of those ligands to the muscarinic receptors (Jakubik et al., 1995). This indicates that all four mACh receptor subtypes investigated are capable of constitutively activating their most efficiently coupled signalling cascades and that at least in some cases, the four ‘antagonists’ tested can display inverse agonism (Jakubik et al., 1995). The inability of AF-DX 116 and QNB consistently to reduce basal activity at all receptor subtypes/in all systems might indicate differences in the negative efficacy of these ligands, but this remains to be conclusively proven.

Several other studies have demonstrated constitutive activity of mACh receptors when expressed in recombinant cell systems. For instance, Migeon & Nathanson (1994) reported that over-expression of the chick M₄ receptor in JEG-3 cells caused pronounced spontaneous inhibition of adenylate cyclase activity, which was reversed by atropine treatment. Vogel et al. (1995) over-expressed the M₂ mACh receptor in CHO cells and found that NMS, QNB and hyoscyamine were all equally capable of increasing the basal cyclic AMP level, suggesting that the M₂ receptors were constitutively active in this system. Over-expression of the M₂ receptor with either Gᵢ or Gₛ in S²⁹ insect cells caused similar levels of constitutive [³⁵S]-GTPyS binding, which was sensitive to atropine, scopolamine and NMS, indicating that the M₂ receptor can spontaneously couple to either Gᵢ or Gₛ, apparently without preference (Uustare et al., 2004).

In support of the early studies on the M₂ receptor population expressed in cardiac tissue from a variety of species, atropine and S(-)-hyoscyamine both enhanced forskolin-stimulated cyclic AMP synthesis in rat cardiac membranes by up to 24 %
The inability of R-(+)-hyoscyamine to influence cyclic AMP synthesis confirmed that this was a receptor-mediated effect, while inclusion of acetylcholine esterase did not affect atropine-mediated enhancement of cyclic AMP accumulation, ruling out the involvement of endogenous ACh (Ricny et al., 2002).

However, not all studies agree on the inverse agonist activities of certain mACh receptor ligands. Daeflfer et al. (1999a) measured GTP hydrolysis in porcine atrial sarcolemma and found that pirenzepine, but not atropine or AF-DX 116, decreased the $V_{max}$ of basal GTP hydrolysis. Although unable to influence basal GTP hydrolysis themselves, the apparent neutral antagonists atropine and AF-DX 116 were able to concentration-dependently inhibit the inverse agonist effects of pirenzepine (Daeflfer et al., 1999a). This report is at odds with the majority of studies discussed above, where atropine has been used as a standard tool to probe for constitutive activity. In a subsequent study, the $M_2$-selective muscarinic antagonist methoctramine was also found to inhibit basal GTPase activity in porcine atrial sarcolemma (Daeflfer et al., 1999b). However, this effect was not blocked by either atropine or AF-DX 116, suggesting that methoctramine interacts directly with G-proteins to inhibit GTP hydrolysis, rather than acting as an inverse agonist at the $M_2$ receptor (Daeflfer et al., 1999b). However, as will be discussed later, the sensitivity of the system employed is critical to the adequate detection of inverse agonism and the failure to observe inverse agonism does not necessarily indicate that the ligand in question does not possess negative efficacy (Kenakin, 2004).

1.5.3.2 Constitutively-active mutant mACh receptors

One of the first studies to identify a constitutively activating mutation in the muscarinic receptors was that by Hogger et al. (1995), who found that mutating residue E360 in the extreme C-terminus of the third intracellular (3) loop of the $M_1$ receptor to alanine, led to enhanced phosphoinositide turnover in the absence of agonist when expressed in HEK-293 cells. This residue is directly N-terminal to the well-defined 'BBXXB' motif (where 'B' = basic amino acid and 'X' = any amino acid), which was shown to be a crucial sequence in the G-protein-activating peptides described by Okamoto & Nishimoto (1992). Mutations in and around this region increase basal receptor activity in a number of different GPCRs, including the $\alpha_1$-adrenoceptors (Cotecchia et al., 1990; Kjelberg et al., 1992). In the muscarinic receptor family, Liu et al. (1996) reported that insertion of between 1 and 4 alanine
residues into TM VI, just 3 residues C-terminal to the BBXXB motif of the M₃ receptor, significantly enhanced constitutive inhibition of adenylate cyclase activity. The authors proposed that the effect of inserting alanine residues into the sixth transmembrane domain might mimic the effect of agonist binding to the receptor, causing a movement of TM VI towards the cytoplasm, opening up the VTIL motif for G-protein interaction (Liu et al., 1996).

More recently, Schmidt et al. (2003) identified the mutation of Q490 to leucine in the M₃ receptor as a constitutively activating mutation, by using a yeast genetic screen. Expression of this mutant M₃ receptor led to robust constitutive activity and increased carbachol potency in both yeast and mammalian cells (Schmidt et al., 2003). Q490 is found within the BBXXB motif of the M₃ receptor (KKAAQ), so this study supports the notion that this region is involved in maintaining the receptor in its inactive state and/or important for G-protein coupling.

Mutation of the first residue of the highly conserved D/ERY motif at the cytoplasmic end of TM3 has been demonstrated to generate constitutively-active, mutant receptors for a variety of GPCRs, including rhodopsin, α₁B- and β₂-adrenoceptors (see Parnot et al., 2002). However, mutation of any of the three residues (DRY) in the human M₁ mACh receptor failed to enhance constitutive activity relative to wild-type receptors, although mutation of Asp122 or Tyr124 severely reduced receptor expression, perhaps indicating that these residues are crucial for maintaining the stability of the receptor (Lu et al., 1997). Indeed, reduced expression has been observed for a number of CAMs, so although not detected at the expression levels achieved by Lu et al., this might be taken as evidence that mutation of Asp122 or Tyr124 generates a constitutively-active, mutant M₁ receptor.

Spalding et al. (1998) performed systematic mutations of several regions of the M₃ mACh receptor in a search for residues involved in receptor function and found a number of mutations that led to significant constitutive activity. The majority of these residues were found in TM6, although high levels of constitutive activity were also observed in receptors mutated in the second intracellular loop (Spalding et al., 1998). Analysis of the constitutively-active, mutant receptors generated by mutations within TM6 identified 5 key residues, mutation of which consistently enhanced the basal activity of the receptor (Spalding et al., 1998). These include Asn459, which supports earlier evidence in which mutation of the homologous asparagine residue of the M₃
receptor generated a mutant receptor that displayed a 2-fold higher basal inositol phosphate accumulation than wild-type M₃ receptors expressed in COS-7 cells (Bluml et al., 1994).

However, it was the mutation of either Phe451 or Ser465 that generated the M₃ mACh receptors with the highest basal activity (Spalding et al., 1998). The importance of the latter residue had earlier been identified when the same group isolated a CAM M₃ receptor with a tyrosine residue substituted for Ser465 and a proline residue in place of Thr466 (Spalding et al., 1995). The CAM M₃ receptor exhibited enhanced agonist affinity and potency, and the enhanced basal activity of the receptor was suppressed by a range of antagonists (atropine, pirenzepine, QNB, NMS and 4-DAMP) with affinities consistent with their binding affinities at both wild-type and mutant receptor (Spalding et al., 1995). In a later study, Spalding and colleagues examined the effects of 13 different amino acid substitutions at Ser465 alone in the M₃ receptor and found that 11 of the mutations produced significant increases in constitutive activity (Spalding et al., 1997). In receptors mutated at this residue and the adjacent threonine, it is therefore likely that it is the serine mutation that contributes most to enhancing basal activity. Substitution of large or basic residues at Ser465 enhanced constitutive activity of the M₃ receptor by between 55 and 110 %, while replacing Ser 465 with small or acidic amino acids produced little or no increase in basal activity (Spalding et al., 1997). The authors modelled the data obtained from these studies and concluded that the primary effect of the mutation of Ser465 was to alter the equilibrium between Rᵣ and Rₐ, destabilising the inactive (Rᵣ) state and the favouring the formation of the active (Rₐ) state in the absence of agonist stimulation.

Subsequent studies demonstrated that the homologous mutations of Ser388 and Thr389 in the M₁ mACh receptor also produced a constitutively active receptor (Huang et al., 1998b). Enhanced potency and efficacy of both full and partial agonists and increased agonist binding affinities also characterised the mutant M₁ receptor (Huang et al., 1998b). Mutation of Ser388 and Thr389 individually indicated that it is the replacement of Ser388 that is primarily responsible for the enhanced agonist binding affinity and potency (Huang et al., 1999). Taken together with the data from the M₃ receptor studies, it would therefore seem that the conserved serine residue at the junction between TM6 and the third extracellular loop is critical in constraining the receptor in the inactive state, as mutation of this residue can induce constitutive
activity and increase sensitivity to agonist. Interestingly, mutation of Thr389 introduced multiple sub-states, with the high affinity site being guanine nucleotide-sensitive (Huang et al., 1999). This would suggest that mutation of this residue alters receptor-G-protein coupling in some way, though this requires more detailed investigation.

More recently, the homologous SerThr (or AsnThr in the case of the M2 and M4 receptors) to TyrPro double mutation was introduced into all five subtypes of the mACh receptor family (Ford et al., 2002). The mutant receptors displayed an increased agonist affinity of between 5 and 40 fold when expressed in COS-7 cells (Ford et al., 2002). In functional assays in NIH-3T3 cells, all five mutant receptors (but not wild-type receptors) displayed constitutive activity of between 30 and 80% of the maximal agonist-stimulated response, with the M5 receptor exhibiting the greatest and the M1 subtype the least constitutive activity (Ford et al., 2002). The level of basal activity was found to be proportional to the mutant receptor expression level, as was the potency of carbachol. In most cases, the carbachol potency in functional assays was significantly higher in mutant relative to wild-type receptors. The only exception to this was for the M2 receptor mutant, which displayed no significant agonist response above basal. However, like all of the mutants tested, atropine was capable of significantly and concentration-dependently reducing the basal functional activity of the M2 mutant receptor (Ford et al., 2002). It is therefore clear that mutation of these conserved residues on the boundary between TM6 and the third extracellular loop generates constitutively active receptor mutants of all five muscarinic receptor subtypes, although the extent of the effect of these homologous mutations does appear to vary between receptor subtypes (Ford et al., 2002).

Recently, Zeng et al. (2003) reported an alternative approach to the generation of constitutively-active muscarinic receptors. They modified the rat M3 mACh receptor by swapping its i3 loop for the corresponding region of the CAM β2-adrenoceptor and also attaching the Renilla reniformis luciferase to the C terminus of the M3 receptor (Zeng et al., 2003). The chimeric receptor displayed similar pharmacological properties to the wild-type M3 receptor but was expressed at a much lower level (Zeng et al., 2003). Chronic treatment with agonists, antagonists and inverse agonists all resulted in a concentration-dependent up-regulation of receptor expression, consistent with what has previously been reported for the CAM β2-adrenoceptor (Gether et al., 2002).
1997). Indeed, the chimeric M3 receptor displayed an enhanced basal Gq activation in HEK-293 cells, as determined by an NFAT reporter gene assay, which was sensitive to atropine treatment (Zeng et al., 2003).

1.5.4 How common is inverse agonism?

When Costa & Herz first reported inverse agonism, it was treated with scepticism and understandably was considered to be an exceptional case. However, with the development of the extended ternary complex model (see Figure 1.5), it became apparent that in order for a ligand to behave as a neutral antagonist (i.e. as all 'antagonists' had been originally classified) it would be required to recognise at least two (Rq and Rg) and probably 3 (RqG) distinct receptor conformations with identical affinities (i.e. α would have to equal 1; see Figure 1.5) (Kenakin, 2004). The association of ligand and active receptor would also be required to have no influence upon the affinity of the receptor for its cognate G-protein (i.e. γ would have to equal 1; see Figure 1.5), as the magnitudes and vector properties (i.e. positive or negative) of both α and γ determine the efficacy, be it positive or negative, of the ligand in question (Kenakin, 2004).

There is also good evidence that the binding of ligands to receptors alters the conformation of the latter and that in fact multiple 'active' receptor states exist in the form of micro-conformations (Kenakin, 2002). It has been speculated that ligands influence the prevailing ensemble of conformations and in so doing induce the observed receptor behaviour, which may encompass not only receptor activation, but also internalisation, dimerisation, etc (Kenakin, 2002). The use of fluorescent probes bound directly to the β2-adrenoceptor has provided evidence that the binding of ligands (even proposed neutral antagonists) can alter the range of micro-conformations adopted by the receptor (i.e. induces a conformational change) (Ghanouni et al., 2001).

It might therefore be anticipated that truly 'neutral' antagonists would form the minority and that the vast majority of GPCR ligands would display some degree of efficacy. Indeed, Kenakin recently surveyed a total of 380 antagonist-receptor pairings and found that in fact 322 (85 %) of them were inverse agonists and only 58 (15 %) were classified as neutral antagonists (Kenakin, 2004). It is important to note that this survey covered only those studies in which significant constitutive activity
was present in the system to allow inverse agonism to be observed for at least one ligand (Kenakin, 2004). This highlights a crucial point regarding the classification of ligands as inverse agonists or neutral antagonists: negative efficacy is a molecular property, inherent to the ligand of study, but inverse agonism is a phenotypic behaviour, whose observation is largely dependent upon the set-point of the system. This set-point is defined in the ETC model by the equilibrium constant L (i.e. the propensity of receptors to spontaneously undergo the conformational switch between $R_i$ and $R_a$), such that the degree of inverse agonism observed is dependent upon the level of constitutive activity in the system (Kenakin, 2004). In a non-constitutively active system, inverse agonists will behave as neutral, competitive antagonists, so the failure to observe inverse agonism does not preclude a ligand possessing negative efficacy.

The manipulations described earlier that are designed to enhance the constitutive activity of the system largely do so by altering the set-point of the system. Increasing receptor expression levels, for instance, can allow moderate L values to produce detectable spontaneous activity, while certain mutations in the mACh receptors have been proposed to directly alter the equilibrium constant L (Huang et al., 1998b, 1999). However, one caveat attached to the use of CAM receptors in the study of receptor activation is that it is not yet clear how closely the ‘active’ state(s) induced by these mutations resemble the natural active state(s) of the receptor (Parnot et al., 2002). Further information in this regard, perhaps by obtaining the crystal structure of agonist- and mutation-induced active states, might expand the utility of CAMs in the study of GPCR signalling.

1.5.5 Endogenous constitutive activity, CAM receptors and inverse agonists

The role of naturally occurring CAMs in human disease was first identified for rhodopsin, where mutation of residue K296 in TM6 results in persistent activation of rhodopsin and potentially the degeneration of photoreceptor cells associated with retinitis pigmentosa (Robinson et al., 1992). Other activating mutations in TMs 2 and 7 of rhodopsin have been linked to congenital night blindness (Dryja et al., 1993). CAMs have also been implicated in hyperfunctioning thyroid adenoma (TSH receptor), male precocious puberty (LH receptor) and autosomal-dominant hypocalcemia (Ca$^{2+}$ sensing receptor), amongst others (see Parnot et al., 2002; Seifert & Wenzel-Seifert, 2002).
In addition to the contribution of constitutively-active, mutant receptors to such rare genetic diseases, it is likely that receptors that are particularly prone to spontaneous activity, including many neurotransmitter and hormone receptors, might be tonically active in vivo. This has been demonstrated for the cannabinoid CB1 receptor, which is involved in the regulation of the thermal nociceptive threshold in mice. Administration of the CB1 receptor inverse agonist SR141716A was shown to produce hyperalgesia via an inhibition of the constitutive activity of the CB1 receptor (Richardson et al., 1997). Care was taken in this study to eliminate endogenous agonists from the system, indicating that the basal tone of this system is mediated by constitutive activity of the CB1 receptor population (Richardson et al., 1997). A further example of physiological constitutive activity comes from the study of histamine H3 receptors. When expressed in recombinant systems, H3 receptors display robust constitutive activity, but perhaps more importantly, they appear to demonstrate similar characteristics in rat brain, where they act as autoreceptors to regulate the activity of histaminergic neurons (Morisset et al., 2000).

Perhaps the only known example where an endogenous agonist and endogenous inverse agonist have been identified to contribute to the physiological regulation of a GPCR system is in the melanocortin system (Adan & Kas, 2003). Recombinant expression of the MC4 receptor has provided evidence that the receptor may be constitutively-active, while α-melanocyte-stimulating hormone (α-MSH) acts as an agonist and Agouti-related protein (AgRP) demonstrates inverse agonism at the MC4 receptor (Nijenhuis et al., 2001; Haskell-Luevano & Monck, 2001). MC4 receptors expressed in the brain are involved in the regulation of body weight and it has been proposed that stimulation of these receptors by α-MSH causes weight loss, while a reduction in the constitutive activity of the MC4 receptors by AgRP might relieve the tonic inhibition of food intake and stimulation of energy expenditure, leading to a gain in weight (Adan & Kas, 2003). Evidence for this hypothesis includes the observation that MC4 receptor mutants found only in obese individuals have lost the constitutive activity associated with wild-type receptors (Vaisse et al., 2000).

Recent evidence also implicates the constitutive activity of the ghrelin receptor in the hormonal modulation of appetite (see Holst & Schwartz, 2004). Ghrelin is the major orexigenic (appetite-stimulating) hormone released from the gastrointestinal tract prior to feeding and it has been appreciated for some time that a ghrelin receptor
antagonist may be capable of suppressing meal-associated food intake. However, the recent observation that the ghrelin receptor displays constitutive activity of approximately 50% of that in response to a maximal ghrelin concentration, suggests that an inverse agonist targeted to this receptor might also lower the set-point for ‘between-meals’ food intake, which may be due to basal activity of the receptor even after ghrelin levels have returned to baseline (Holst et al., 2003).

1.5.6 Implications of inverse agonism and constitutive activity for drug discovery

The appreciation that most, if not all, ligands with affinity for a given GPCR are also likely to possess efficacy, necessitates a fuller understanding of these properties in any candidate therapeutic. The use of constitutively-active systems in high-throughput screening of chemical libraries is therefore likely to form an integral part of the drug discovery process in the coming years. Chen et al. (2000) characterised such a system, using melanophores transiently expressing a variety of different GPCRs that can be expressed at sufficiently high levels as to promote constitutive activity, yet still allow for further agonist-mediated responses. Such a system, whereby both agonists and inverse agonists may be detected in a high-throughput screen, is likely to provide the most versatile and sensitive means of screening large numbers of ligands of unknown efficacy.

Recent reports by Milligan (2003) and Kenakin (2004) have indicated that the vast majority of currently marketed therapeutic ‘antagonists’ are in fact inverse agonists. Where the target receptor system is not constitutively-active, an inverse agonist will have similar effects to a neutral antagonist, but where there is some degree of spontaneous activity in the system, inverse agonism may either be a useful property or a deleterious one (Kenakin, 2004). Where pathologies involve constitutively-active receptors (see above and Parnot et al., 2002), a ligand with negative efficacy might be preferable to a neutral antagonist. However, long-term treatment with inverse agonist is likely to cause an up-regulation of receptor expression in a tonically active system (Milligan & Bond, 1997). This has been suggested to contribute to the tolerance developed to the H2 histamine receptor inverse agonists, such as cimetidine and ranitidine, in the control of gastric acid secretion (Smit et al., 1996). In contrast, the neutral antagonist burimamide causes no receptor up-regulation and it might therefore be expected that long-term treatment with such a neutral antagonist might be more effective (Smit et al., 1996). Inverse agonist-mediated receptor up-regulation has been
widely reported for a number of GPCRs (Milligan & Bond, 1997; see also section 5.3) and the relevance of this phenomenon to tolerance and withdrawal effects in drug therapy has obvious implications for future drug development.

1.6 Thesis Aims

The general aims of this study are two-fold: to further characterise the pharmacology of certain muscarinic antagonists currently used or proposed for use in the treatment of overactive bladder; and to investigate the phenomena of constitutive activity and inverse agonism at the muscarinic receptors. Specific questions relating to these two broad aims will be addressed in each of four results chapters. The specific questions posed in each case are outlined below.

1). The binding and functional selectivity profiles of atropine, darifenacin, oxybutynin and tolterodine at M₂ and M₃ mACh receptors will be determined, to clearly define the muscarinic receptor subtype selectivities of these ligands. By investigating binding and functional affinities at the M₂ and M₃ mACh receptors, both stably expressed in similar CHO cell backgrounds, a valid comparison of the affinities of these antagonists will be possible.

2). Previous reports, particularly from in vivo studies, have indicated that darifenacin and other muscarinic antagonists display functional selectivity for the inhibition of responses in the urinary bladder, relative to those in the salivary glands. The aim of this study will be to investigate the relative abilities of darifenacin, oxybutynin, tolterodine and atropine to inhibit M₃ receptor-mediated phosphoinositide turnover in guinea-pig urinary bladder and submandibular salivary glands, in order to determine whether certain muscarinic antagonists are capable of displaying tissue-dependent pharmacologies at the level of second messenger turnover.

3). The effect of a single point mutation at the junction of TM 6 and the third extracellular loop (N410Y), known to enhance constitutive activity in other muscarinic receptor subtypes, will be examined in the M₂ mACh receptor. The
constitutive activity of transiently expressed wild-type and N410Y mutant M₃ mACh receptors will be assessed and, together with the previously characterised, homologously-mutated M₃ mACh receptor, the ability of a range of muscarinic antagonists to exhibit inverse agonism will be investigated. Of particular relevance will be those ligands with clinical utility in the management of overactive bladder, as the potential for these ligands to display inverse agonism has not previously been investigated.

4). CHO cell lines stably expressing wild-type or N410Y mutant M₂ mACh receptors will be generated, to facilitate the pharmacological characterisation of the effect of the N410Y mutation. In particular, the agonist pharmacology of the N410Y mutant will be examined, as other characteristics such as enhanced agonist potency and increased partial agonist efficacy would be consistent with a constitutively-active, mutant receptor.
Chapter 2: Materials and Methods

2.1 Materials

Wild-type human M₃ mACh receptor in pcDNA3 and wild-type human M₂ receptor in pCD were both kindly provided by Dr A. B. Tobin (University of Leicester, UK). Gₛ in pCDN was a generous gift from Dr S. Rees (GlaxoSmithKline, Stevenage, UK). Gₒ₄-specific antiserum ('IQB') was generously provided by Prof. G. Milligan (University of Glasgow, UK).

[^H]-NMS, [^H]-myo-inositol and [^H]-cyclic AMP were obtained from Amersham Biosciences UK Ltd (Little Chalfont, UK), while [³⁵S]-GTPγS and [^H]-Ins(1,4,5)P₃ were from N.E.N. Life Science Products Ltd (Boston, USA). Darifenacin (UK 88,525) and tolterodine (UK-233,743) were kindly provided by Pfizer. AF DX 384 was purchased from Tocris-Cookson Ltd (Bristol, UK). MT-7 was purchased from Peptides Int. (Kentucky, USA).

QuickChange™ site-directed mutagenesis kit was from Stratagene Inc. (La Jolla, CA, USA). Gene Juice™ transfection reagent and all other kits used for molecular biology were purchased through Novagen (Merck Biosciences Ltd, Nottingham, UK). All materials for cell culture were supplied by Life Technologies Inc (Paisley, UK).

Atropine sulphate, lithium chloride, oxybutynin chloride, QNB, N-methyl scopolamine, methoctramine, pirenzepine, 4-DAMP, acetyl-β-methylcholine (methacholine), carbamylcholine (carbachol), pilocarpine, oxotremorine, oxotremorine-M, forskolin, adenosine 3', 5'-cyclic monophosphate (cyclic AMP), Ins(1,4,5)P₃, G418 (geneticin), GDP, GTP, GTPγS, trichloroacetic acid, EDTA, EGTA, tri-n-octylamine, 1,1,2-trichlorotrifluoroethane, pertussis toxin, HEPES, dimethyl sulphoxide (DMSO), dithiothreitol, sodium dodecyl sulphate, Tween-20, Triton X-100 and TEMED were purchased from Sigma-Aldrich Ltd (Poole, UK). ECL+ was obtained from Amersham Biosciences UK Ltd (Little Chalfont, UK). All other reagents were of analytical grade and were purchased from Fisher Scientific UK Ltd (Loughborough, UK).
2.2 Animals
Male Dunkin Hartley guinea pigs (supplied by David Hall Guinea Pigs, Staffordshire, UK), approximately 300g-500g in weight, were killed by injection of 1g kg\(^{-1}\) pentobarbitone.

2.3 Cell culture
2.3.1 Culture of recombinant cell lines
Chinese hamster ovary cells stably expressing cloned human M\(_1\), M\(_2\) or M\(_3\) receptors (CHO-m1, CHO-m2 and CHO-m3 respectively) were grown in minimum essential medium-\(\alpha\) (MEM-\(\alpha\)) supplemented with 10 % newborn calf serum, 100 IU\(^{-1}\) penicillin, 100 \(\mu\text{g ml}^{-1}\) streptomycin and 2.5 \(\mu\text{g ml}^{-1}\) amphotericin B. CHO-K1 and HEK-293 cells were cultured in similar media, but with newborn calf serum replaced with 10 % foetal calf serum. CHO-K1 cells stably expressing cloned human wild-type M\(_2\) or N410Y mutant M\(_2\) receptors generated in this project were grown in identical media to that used in the culture of CHO-K1 cells, supplemented with 500 \(\mu\text{g ml}^{-1}\) Geneticin (G418; Sigma-Aldrich Ltd) selection agent (see section 2.3.3). CHO-m3 CAM cells were also cultured in geneticin-supplemented MEM-\(\alpha\) medium, containing foetal calf serum, penicillin, streptomycin and amphotericin B as described above.

All cells were maintained at 37°C in a humidified atmosphere of O\(_2\)/CO\(_2\) (19:1) and were routinely split 1:5 (newborn calf serum) or 1:10 (foetal calf serum) every 3-4 days, using trypsin-EDTA. Where required, cells were treated with pertussis toxin (PTx; 100 ng ml\(^{-1}\)) 20-24 h prior to experiments in normal media.

2.3.2 Transient transfection of CHO-K1 and HEK-293 cells
Cells were plated out approx. 72 h prior to experimenting, such that 24 h later, cells would be approx. 50 % confluent to allow optimal transfection. Immediately prior to transfection, media bathing the cells was changed. Gene Juice\(^{\text{TM}}\) transfection reagent (4:1 ratio of Gene Juice\(^{\text{TM}}\) (\(\mu\text{l}\)): cDNA (\(\mu\text{g}\)) was added dropwise to serum-free MEM-\(\alpha\) medium (containing 100 IU\(^{-1}\) penicillin, 100 \(\mu\text{g ml}^{-1}\) streptomycin and 2.5 \(\mu\text{g ml}^{-1}\) amphotericin B). The volume of serum-free medium used, was generally adjusted to allow 25 or 50 \(\mu\text{l}\) of the resulting transfection mixture to be added to each well for
transfection. 5-10 min later, the Gene Juice™ in serum-free medium was added dropwise to the appropriate amount of cDNA (in sterile water) and allowed to incubate at room temperature for 15-20 min. At this point, the DNA/Gene Juice™ mixture was added directly to the appropriate wells. Approx. 6 h later, media bathing transfected cells was changed and experiments were performed approx. 48 h post transfection.

2.3.3 Generation of stable CHO cell lines

A confluent monolayer of CHO-K1 cells in a 175 cm² flask was washed with PBS and lifted in 5 ml PBS-EDTA (PBS containing 1 mM EDTA). 0.5 ml of the resulting cell suspension was seeded into each of four 10 cm diameter petri dishes containing 10 ml CHO-K1 medium (see section 2.3.1). The following day, the medium was changed and cells were transfected with either 5 or 10 µg of wild-type or N410Y mutant M₃ cDNA (petri dishes # 1 - # 4 respectively). For transfection method, see Section 2.3.2.

Two days later, medium was changed again and the following day cells were washed with PBS and lifted in 5 ml PBS-EDTA. Cells were left for 15 min at 37°C to allow all cells to be lifted off the plate. Either 5 or 10 µl of the resulting cell suspension was then seeded into each of two 10 cm diameter petri dishes, containing 10 ml of CHO-K1 medium (see section 2.3.1) supplemented with G418 (500 µg ml⁻¹).

Approx. 2 weeks later, when numerous large isolated colonies were present on all eight plates (4 x wild-type; 4 x N410Y mutant), 24 colonies were picked from each plate and seeded into wells (each containing 1 ml of G418-supplemented CHO-K1 medium) on each of eight 24-well plates. 7-10 days later, when a number of wells were approaching confluency, the medium was changed. 2 days later, medium was removed and 500 µl PBS-EDTA was added to each well. Cells were left for 15 min at 37°C before removing 250 µl of the resulting cell suspension into each of 2 wells (each containing 1 ml of G418-supplemented CHO-K1 medium) in separate plates.

2 days later, the primary [³H]-NMS binding screen was performed on one set of eight 24-well plates (one well containing each clone). Based on the results of this primary screen, clones selected for a secondary [³H]-NMS binding screen were split from the
remaining well (on the second set of 8 plates) into 6 wells (4 wells for \[^{3}H\]-NMS binding and 2 wells for protein analysis) and a small flask (for passaging and freezing down).

2.4 Membrane preparation

2.4.1 Recombinant cell lines

Confluent monolayers of CHO or HEK-293 cells were briefly washed with HBS and cells lifted from the flask by the addition of HBS-EDTA (10 mM HEPES, 0.9% NaCl, 0.2% EDTA, pH 7.4) for approx. 15 min. A cell pellet was recovered by centrifugation at 1700 r.p.m. for 5 min. The pellet was homogenised on ice in lysis buffer (10 mM HEPES, 10 mM EDTA, pH 7.4) using a polytron homogeniser (1 x 20 s burst). The homogenate was centrifuged (18,000 r.p.m., 15 min, 4°C), re-homogenised and re-centrifuged as described above in freezing buffer (10 mM HEPES, 0.1 mM EDTA, pH 7.4). The final membrane pellet was re-suspended in freezing buffer at a concentration of 2 mg protein ml\(^{-1}\) and stored at -80°C until required for radioligand binding.

2.4.2 Guinea pig tissue

The frozen tissues were finely chopped with scissors in 20 mM HEPES (pH 7.4) buffer and any excess fat decanted off. Tissue was then homogenised at 14,000 r.p.m. for approx. 20 s. Homogenates were centrifuged at 5000 r.p.m. for 10 min and the resulting supernatant poured through Miracloth (Calbiochem) into fresh beakers. This was then centrifuged at 20,000 r.p.m. for 20 min. The resultant pellet was re-homogenised in buffer before a second high speed spin as before. This final pellet was re-suspended in 20 mM HEPES buffer and aliquots frozen down at -80°C at a concentration of either 2 or 3 mg protein ml\(^{-1}\).
2.5 Radioligand binding

2.5.1 Membrane homogenates

Saturation binding was performed using a range of concentrations of $[^{3}H]$-NMS (0.03 - 6.0 nM; specific activity 81-84 Ci mmol$^{-1}$) in the absence and presence of atropine (10 μM) to define non-specific binding. Binding assays were performed in final volumes of between 200 and 1000 μl (assay buffers: 20 mM HEPES, pH 7.4; or 10 mM HEPES, 100 mM NaCl, 10 mM MgCl$_2$, pH 7.4 ['+ salt' buffer]) containing between 25 and 100 μg membrane protein and were incubated either for 60 min at 37°C or 120 min at 25°C. Bound radioligand was separated from free by rapid vacuum filtration through Whatman GF/B grade filters on a 24 well Brandel cell harvester and radioactivity quantified by liquid scintillation counting. Total membrane binding was always < 10% of total $[^{3}H]$-NMS added.

Competition binding experiments were performed using a fixed concentration of $[^{3}H]$-NMS (approximately 0.3-0.5 nM) in the absence and presence of a range of ligand concentrations (added 10 min prior to addition of radiolabel). Where the effect of guanine nucleotides upon competition binding curves was being investigated, GTP (100 μM) was added at the same time as the competitor. Bound radioligand was separated from free by rapid vacuum filtration, either through Whatman GF/B Unifilter Plates on a 96 well Packard Unifilter Harvester or through Whatman GF/B grade filters on a 24 well Brandel cell harvester. All other conditions were as stated above for saturation binding assays.

2.5.2 Intact cells

Intact cell binding assays were performed on cell monolayers on 24-well plates, in Krebs-Henseleit buffer (KHB; composition in mM: NaCl 118, KCl 4.7, CaCl$_2$ 1.3, KH$_2$PO$_4$ 1.2, MgSO$_4$ 1.2, Na HCO$_3$ 25, HEPES 5, Glucose 10). Medium was aspirated from each well and the cells washed 3 x 1 ml with warm, gassed KHB. Where applicable, antagonists were pre-incubated for 10 min before the addition of $[^{3}H]$-NMS, in a total assay volume of 500 μl. For measurement of 'non-agonist' ligand binding affinities, cells were incubated for 60 min at 37°C. For agonist affinity determinations, cells were maintained on ice at 4°C overnight. Following incubation,
cells were washed 3 times with warm, gassed KHB before digestion of cells with NaOH (0.1 M) and subsequent liquid scintillation counting.

When measuring the effects of chronic antagonist/agonist exposure on the mACh receptor expression levels in intact cells, ligands were made up (where possible) and diluted in sterile water and added directly to the medium bathing cells in 24-well plates, at the appropriate time-point prior to assaying. Prior to the addition of [3H]-NMS, cells were thoroughly washed 3 x 1 ml with warm, gassed KHB before being incubated in 1 ml warm KHB at 37°C for 20 min. After this time, KHB was aspirated and cells were washed once more with 1 ml warm, gassed KHB before being assayed as detailed above. In these assays, as when monitoring the receptor expression levels following transient transfections, a single high concentration (3-4 nM) of [3H]-NMS (performed in duplicate) was generally used to approximate the maximal level of binding.

2.6 Total [35S]-GTPγS binding

[35S]-GTPγS binding assays were performed essentially as described by Lazareno et al. (1993). Briefly, assays were carried out in a total volume of 1 ml, containing membrane protein (100 μg), approx. 100 pM [35S]-GTPγS (1250 Ci mmol⁻¹), GDP (1 μM), ± agonist (MCh) in assay buffer (10 mM HEPES, 100 mM NaCl, 10 mM MgCl₂, pH 7.4). Where effects of atropine on basal [35S]-GTPγS binding were investigated, atropine (10 μM) was pre-incubated with membrane protein for 15 min prior to addition of [35S]-GTPγS. Assays were incubated for 60 min at 30°C and the reaction was terminated by rapid filtration through pre-wetted Whatman GF/B grade filters. This was followed by 5 x 1 ml washes with ice-cold assay buffer. Radioactivity was quantified by liquid scintillation counting, with non-specific binding defined in the presence of 10 μM GTPγS.

2.7 Immunoblot analysis

Cells were seeded into 6-well plates and transfected 24 h later (see section 2.3.2). 48 h post-transfection, cells were washed with PBS and lysed with a Triton-X based lysis buffer (20 mM HEPES, 200 mM NaCl, 10 mM EDTA, 1 % Triton-X 100, pH 7.4).
Lysis buffer (300 µl) was added to each well and incubated on ice for 10 min. Cells were scraped and the resulting suspension was added to an equal volume of 2 x sample buffer (125 mM Tris buffer, 4 % SDS, 20 % glycerol, 50 µM dithiothreitol, 0.01 % bromophenol blue, pH 6.8) and boiled at 90°C for 5 min. Samples were electrophoresed on 10 % SDS-PAGE minigels (5 % stacking gels) and run at constant voltage (120 V) for 90 min (running buffer composition: 25 mM Tris, 250 mM glycine, 0.1 % SDS, pH 8.0). Transfer to nitrocellulose was achieved using a semi-dry apparatus (transfer buffer composition: 48 mM Tris, 39 mM glycine, 0.037 % SDS, 20 % methanol, pH 8.3). Nitrocellulose membrane washing steps were performed using a modified high stringency TBS Tween buffer (20 mM Tris, 1 M NaCl, 1 % Tween-20, pH 7.5) and blocking was performed with 20 % milk in this modified TBS Tween buffer. Immunoblotting was performed using a rabbit polyclonal Gaq antiserum ('IQB') (1:1000) raised against amino acids 119-134 of Gaq. Immunoreactive proteins were detected using a horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (Sigma-Aldrich Ltd, Poole, UK) and enhanced chemiluminescence (ECL+) reagents.

2.8 cAMP accumulation in CHO and HEK-293 cells

cAMP accumulation assays were performed on intact cell monolayers on 24-well plates at 37°C. For agonist/antagonist experiments, cells were stimulated with forskolin (10 µM) for 10 min in the presence of agonist (added 10 min prior to forskolin addition) ± a range of antagonist concentrations (added 30 min prior to forskolin addition). When investigating the potential for inverse agonism in cAMP responses, forskolin (3 or 10 µM) was added either 10 min prior to, 20 min after or simultaneously with the putative inverse agonist and incubations were extended for between 1 and 25 min subsequent to this. In each case, the assay was stopped by the addition of 0.5 M trichloroacetic acid (TCA; 400 µl) to each well following aspiration. Samples were left on ice to extract for 20 min before 400 µl was removed from each well into microfuge tubes containing 10 mM EDTA (50 µl, pH 7.0) (blank buffer was also generated by the addition of 0.5 M TCA (400 µl) to each of 12 tubes containing 10 mM EDTA (50 µl) and subsequent treatment identical to sample tubes). This was followed by the addition of 1,1,2-trichloro-trifluoroethane/tri-n-octylamine.
Tubes were then vortexed several times over a 15 min period, before centrifugation for 2 min at 13,000 r.p.m. 200 µl samples of the upper phases were recovered into microfuge tubes containing 60 mM NaHCO₃ (50 µl). Where extracellular cAMP accumulations were measured, samples (100 µl) of extracellular solution were removed immediately prior to aspiration, added to equal volumes of 0.5 M TCA and processed as described above.

Mass determination of cAMP levels was then performed according to the method of Brown et al. (1971). Briefly, samples (50 µl) or cAMP standards (50 µl, in blank buffer) were added to microfuge tubes containing approximately 3 nM [³H]-cAMP (100 µl) and cAMP binding protein (150 µl) in a final assay volume of 300 µl of T/E buffer (50 mM Tris / 4 mM EDTA). All tubes were then vortexed and incubated for 90 min at 4°C. The assay was stopped by the addition of charcoal suspension (5 mg ml⁻¹ charcoal; 2 mg ml⁻¹ BSA, in T/E buffer; 250 µl per tube) and left to stand on ice for 12 min before vortexing and centrifuging (13,000 r.p.m.; 4 min). Supernatant (400 µl) was then removed and radioactivity within this sample quantified by liquid scintillation counting.

### 2.9 Total [³H]-inositol phosphate accumulation in CHO and HEK-293 cells

Total [³H]-inositol phosphate accumulation assays were performed on [³H]-myo-inositol labelled cells (2.5 µCi ml⁻¹; specific activity 88 Ci mmol⁻¹; 24 h) under 10 mM LiCl block. Cells were stimulated with agonist for 10 min at 37°C, in the absence and presence of a range of antagonist concentrations. Cells were pre-incubated with antagonists for 30 min and with LiCl for 15 min, prior to agonist stimulation. Incubations were terminated after 10 min by aspirating and adding ice-cold 0.5 M TCA (500 µl). Samples were left on ice to extract for 20 min before 500 µl samples were transferred to 1.5 ml microfuge tubes containing 10 mM EDTA (100 µl). This was followed by the addition of 1,1,2-trichloro-trifluoroethane/tri-n-octylamine (1:1 v/v, 500 µl). Tubes were then vortexed several times over a 15 min period, before centrifugation for 2 min at 13,000 r.p.m. 450 µl samples of the upper phase were recovered into microfuge tubes containing 60 mM NaHCO₃ (100 µl).
Inositol phosphates were separated according to their charge by anion exchange chromatography over a 1 ml depth of Dowex 1 – X8 (200-400 mesh, formate form; Sigma-Aldrich Ltd, Poole, UK), as described previously (Challiss et al., 1992). Columns were first regenerated with regeneration buffer (2 M ammonium formate [CH$_2$O$_2$NH$_3$] / 0.1 M formic acid [CO$_2$H$_2$]; 10 ml), followed by excess deionised water. Samples were then washed onto columns with approx. 5 ml water. Glycerophosphoinositol was then removed from each sample by washing with 60 mM ammonium formate/ sodium tetraborate (Na$_2$B$_4$O$_7$) (10 ml). $[^3]$H-InsP$_{1,3}$ were then eluted into 20 ml scintillation vials, with 0.75 M ammonium formate / 0.1 M formic acid (10 ml). Radioactivity in a 5 ml sample of the eluate was determined by scintillation counting.

2.10 Functional assays in guinea-pig tissue

2.10.1 Preparation of tissue for functional experiments

For each experiment, urinary bladder, submandibular and/or sublingual salivary glands were extracted from each of four animals. The tissue was cleared of connective tissue and immediately transferred into ice-cold Krebs-Henseleit buffer (KHB) and kept on ice prior to chopping. Each tissue was cross-chopped (300 μm x 300 μm) with a McIlwain tissue chopper. The resulting slices were transferred to 30 ml tubes containing approx. 20 ml warm KHB, gassed with O$_2$ / CO$_2$ (19:1). Slices were shaken vigorously at 37°C and washed 3-4 times with warm KHB over a 30 min period.

2.10.2 Total $[^3]$H-Inositol phosphate accumulation in bladder slices

Following the 30 min washing period, bladder slices were transferred to a 50 cm$^3$ culture flask containing MEM-α medium (10 ml), supplemented with 100 IU$^{-1}$ penicillin and 100 μg ml$^{-1}$ streptomycin. Myo-$[^3]$H]-inositol (25 μCi; specific activity 88 Ci mmol$^{-1}$) was added to the flask and incubated for 24 hr at 37°C in O$_2$ / CO$_2$ (19:1).

Following 24 hr incubation, the slices were transferred to a 30 ml sterilin containing warm KHB. The slices were incubated at 37°C with vigorous shaking for 20 min. During this period the buffer was changed 3-4 times to ensure complete removal of
medium. Gravity-packed slices (50 μl) were transferred to insert vials containing KHB (175 μl). Vials were then gassed with O₂/CO₂, capped and incubated at 37°C. At this point, antagonists (25 μl) were added where appropriate. LiCl (5 mM; 25 μl) was then added to each vial. Incubations were initiated, 15 min later, by the addition of CCh (25 μl), to give a final assay volume of 300 μl.

Incubations were terminated after 30 min by the addition of ice-cold 1M TCA (300 μl). Samples were left on ice to extract for 20 min. At the end of this period, vials were centrifuged for 15 min at 5000 r.p.m. 500 μl samples of the resulting supernatants were transferred to 1.5 ml microfuge tubes containing 10 mM EDTA (100 μl) and inositol phosphates were separated as described above for cell lines.

2.10.3 IP₃ mass determinations in salivary gland slices

IP₃ generation in salivary gland slices was determined essentially according to Batty et al., 1997. Following the washing period, gravity packed slices (50 μl) were transferred to insert vials containing KHB (150 μl). Vials were then gassed with O₂/CO₂, capped and incubated at 37°C. At this point, antagonists (50 μl) were added where appropriate. Incubations were initiated, 15 min later, by the addition of CCh (50 μl), to give a final assay volume of 300 μl. Ca²⁺-free experiments were performed in KHB without CaCl₂ and supplemented with EGTA (100 μM). Slices for Ca²⁺-free experiments were pre-incubated in this Ca²⁺-free KHB for approx. 20 min prior to aliquoting into vials.

Incubations were terminated after 5 min by the addition of ice-cold 1M TCA (300 μl). Samples were left on ice to extract for 20 min. At the end of this period, vials were centrifuged for 15 min at 5000 r.p.m. 500 μl samples of the resulting supernatants were transferred to 1.5 ml microfuge tubes containing 10 mM EDTA (100 μl) (Blank buffer was also generated by the addition of 1M TCA (500 μl) to each of 12 tubes containing 10 mM EDTA (100 μl) and subsequent treatment identical to sample tubes). This was followed by the addition of 1,1,2-trichloro-trifluoroethane/tri-n-octylamine (1:1 v/v; 500 μl) to each tube. Tubes were then vortexed several times over a 15 min period, before centrifugation for 2 min at 13,000 r.p.m. A 200 μl
sample of the upper phase was recovered into microfuge tubes containing 60 mM NaHCO₃ (50 µl).

Standard or sample (30 µl) was added to 100 mM Tris·HCl / 4 mM EDTA pH 8.0 (30 µl), and [³H]-Ins(1,4,5)P₃ (approx. 8000 d.p.m./assay; 30 µl) on ice. A standard curve for D-Ins(1,4,5)P₃ was constructed using concentrations corresponding to 0.036 – 36 pmol Ins(1,4,5)P₃ in blank buffer. The assay was initiated by addition of of Ins(1,4,5)P₃ binding protein (30 µl), prepared from bovine adrenal cortex (Batty et al., 1997). Samples were incubated on ice for 30 min, with intermittent vortexing. Bound and free radioligand were separated by vacuum filtration through Whatman GF/B filters on vacuum filtration manifolds. Samples were diluted with 3 ml wash buffer (25 mM Tris·HCl; 1 mM EDTA; 5 mM NaHCO₃; pH 8.0), immediately filtered, and the sample tube rapidly washed with 2 x 3 ml wash buffer. Following filtration, Whatman GF/B filter discs were transferred to vials and 4 ml scintillant (Safefluor, Packard Bioscience Ltd) added. Samples were allowed to extract overnight prior to scintillation counting.

2.11 Molecular biology

2.11.1 Sub-cloning of the M₂ mACh receptor gene

The individual primers designed to amplify the M₂ receptor gene are detailed in section 5.2.2.1.

2.11.1.1 Polymerase chain reaction (PCR)

The M₂ receptor gene was amplified from the pCD template using a NovaTaq™ DNA polymerase kit. Four thin-walled PCR tubes were prepared according to the following recipe:

<table>
<thead>
<tr>
<th>Volume</th>
<th>Component</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 µl</td>
<td>Template pCD M₂ – (50 ng)</td>
</tr>
<tr>
<td>1 µl</td>
<td>dNTP Mix (10 mM each)</td>
</tr>
<tr>
<td>3.1 µl</td>
<td>Primer 1 (200 µg ml⁻¹)</td>
</tr>
<tr>
<td>3.1 µl</td>
<td>Primer 2 (200 µg ml⁻¹)</td>
</tr>
<tr>
<td>5 µl</td>
<td>10 x NovaTaq Buffer with MgCl₂</td>
</tr>
</tbody>
</table>
Following gentle mixing of the contents, each tube was subjected to the following thermal cycling: 30 cycles, 1 min at 94°C, 1 min at 60°C, 2 min at 72°C followed by a final extension for 5 min at 72°C. Samples were then held at 4°C. Negative controls were performed using an identical recipe lacking only the template.

2.11.1.2 Gel electrophoresis

DNA gels were cast from 1.5% agarose (wt/vol) in TAE buffer, supplemented with 0.5 μg ml⁻¹ ethidium bromide to allow detection of DNA fragments under UV illumination. Prior to gel loading, DNA samples (10 μl) were added to 5 x DNA loading buffer (2.5 μl). Gels were run at constant voltage (110 mV) for 60-90 min. DNA was visualised under UV illumination and the image captured using a GeneGnome™ bio-imaging system (Syngene, Cambridge, UK).

2.11.1.3 PCR product purification

PCR products were purified to remove unwanted amplification products using the SpinPrep™ PCR clean-up kit, according to manufacturer's instructions. Briefly, PCR products were pooled and added to SpinPrep-Bind buffer. Following high speed (10,000 g) centrifugation through a SpinPrep PCR filter, the supernatant was discarded. PCR products were then washed and centrifuged sequentially with SpinPrep-Bind buffer and wash buffer, before elution in 50 μl of pre-warmed (50°C) elution buffer via centrifugation.

2.11.1.4 Restriction endonuclease digestion

Both the purified M₂ receptor PCR product and pcDNA3 (the vector into which the gene will be ligated) were subjected to double digestion with the restriction endonucleases Eco RI and Xho I to form complementary ‘sticky ends’ to facilitate ligation. Digests were set up as follows:

0.25 μl NovaTag DNA Polymerase (1.25 U)
36.55 μl PCR Grade H₂O
<table>
<thead>
<tr>
<th>Component</th>
<th>M2 PCR product</th>
<th>pcDNA3</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA</td>
<td>15 µl</td>
<td>5 µl</td>
</tr>
<tr>
<td>10 x buffer</td>
<td>2 µl</td>
<td>2 µl</td>
</tr>
<tr>
<td>Eco RI (20,000 Units/ml)</td>
<td>1 µl</td>
<td>1 µl</td>
</tr>
<tr>
<td>Xho I (20,000 Units/ml)</td>
<td>1 µl</td>
<td>1 µl</td>
</tr>
<tr>
<td>ddH&lt;sub&gt;2&lt;/sub&gt;O</td>
<td>1 µl</td>
<td>11 µl</td>
</tr>
</tbody>
</table>

Restriction digests were performed at 37°C for 2 h, with 1 µl alkaline phosphatase added to the pcDNA3-containing tube 30 min from the end of the incubation. This is designed to minimise the potential for the vector to re-ligate itself. After 2 h incubation, digest products were added to an appropriate volume (5 µl) of 5 x DNA loading buffer and subjected to gel electrophoresis as detailed in Section 2.11.1.2.

### 2.11.1.5 Extraction of DNA from agarose gels

DNA fragments were excised from the gel using a scalpel blade and transferred to pre-weighed microfuge tubes. Tubes were re-weighed to determine the mass of each gel slice. A SpinPrep™ gel DNA kit was used to recover the DNA from each gel slice. Briefly, the appropriate volume of gel melt solution was added to each tube and the gel melted by 10 min incubation at 50°C. The resulting solution was filtered through a SpinPrep filter by high speed (10,000g) centrifugation and the flow through discarded. The filter was then washed and centrifuged sequentially with gel melt solution and wash buffer, before elution in 50 µl of pre-warmed (50°C) elution buffer via centrifugation (after 3 min incubation at 50°C).

### 2.11.1.6 Ligation of DNA

In order to optimise the potential for successful ligation, different ratios of vector:insert were set up in separate ligation reactions. The mass of DNA recovered from the gel slices was determined by the absorbance measured at wavelength (λ) = 260/280 nm in a spectrophotometer (Shimadzu UV-1201; Shimadzu UK, Milton).
Keynes, UK), allowing the molar ratios of vector:insert to be calculated according to the following equation:

\[
[DNA] \text{ (pmol)} = \frac{\text{mass of DNA (pg)}}{[\text{DNA fragment length (bp)}] \times 650 \text{ pg/pmol/bp}}
\]

To achieve vector:insert ratios of 1:2, 1:3 and 1:4 the amount of pcDNA3 vector was kept constant at 0.025 pmol and the quantity of insert (M2 receptor PCR product) was varied from 0.05 to 0.1 pmol. Ligations were performed using a Clonables™ kit according to manufacturers instructions. Briefly, each of the mixtures of vector and insert cDNAs were made up to 5 μl in volume (with nuclease-free water) and added to 5 μl of 2 x ligation mix (pre-mixed, containing DNA ligase and appropriate buffer). The reaction was maintained at 16°C for 15 min and then stored at 4°C until ready to proceed with transformation.

2.11.1.7 Preparation of LB-ampicillin plates

1 litre of LB agar was prepared and autoclaved. When cool to the touch, sterile-filtered ampicillin was added to a final concentration of 100 μg ml⁻¹. 20-25 ml of LB-ampicillin was poured into sterile 10 cm diameter plates under aseptic conditions and left to solidify. Plates were then stored at 4°C until use.

2.11.1.8 Transformation

Transformation of ligation products was performed using NovaBlue Strain Singles™ competent cells, with 50 μl of cells thawed on ice for each transformation reaction. 1 μl of ligation product was added directly to the competent cells which, following gentle agitation, were maintained on ice for 5 min. Cells were then heat-shocked at 42°C for 30 s before returning the tubes to ice for 2 min. S.O.C. medium (250 μl, room temperature) was added to each tube before incubating the cells at 37°C for 30 min, with shaking at 250 r.p.m.

For each transformation reaction two LB-ampicillin plates were dried at 37°C for 10-15 min (to remove condensation) and either 10 or 90 μl of transformation product was spread onto each plate. Plates were then transferred to an incubator at 37°C for 15 min.
to allow the freshly spread transformation mixture to dry. After this time, plates were inverted and maintained at 37°C for 18-24 h.

2.11.1.9 Small-scale preparation of DNA

Between 12 and 24 isolated colonies (where possible) were picked using aseptic technique and transferred to 3 ml of LB-ampicillin broth. These cultures were maintained at 37°C for 16 h. DNA was then isolated using a SpinPrep™ plasmid kit according to the manufacturer’s instructions. Briefly, 1.5 ml of overnight cultures (the remaining 1.5 ml was stored at 4°C until required for large-scale preparation of DNA; see section 2.11.1.11) were centrifuged and the pellet re-suspended in re-suspension buffer (100 µl). Cells were lysed by the addition of lysis buffer (200 µl) and gentle inversion of the tubes. This was followed by the addition of neutralisation buffer (400 µl) and gentle inversion. Bacterial genomic DNA was then pelleted by centrifugation, with 600 µl of supernatant being transferred into SpinPrep columns, which were then centrifuged at 12,000 g for 30 s and the flow-through discarded. Columns were washed with wash buffer (650 µl), followed by centrifugation, and the DNA eluted into a fresh collection tube with pre-warmed elution buffer (50 µl), again by centrifugation.

2.11.1.10 Restriction endonuclease digests

Double restriction digests with Eco RI and Xho I were performed on samples of DNA prepared from all selected colonies and controls, as detailed in section 2.11.1.4. Digestion products were then subjected to gel electrophoresis as described in section 2.11.2.

2.11.1.11 Large-scale preparation of DNA

The residual 1.5 ml of bacterial culture from the selected colonies was transferred to an autoclaved 500 ml conical flask containing 100 ml LB-ampicillin broth and maintained at 37°C for 16-18 h, with shaking at 250 r.p.m. Glycerol stocks were prepared from the bacterial culture by the addition of an equal volume of warmed
glycerol to 0.75 ml of culture. Glycerol stocks were then stored at -80°C to allow
future DNA preparation.

The remaining DNA was isolated using a Mobius™ 1000 plasmid kit, according to
manufacturer's instructions. Briefly, bacterial cultures were centrifuged at 5000 g for
10 min and the pellet was vigorously re-suspended in bacterial re-suspension buffer (8
ml). Cell lysis was achieved by the addition of bacterial lysis buffer (8 ml) and gentle
inversion of the tubes. Neutralization buffer (8 ml) was added 5 min later and again
the tubes were gently inverted before incubation on ice for a further 5 min. The
resulting flocculant precipitate was separated by centrifugation at 10,000 g for 2 min
and the cleared supernatant was filtered through a ClearSpin filter by centrifugation at
2000 g for 3 min. The resulting filtrate was transferred to a Mobius 1000 column and
allowed to pass through by gravity. DNA bound to the column was washed with wash
buffer (20 ml) before eluting DNA in 5 ml of elution buffer into a sterile centrifuge
tube. DNA was precipitated by the addition of isopropanol (3.5 ml) and immediately
pelleted by centrifugation at 15,000 g for 20 min. The resulting pellet was washed
with 70 % ethanol (3 ml), to remove residual isopropanol, before a further
centrifugation at 15,000 g for 20 min. Ethanol was carefully removed and the DNA
pellet re-suspended in DNAase-free ddH₂O (500 μl). DNA was then quantified as
described in section 2.11.1.6 and stored at -20°C. Sequences of each clone were then
verified by dideoxy nucleotide sequencing.

2.11.2 Generation of the N410Y mutant M₂ mACh receptor

Specific primers were designed to incorporate the N410Y mutation into the human M₂
receptor gene in pcDNA3 and these are detailed in section 5.2.3. Mutagenesis was
performed using the QuikChange® site-directed mutagenesis kit (Stratagene),
according to the manufacturer's instructions, but the process is briefly described
below.

2.11.2.1 Mutant strand synthesis

All primers were made up to a concentration of 125 μg ml⁻¹ in ddH₂O. The following
reactions were set up in thin-walled PCR tubes:
Mutagenesis Control reaction:  
5 μl  10x reaction buffer  
2 μl  pWhitescript 4.5-kb control plasmid  
1.25 μl  Control primer #1  
1.25 μl  Control primer #2  
1 μl  dNTP mix

\[ \begin{array}{ll}
\text{mutagenesis control reaction:} & 5 \mu l \text{ } 10x \text{reaction buffer} \\
 & 2 \mu l \text{ pwhitescript 4.5-kb control plasmid} \\
 & 1.25 \mu l \text{ control primer #1} \\
 & 1.25 \mu l \text{ control primer #2} \\
 & 1 \mu l \text{ dNTP mix} \\
\end{array} \]

\[ \begin{array}{ll}
\text{N410Y M2 sample reaction:} & 39.5 \mu l \text{ ddH}_2 \text{O} \\
 & 5 \mu l \text{ 10x reaction buffer} \\
 & 2 \mu l \text{ pcDNA-M2 template (25 μmol ml}^{-1} \text{)} \\
 & 1.25 \mu l \text{ sense primer} \\
 & 1.25 \mu l \text{ anti-sense primer} \\
 & 1 \mu l \text{ dNTP mix} \\
 & 39.5 \mu l \text{ ddH}_2 \text{O} \\
\end{array} \]

Mixtures in each tube were gently mixed before the addition of 1 μl of \textit{PfuTurbo} DNA polymerase. Samples were then subjected to the following thermal cycling: 1 cycle of 30 s at 95°C; 16 cycles of 30 s at 95°C, 1 min at 55°C and 7 min at 68°C. Samples were then held at 4°C.

2.11.2.2 \textit{Dpn I} digestion of PCR products

1 μl of \textit{Dpn I} restriction endonuclease was added to each tube following PCR and incubated for 1 h at 37°C. \textit{Dpn I} endonuclease is specific for methylated and hemimethylated DNA. DNA synthesised in most \textit{E. coli} strains is dam methylated as a result of the actions of endogenous dam methylase enzymes. Mutated DNA strands generated \textit{in vitro} in the PCR reaction will not, however, be dam methylated. Treatment of PCR products with \textit{Dpn I} will therefore digest the parental DNA template and thus select for newly synthesised DNA incorporating the mutation.
2.11.2.3 Transformation of PCR products

_Dpn I_ digested PCR products were used to transform XL1-Blue super-competent cells, by the addition of 1 μl of PCR product to 50 μl aliquots of XL1-Blue cells in microfuge tubes. After gentle mixing, cells were left on ice for 30 min and then heat-shocked at 42°C for 2 min. 0.5 ml of S.O.C medium, pre-warmed to 37°C, was added to each tube before incubation at 37°C for 1 h.

2.11.2.4 Colony screening

The pWhitescript™ control plasmid contains a mutation in β-galactosidase gene (Glutamine to STOP, abolishing β-galactosidase activity), such that cells transformed with this control plasmid appear white (β-gal−) on LB-ampicillin plates containing IPTG and X-gal. However, successful mutagenesis, encoded by the control oligonucleotide primers, reverts the STOP codon to a glutamine codon, re-instating β-galactosidase activity. Following transformation with successfully mutated DNA, colonies appear blue (β-gal+) on LB-ampicillin plates containing IPTG and X-gal. This control therefore provides an indication of the efficiency of mutagenesis, with a high proportion of blue:white colonies indicating a high efficiency of mutagenesis.

LB-ampicillin plates were prepared as detailed in section 2.11.1.7. For the sample plates, the entire volume (500 μl) of bacterial suspension was spread onto LB-ampicillin plates and treated as described in section 2.11.1.8. For control plates, 10 mM IPTG (100 μl) and 2 % X-gal (100 μl) were spread onto LB-ampicillin plates 30 min prior to bacterial plating. 250 μl of control bacterial suspension was then plated out and plates were treated as described in section 2.11.1.8.

Following 18-20 h incubation at 37°C, between 12 and 16 isolated colonies were picked and cultured for small-scale DNA preparation, as described in section 2.11.1.9.

2.11.2.5 Restriction endonuclease digestion

The mutagenesis strategy for the N410Y M2 receptor included the incorporation of a silent mutation, designed to produce a distinct restriction fragment profile for the N410Y mutant M2 receptor gene, compared with the wild-type gene, when digested
with the restriction endonuclease Ava II (see section 3.2.3). A preliminary assessment of the success of the mutagenesis in the clones selected for small-scale DNA preparation was achieved by single restriction digest on both wild-type and a number of clones of N410Y mutant M2 receptor in pcDNA3 with Ava II. Restriction digests were performed in 20 µl volumes, containing 10 x buffer (2 µl), Ava II enzyme (10,000 Units/ml; 1 µl) and cDNA (1 or 10 µl for wild-type and mutant respectively; [DNA] < 100 µg ml⁻¹), made up with ddH₂O. Digests were incubated for 2 h at 37°C and the resulting DNA fragments were assessed by gel electrophoresis, as described in section 2.11.1.2.

Two successfully mutated clones (based upon restriction digest fragments) were then selected for large-scale DNA preparation (as described in section 2.11.1.11) and the sequences verified by dideoxy nucleotide sequencing.

2.12 Determination of proteins

In all cases where data are expressed 'per milligram of protein', protein levels in all samples (or a representative number of samples) were determined, according to the method of Lowry et al. (1951), and data normalised to these values.

2.13 Data analysis

Data are shown as means ± standard error of mean (s.e.m.) for the indicated number of experiments.

2.13.1 Radioligand binding experiments

Saturation binding data were fitted with hyperbolae (one-site binding) using GraphPad Prism 3.0 (GraphPad software, San Diego, USA). Bₘᵦₓ and Kᵢ values were derived from these curves. Competition binding curves were fitted to the “four parameter logistic equation”:

\[ Y = \text{Bottom} + \frac{\text{Top} - \text{Bottom}}{1 + 10^{\log EC_{50} - X \cdot \text{Hill coefficient}}} \]

using GraphPad Prism 3.0. The best fit between a variable Hill coefficient and a Hill coefficient fixed to unity was determined using an F-test. IC₅₀ values, generated by
these inhibition curves, were corrected to give binding constant (K_i) values for each test compound, using the Cheng-Prusoff equation (Cheng & Prusoff, 1973).

2.13.2 Functional experiments

Sigmoidal concentration-response curves for mACh receptor agonists, as well as all antagonist inhibition curves, were fitted to the "four parameter logistic equation" using GraphPad Prism 3.0. IC_{50} values, generated by these inhibition curves, were corrected to give binding constant (K_B) values for each test compound, using the functional equivalent of the Cheng-Prusoff equation (Craig, 1993; Lazareno & Birdsall, 1993): K_B = IC_{50}/[1 + (L/EC_{50})], where L = [Agonist] and EC_{50} = Agonist EC_{50} (derived from agonist concentration-response curve). Relative efficacies were calculated according to the method of Ehlert (1985):

$$ e_{rel} = (0.5 \times E_{max}/E_{maxA}) \times (1 + [K_B/EC_{50}]) $$

Where E_{max} and E_{maxA} are the maximal responses to test agonist and reference agonist (methacholine) respectively; K_B represents the apparent binding affinity of the test agonist and EC_{50} is the concentration of test agonist required to elicit 50 % of the maximal response.

2.13.3 Statistical analysis

The statistical significance of differences between data was determined using either an un-paired, two-tailed Student’s t test, with K_i and K_B values first being converted to the respective normally distributed negative logarithm (pK_i or pK_B), or one-way analysis of variance (ANOVA) with Dunnett's post test or Bonferroni’s test for multiple comparisons. Statistical significance was considered for p values less than 0.05 and is indicated throughout by * (unless otherwise indicated). Where normalized data are presented, statistical analysis was performed on raw data, prior to normalization.
Chapter 3: Characterisation of the binding and functional affinities of muscarinic acetylcholine (mACh) receptor antagonists at the M2 and M3 mACh receptors, stably expressed in Chinese hamster ovary (CHO) cells.

3.1 Introduction

Molecular biological techniques have identified five distinct genes encoding muscarinic acetylcholine (mACh) receptors, defined as m1-m5 (Bonner et al., 1987). Pharmacologically defined M1-M4 receptors corresponding to the m1-m4 gene products have been extensively described, but a full characterization of the M5 receptor is still lacking (Caulfield & Birdsall, 1998). The five mACh receptor subtypes display ≥90% sequence homology (Felder et al., 1995), which may explain the paucity of subtype-selective muscarinic ligands. Many tissues co-express multiple mACh receptor subtypes and given the highly conserved amino acid sequences of the five subtypes it is perhaps not surprising that in many cases the clear pharmacological identification of the muscarinic receptor subtype(s) mediating a given physiological response has proven difficult.

The lack of subtype-selective agonists and the dearth of sufficiently subtype-selective antagonists have also significantly constrained the use of mACh receptor ligands in the treatment of a variety of medical disorders (Caulfield & Birdsall, 1998). One such disorder is overactive bladder (OAB), characterised by symptoms of frequency, urgency and urge incontinence. Anti-muscarinic therapy is a mainstay in the treatment of OAB (Wyndaele, 2001), as mACh receptors are the predominant receptor system controlling bladder contraction (Andersson, 1993). The M2 mACh receptor is the predominant muscarinic receptor subtype expressed in the bladder, comprising approximately 80% of the total population (see Eglen et al., 1996a). Roles for the majority M2 receptor population in the contraction of various smooth muscle types (including bladder detrusor) have been proposed, based upon observations in tissues treated with N-2-chloroethyl-4-piperidinyl diphenylacetate (4-DAMP mustard) selectively to inactivate the M3 receptor population. Under these conditions M2 receptor activation may potentiate M3-stimulated contraction (via activation of non-selective cation currents, or inhibition of Ca^{2+}-dependent K' efflux), in addition to
opposing the relaxation mediated by agents that increase cyclic AMP (Ehlert, 2003). When recombinantly expressed at high levels, M2 receptors have been reported to initiate a weak phosphoinositide response through Gi activation (Ashkenazi et al., 1987; Lai et al., 1991; Dell’Acqua et al., 1993), but there is little evidence that this can occur in smooth muscle (Sawyer & Ehlert, 1999).

In contrast, M3 receptor stimulation has been linked to phosphoinositide hydrolysis in bladder smooth muscle (Noronha-Blob et al., 1989) and pharmacological characterization of the mACH receptors mediating contraction in detrusor smooth muscle indicate the predominant involvement of the minority M3 receptor population in a variety of species (Longhurst et al., 1995; Wang et al., 1995; Chess-Williams et al., 2001). Antagonists with high affinity for the M3 receptor have therefore been used in the management of OAB, but the lack of subtype-selectivity of these compounds can lead to debilitating side-effects including dry mouth, tachycardia, blurred vision and cognitive defects (Wallis & Napier, 1999). It has been proposed that ligands selective for the M3 receptor subtype would antagonize smooth muscle contractility in the absence of (in particular) M1- and M2-mediated side-effects (Wallis & Napier, 1999), prompting the search for more subtype-selective mACh receptor antagonists.

The aim of this study was to investigate the relative selectivity for M2 and M3 muscarinic receptors of a number of clinically important muscarinic antagonists. Oxybutynin and tolterodine are currently widely administered anti-muscarinic agents for OAB and have previously been characterized in radioligand binding studies as being approximately 10-fold selective for M3 versus M2 receptors (Nilvebrant et al., 1997a) and non-selective (Nilvebrant et al., 1997a; Gillberg et al., 1998) respectively. Darifenacin, a recently FDA-approved OAB therapy, has been reported to display up to 60-fold selectivity for M3 versus M2 receptors in radioligand binding assays (Wallis & Napier, 1999). All three antagonists potently inhibit muscarinic agonist-induced urinary bladder contractions in vitro and in vivo (Nilvebrant et al., 1997a; Gillberg et al., 1998).

In this study, binding affinity and ‘functional’ affinity (measured by the inhibition of muscarinic agonist-mediated alterations in second messenger signalling pathways) constants were derived for darifenacin, oxybutynin, tolterodine and the classical non-selective muscarinic antagonist atropine, in Chinese hamster ovary (CHO) cells stably expressing the cloned human M2 and/or M3 muscarinic receptors. This provides a
comprehensive account of the relative selectivities of these clinically relevant mACh receptor antagonists for the human M₂ and M₃ receptor subtypes expressed in a recombinant cell background.
3.2. Results

3.2.1 Radioligand binding studies in CHO-m2 and CHO-m3 cells

3.2.1.1 Membrane homogenates of CHO-m2 and CHO-m3 cells

Representative [³H]-NMS saturation binding curves at membrane homogenates prepared from CHO-m2 (a) and CHO-m3 (b) cells are shown in Figure 3.1. Mean expression (Bmax) and binding affinity constant (Kd) estimates for each cell line are given in Table 3.1. [³H]-NMS displayed significantly higher affinity (3.0 fold; p < 0.05) for the M3 over the M2 muscarinic acetylcholine (mACh) receptor.

The relative selectivities for M2 and M3 mACh receptor subtypes of atropine, darifenacin, oxybutynin and tolterodine were determined at the level of receptor binding affinity in competition radioligand binding assays, performed in 20 mM HEPES buffer (pH 7.4). [³H]-NMS competition binding curves were generated for each ligand by measuring the binding of a fixed concentration of radioligand ([³H]-NMS) in the absence and presence of a range of concentrations of competitor ligand. The resulting competition curves for each mACh receptor subtype are shown in Figure 3.2. IC₅₀ values derived from these curves were corrected to binding constant (Kᵢ) values according to the Cheng-Prusoff equation, using the [³H]-NMS Kd estimates determined in saturation binding experiments. Binding constant estimates (expressed as pKᵢ values) and Hill slope (nᵢ) values for each ligand at M2 and M3 receptors are summarized in Table 3.2, along with the relative selectivities of each ligand for M3 versus M2 receptor.

Darifenacin displayed marked selectivity (31.3 fold; p<0.001) for the M3 over the M2 mACh receptor. Oxybutynin was also M3 selective (8.0 fold; p < 0.001), while both atropine and tolterodine displayed approximately equivalent affinities at each receptor subtype. The mean slope factor associated with the tolterodine competition binding curve at the M2 receptor is significantly less than unity (0.84; p < 0.05), while all other slope factors were not significantly different from unity.

Similar competition binding experiments were performed with darifenacin at M2 and M3 receptors in 20 mM HEPES buffer containing 100 mM NaCl and 10 mM MgCl₂ (data not shown), as buffer ionic composition has previously been demonstrated to influence binding affinities (Pedder et al., 1991). The mean pKᵢ value calculated for
Figure 3.1
$[^3H]$-NMS saturation binding curves for membrane homogenates of CHO-m2 (a) and CHO-m3 (b) cells. Data are expressed as specific binding in pmol receptor expression mg$^{-1}$ protein and are representative curves of 3 or more experiments.
Table 3.1 Binding affinity constant (K_D) and Hill slope estimates for [3H]-NMS at M_2 and M_3 muscarinic receptors stably expressed in CHO cells. Data are expressed as mean (s.e.m.) values from n ≥ 3 experiments.

<table>
<thead>
<tr>
<th></th>
<th>membranes</th>
<th>intact cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>K_D (nM)</td>
<td>B_{max} (pmol mg^-1 protein)</td>
</tr>
<tr>
<td>CHO-m2</td>
<td>0.36 (0.04)</td>
<td>0.96 (0.14)</td>
</tr>
<tr>
<td>CHO-m3</td>
<td>0.12 (0.01)</td>
<td>1.84 (0.10)</td>
</tr>
</tbody>
</table>
Figure 3.2
[\textsuperscript{3}H]-NMS competition binding curves for CHO-m2 and CHO-m3 cell membranes for the mACH receptor antagonists: atropine (a), darifenacin (b), oxybutynin (c) and tolterodine (d).
Data are expressed as mean percent of control specific binding ± s.e.m., n ≥ 3.
Figure 3.2 (cont.)

$[^3]$H-NMS competition binding curves for CHO-m2 and CHO-m3 cell membranes for the mACH receptor antagonists: atropine (a), darifenacin (b), oxybutynin (c) and tolterodine (d).

Data are expressed as mean percent of control specific binding ± s.e.m., n ≥ 3.
Table 3.2 Binding affinity constant ($K_i$) and Hill slope estimates for mACh receptor antagonists at membrane homogenates and intact CHO-m2 and CHO-m3 cells. Data are expressed as mean (s.e.m.) values from $n \geq 3$ experiments.* indicates value significantly different from unity ($p < 0.05$; F-test).

<table>
<thead>
<tr>
<th></th>
<th>membranes</th>
<th></th>
<th>intact cells</th>
<th>Selectivity m3 vs m2</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CHO-m2</td>
<td>CHO-m3</td>
<td>$K_i$, nh</td>
<td>CHO-m2</td>
<td>CHO-m3</td>
<td>$K_i$, nh</td>
</tr>
<tr>
<td>Atropine</td>
<td>9.02 (0.09)</td>
<td>1.05 (0.05)</td>
<td>1.28</td>
<td>9.13 (0.10)</td>
<td>0.91 (0.06)</td>
<td>1.02 (0.05)</td>
</tr>
<tr>
<td>Darifenacin</td>
<td>7.83 (0.05)</td>
<td>0.93 (0.09)</td>
<td>31.3</td>
<td>6.97 (0.07)</td>
<td>0.98 (0.07)</td>
<td>14.8</td>
</tr>
<tr>
<td>Oxybutynin</td>
<td>8.01 (0.09)</td>
<td>0.95 (0.09)</td>
<td>8.04</td>
<td>7.21 (0.09)</td>
<td>0.99 (0.01)</td>
<td>1.13 (0.03)</td>
</tr>
<tr>
<td>Tolterodine</td>
<td>8.69 (0.05)</td>
<td>0.84 (0.02)</td>
<td>0.98</td>
<td>8.49 (0.12)</td>
<td>0.92 (0.03)</td>
<td>0.15</td>
</tr>
</tbody>
</table>
Darifenacin at M2 receptors was 7.60 ± 0.18 in the presence of NaCl/MgCl2, compared with 7.83 ± 0.05 in HEPES alone. At the M3 receptor, darifenacin displayed a mean pKi value of 9.25 ± 0.07 in the presence of NaCl/MgCl2, while in HEPES alone its affinity was 9.33 ± 0.07. The presence of salt in the buffer solution therefore seemed to have a negligible influence on the binding affinity of darifenacin for the M2 and M3 mACh receptors.

3.2.1.2 Intact, adherent CHO-m2 and CHO-m3 cells

Figure 3.3 displays representative [3H]-NMS saturation binding curves at intact CHO-m2 (a) and CHO-m3 (b) cells. Mean expression level (Bmax) and binding affinity constant (Kd) estimates for each cell line are given in Table 3.1. In contrast to data obtained in membrane homogenates of the same cells (see section 3.2.1.1), [3H]-NMS failed to display any significant selectivity between M2 and M3 mACh receptor subtypes. However, Bmax estimates obtained in intact cells were in good agreement with those determined in membrane homogenates (see Table 3.1).

[3H]-NMS competition assays were performed with both intact CHO-m2 and CHO-m3 cells, for each of the mACh receptor antagonists atropine, darifenacin, oxybutynin and tolterodine. Mean competition binding curves are shown in Figure 3.4 and mean binding affinity estimates (pKi), Hill slope values (nH) and receptor subtype selectivities are summarized in Table 3.2. As was found in membrane homogenates, darifenacin displayed marked selectivity (14.8 fold; p < 0.001) for the M3 over the M2 mACh receptor. However, in contrast to data obtained in membranes, oxybutynin displayed no significant selectivity between M2 and M3 receptors expressed in intact, adherent cells. In addition, atropine (5.1 fold; p < 0.01) and tolterodine (6.6 fold; p < 0.01) each exhibited higher affinity for the M2 receptor than for the M3 receptor expressed in intact cells, contrasting with their non-selective profile in membrane homogenates. As seen in experiments performed in membrane homogenates of CHO-m2 and CHO-m3 cells, slope factors estimated in intact cells generally approximated unity.
Figure 3.3
$[^3]H$-NMS saturation binding curves for adherent, intact CHO-m2 (a) and CHO-m3 (b) cells.
Data are expressed as specific binding in pmol receptor expression mg$^{-1}$ protein
and are representative curves of 3 or more experiments.
Figure 3.4
$[^3]H$-NMS competition binding curves for intact CHO-m2 and CHO-m3 cell monolayers for the mACh receptor antagonists: atropine (a), darifenacin (b), oxybutynin (c) and tolterodine (d).
Data are expressed as mean percent of control specific binding ± s.e.m., $n \geq 3$. 

(a) 
% specific binding

-12 -11 -10 -9 -8 -7 -6 -5 -4
[atropine] (log M)

M2
M3

(b) 
% specific binding

-12 -11 -10 -9 -8 -7 -6 -5 -4
[darifenacin] (log M)

M2
M3
Figure 3.4 (cont.)
[3H]-NMS competition binding curves for intact CHO-m2 and CHO-m3 cell monolayers for the mACh receptor antagonists: atropine (a), darifenacin (b), oxybutynin (c) and tolterodine (d).
Data are expressed as mean percent of control specific binding ± s.e.m., n ≥ 3.
3.2.2 Functional (second messenger) assays in intact, adherent CHO-m2 and CHO-m3 cells

Figure 3.5 (a) illustrates the protocol adopted to examine the ‘functional’ affinities of mACh receptor antagonists at the M2 receptor, showing a representative curve for the inhibition of forskolin-stimulated cAMP accumulation by MCh in CHO-m2 cells (EC50 = 0.27 ± 0.03 nM; n = 7). This allowed the estimation of an approx. EC70 concentration (1 nM) (Figure 3.5 (a)) from which antagonist inhibition curves were constructed (Fig. 3.5 (b)). Functional affinity (pKa) estimates were derived from these curves as described earlier (see Methods) and are summarized in Table 3.3, along with Hill slope (nH) estimates for each curve.

Figure 3.6 (a) shows a concentration-response curve for MCh-stimulated accumulation of [3H]-inositol phosphates (in the presence of Li+) in CHO-m3 cells (EC50 = 1.26 ± 0.14 nM; n = 6). An approx. EC70 concentration (3 nM) was estimated from this curve and used to construct antagonist inhibition curves (Figure 3.6 (b)). Affinity estimates were calculated as described above and are summarized as pKi values in Table 3.3. Estimates of Hill slopes (nH) and selectivity for M3 versus M2 receptor-mediated inhibition of functional responses are also summarized in Table 3.3.

Comparing the functional affinity estimates at M2 and M3 mACh receptors, darifenacin was again found to be the most M3-selective antagonist (32.4 fold; p < 0.01), followed by oxybutynin which was modestly, but significantly M3-selective (3.4 fold; p < 0.05). Atropine (2.1 fold) and tolterodine (2.8 fold) were not significantly subtype-selective in functional assays (Table 3.3). Slope factor estimates did not differ significantly from unity in any case.

3.2.3 mACh receptor antagonist affinities for intact, adherent CHO-m2m3 cells

3.2.3.1 Radioligand binding studies in intact CHO-m2m3 cells

Figure 3.7 shows a representative [3H]-NMS saturation binding curve for intact CHO-m2m3 cells (clone B2; see Hornigold et al., 2003). The mean [3H]-NMS binding affinity constant (Kd) for these cells, derived from 3 separate experiments, was 0.27 ± 0.05 nM. The total mACh receptor expression level in CHO-m2m3 cells was also
Figure 3.5 Agonist and antagonist effects on forskolin-stimulated cyclic AMP responses in CHO-m2 cells
(a) Representative curve illustrating the inhibition of forskolin (10 μM)–stimulated cyclic AMP accumulation by MCh. Basal cyclic AMP accumulation (in the absence of forskolin) in CHO-m2 cells was 1.6 ± 0.2 pmol mg⁻¹ protein. Mean forskolin (10 μM)-stimulated cyclic AMP accumulation was 1038.8 ± 100.2 pmol mg⁻¹ protein and this was inhibited by > 90 % by MCh. Arrow indicates MCh concentration (1 μM) selected for generation of antagonist-inhibition curves.
(b) Effect of mACh receptor antagonists on 1 μM MCh-induced inhibition of 10 μM forskolin-stimulated cyclic AMP production in CHO-m2 cells. Results are expressed as mean percent of maximal inhibition of forskolin (10 μM) -induced cyclic AMP accumulation ± s.e.m., n ≥ 3.
Table 3.3 Summary of functional affinity constants (pKₐ) and Hill slopes for the reversal of agonist mediated responses in CHO-m2 and CHO-m3 cells by mACh receptor antagonists. Data are expressed as mean (s.e.m.) values from n ≥ 3 experiments.

<table>
<thead>
<tr>
<th></th>
<th>CHO-m2</th>
<th>CHO-m3</th>
<th>Selectivity m3 vs m2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pKₐ</td>
<td>n_h</td>
<td>pKₐ</td>
</tr>
<tr>
<td>Atropine</td>
<td>9.37 (0.08)</td>
<td>1.07 (0.02)</td>
<td>9.04 (0.08)</td>
</tr>
<tr>
<td>Darifenacin</td>
<td>7.30 (0.17)</td>
<td>1.00 (0.17)</td>
<td>8.81 (0.14)</td>
</tr>
<tr>
<td>Oxybutynin</td>
<td>7.21 (0.11)</td>
<td>0.91 (0.14)</td>
<td>7.74 (0.10)</td>
</tr>
<tr>
<td>Tolterodine</td>
<td>8.59 (0.09)</td>
<td>0.86 (0.03)</td>
<td>8.15 (0.11)</td>
</tr>
</tbody>
</table>
Figure 3.6 Agonist and antagonist effects on [H]-inositol phosphate accumulation in CHO-m3 cells
(a) Representative curve illustrating the stimulation of [H]-IP₃ accumulation in response to MCh in CHO-m3 cells. Basal [H]-IP₃ accumulation in CHO-m3 cells was 396.5 ± 70.9 d.p.m. mg⁻¹ protein and the mean maximal response to MCh (Eₘₐₓ) was 5037.5 ± 1460.2 d.p.m. mg⁻¹ protein. Arrow indicates MCh concentration (3 μM) selected for generation of antagonist-inhibition curves.
(b) Effect of mACh receptor antagonists on [H]-IP₃ accumulation in response to 3 μM methacholine in CHO-m3 cells. Results are expressed as mean percent of maximal stimulation ± s.e.m., n ≥ 3.
Figure 3.7
$[^3\text{H}]-\text{NMS}$ saturation binding curves for adherent, intact CHO-m2m3 cells. Data are expressed as specific binding in pmol receptor expression mg$^{-1}$ protein and is a representative curve of 3 or more experiments.
estimated in each experiment, yielding a mean $B_{\text{max}} = 3.84 \pm 0.17$ pmol mg protein

In order to dissect the relative proportions of $M_2$ and $M_3$ receptors comprising the mACh receptor population in CHO-m2m3 cells, competition radioligand binding assays were performed, using mACh receptor antagonists known to exhibit different affinities for these two subtypes (darifenacin and methoctramine) as well as the non-selective antagonist atropine. The resulting data were fitted to either one- or two-site competition curves and binding affinity constants ($pK_i$) were calculated as described above (see section 3.2.1.1). These $pK_i$ values, along with the proportion of the total mACh receptor sites corresponding to the high affinity population for each ligand, are summarized in Table 3.4. Hill slope ($n_H$) estimates for one-site fits are also included for each antagonist. Darifenacin and methoctramine both exhibit highly significantly shallow slope factors ($p < 0.001$) when fitted with a one-site model, indicative of a heterogeneous receptor population recognized with different affinities by these ligands (Table 3.4).

Figure 3.8 displays mean competition binding curves for atropine (a), methoctramine (b) and darifenacin (one-site fit (c) and two-site fit (d)). Both darifenacin and methoctramine were significantly better fitted to the two-site model than the one-site model ($p < 0.001$). Affinity estimates for high and low affinity sites recognized by methoctramine in CHO-m2m3 cells correspond well with its published affinity estimates for $M_2$ and $M_3$ receptors, respectively (Eglen et al., 1996a). Comparison of high and low affinity sites for darifenacin with both published values and those determined in CHO-m2 and CHO-m3 cells (see section 3.2.1.2), indicate that as expected, the high affinity site correlates best with an $M_3$ population (though it is approximately 10 fold higher than the calculated $pK_i$ for darifenacin in intact CHO-m3 cells [9.10 vs 8.14]) while the low affinity site most closely resembles an $M_2$ receptor population.

The relative proportions of high and low affinity sites for methoctramine and darifenacin differ in their estimation of the ratio of $M_2$:$M_3$ receptor expressed in CHO-m2m3 cells. Based on the competition binding curve for darifenacin, it is estimated that approx. 40 % of the total mACh receptor population is of the $M_3$ subtype (Table 3.4). In contrast, similar analysis of data with methoctramine indicate that approx. 80 % of the muscarinic receptor complement is $M_3$. 

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Table 3.4 Binding affinity constant ($K_i$) derived from one or two site analysis and Hill slope estimates (from one site fits) for mACH receptor antagonists at intact CHO-m2m3 cells. Data are expressed as mean (s.e.m.) values from $n \geq 3$ experiments. * indicates value significantly different from unity ($p < 0.05$; F-test).

<table>
<thead>
<tr>
<th></th>
<th>$pK_i$</th>
<th>% high affinity site</th>
<th>$n_s$ (1 site)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Atropine</strong></td>
<td>8.40 (0.04)</td>
<td>-</td>
<td>0.92 (0.01)</td>
</tr>
<tr>
<td><strong>Darifenacin</strong></td>
<td></td>
<td>9.10 (0.25)</td>
<td>40.2 (4.1)</td>
</tr>
<tr>
<td><strong>Methoctramine</strong></td>
<td></td>
<td>7.42 (0.33)</td>
<td>19.9 (3.4)</td>
</tr>
</tbody>
</table>
Figure 3.8
[^H]-NMS competition binding curves at intact CHO-m2m3 cell monolayers for the muscarinic acetylcholine receptor antagonists: atropine (one-site fit) (a); methoctramine (two-site fit) (b); darifenacin (one-site fit) (c) and darifenacin (two-site fit) (d). Data are expressed as mean percent of control specific binding ± s.e.m., n ≥ 3.
Figure 3.8 (cont.)

$[^{3}H]$-NMS competition binding curves at intact CHO-m2m3 cell monolayers for the muscarinic acetylcholine receptor antagonists: atropine (one-site fit) (a); methoctramine (two-site fit) (b); darifenacin (one-site fit) (c) and darifenacin (two-site fit) (d). Data are expressed as mean percent of control specific binding ± s.e.m., n ≥ 3.
3.2.3.2 Functional (second messenger) studies in intact CHO-m2m3 cells

Figure 3.9 (a) shows a concentration-response curve for MCh-stimulated accumulation of $[^{3}H]$-inositol phosphates (in the presence of Li$^{+}$) in CHO-m2m3 cells ($EC_{50} = 0.54 \pm 0.05 \mu M; n = 3$). An approx. $EC_{50}$ concentration (3 $\mu M$) was estimated from this curve and used to construct antagonist inhibition curves (Figure 3.9 (b)). Affinity estimates were calculated as described above and are summarized as pK$_A$ values in Table 3.5, along with Hill slope ($n_h$) estimates for each ligand.

Comparison of the antagonist functional affinity (pK$_A$) values derived for the inhibition of $[^{3}H]$-inositol phosphate accumulation in CHO-m2m3 cells with those generated in CHO cells expressing M$_3$ receptors alone (Table 3.3), indicates that the co-expression of M$_2$ receptors appears to have no significant effect upon the functional affinity of any of the antagonists investigated. Atropine, darifenacin and oxybutynin all displayed approx. equivalent affinity in CHO-m2m3 cells as in CHO-m3 cells. Tolterodine displayed slightly higher affinity in CHO-m2m3 cells than in CHO-m3 cells ($p < 0.05$). Hill slope estimates for the inhibition curves of all four antagonists showed a tendency towards being greater than unity, but this was only statistically significant in the case of atropine ($p < 0.05$).
Figure 3.9
Agonist and antagonist effects on $[^3]$H-inositol phosphate accumulation in CHO-m2m3 cells (a) Representative curve illustrating the stimulation of $[^3]$H-IP$_x$ accumulation in response to MCh in CHO-m2m3 cells. Basal $[^3]$H-IP$_x$ accumulation in CHO-m2m3 cells was 16451.1 ± 1110.6 d.p.m. mg$^{-1}$ protein, while the mean maximal response to MCh (E$_{max}$) was 330276.3 ± 4833.6 d.p.m. mg$^{-1}$ protein. Arrow indicates MCh concentration (3 μM) selected for generation of antagonist-inhibition curves. (b) Effect of mACh receptor antagonists on $[^3]$H-IP$_x$ accumulation in response to 3 μM methacholine in CHO-m2m3 cells. Results are expressed as mean percent of maximal stimulation ± s.e.m., n ≥ 3.
Table 3.5 Summary of functional affinity constants (pKₐ) and Hill slopes for the reversal of agonist mediated responses in CHO-m2m3 cells by mACh receptor antagonists. Data are expressed as mean (s.e.m.) values from n ≥ 3 experiments. * indicates value significantly different from unity (p < 0.05; F-test).

<table>
<thead>
<tr>
<th></th>
<th>pKₐ</th>
<th>nₙ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atropine</td>
<td>9.02 (0.07)</td>
<td>* 1.58 (0.09)</td>
</tr>
<tr>
<td>Darifenacin</td>
<td>8.84 (0.07)</td>
<td>1.15 (0.30)</td>
</tr>
<tr>
<td>Oxybutynin</td>
<td>7.68 (0.18)</td>
<td>1.42 (0.20)</td>
</tr>
<tr>
<td>Tolterodine</td>
<td>8.55 (0.10)</td>
<td>1.16 (0.13)</td>
</tr>
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</table>
3.3 Discussion

This study has confirmed the selectivity of the mACh receptor antagonist darifenacin for the M₃ receptor subtype. Previous studies have demonstrated that darifenacin binds to the cloned human M₃ muscarinic receptor with sub-nanomolar affinity and displays up to 60 fold selectivity for this subtype over the M₂ receptor (e.g. Wallis & Napier, 1999). Data presented here in membrane homogenates prepared from CHO cells stably expressing the human M₂ or M₃ mACh receptors indicate that the M₃ selectivity of darifenacin is approximately 30-fold. Although our data suggest a slightly lesser M₃/M₂ selectivity for darifenacin, it does compare reasonably well with published selectivity estimates of 59-fold (Wallis & Napier, 1999) and 47-fold (Gillberg et al., 1998) and was clearly the most M₃ selective agent tested in this study.

Oxybutynin was less selective for M₃ versus M₂ receptors, but the 8-fold higher affinity for the M₃ subtype than the M₂ subtype is in reasonable agreement with published estimates. Nilvebrant et al. (1997a) reported that oxybutynin displayed approx. 10-fold M₃/M₂ selectivity in CHO cell lines, while Wallis & Napier (1999) found oxybutynin to have approx. 12-fold higher affinity for the M₃ receptor. The non-selective nature of atropine and tolterodine was confirmed in this study, in line with previous work with these compounds (e.g. Nilvebrant et al., 1997a; Caulfield & Birdsall, 1998).

Comparison of binding affinities derived in membrane homogenates with those found in intact cells highlights some interesting contrasts. Whereas [³H]-NMS was 3-fold selective for the M₃ receptor in membrane homogenates, no such selectivity was observed when similar assays were performed using intact, adherent cells. [³H]-NMS displayed significantly lower affinity for intact CHO-m3 cells than for membranes prepared from the same cells, while little difference was observed in the affinity of [³H]-NMS for the M₂ receptor between membranes and intact cells. Although generally considered to be a non subtype-selective radiolabel, evidence has previously been presented that [³H]-NMS can exhibit some degree of selectivity between muscarinic receptor subtypes, particularly for M₃ over M₂ receptors (Waelbroeck et al., 1986). This is supported by our data in CHO cell membrane homogenates, though the M₃ selectivity of [³H]-NMS is not observed in intact cells.
In general, antagonist binding affinities at both receptor subtypes were lower in intact cells than in membrane homogenates prepared from the same cells. However, affinity estimates were not uniformly affected by the change in environment between intact cell and isolated membranes. For instance, the affinity of the M2 receptor for darifenacin and oxybutynin was greatly enhanced in membrane preparations (by 6-7 fold) compared with intact cells, but the affinity of this receptor subtype for atropine and tolterodine was relatively unaffected. Alterations in affinity were generally more pronounced at the M3 receptor with at least 10 fold shifts observed between membranes and intact cells for darifenacin, oxybutynin and tolterodine. The difference in affinity between the two preparations therefore appears to be specific to the ligand/receptor pairing and this is reflected in the differences in M3/M2 selectivity observed between intact cell and isolated membrane binding assays (see Table 3.2).

Thus, although less pronounced than in membrane homogenates, darifenacin displayed marked selectivity for the M3 receptor (14.8 fold) in intact cells. Oxybutynin, however, was non-selective in intact cells in contrast to the 8 fold M3 selectivity observed in membranes. Atropine and tolterodine also both exhibited altered selectivity profiles, displaying significantly higher affinity for the M2 receptor than the M3 subtype in intact cells. This differs somewhat from the majority of published work that reports these compounds to be non-selective (e.g. Nilvebrant et al., 1997a; Caulfield and Birdsell, 1998). However, some degree of receptor subtype selectivity has been observed for both atropine and tolterodine in binding studies, albeit selectivity for the M3 subtype and therefore the opposite of that found in this study (Buckley et al., 1989; Wallis & Napier, 1999). It should be noted that the majority of studies relating to binding affinities of muscarinic antagonists employ radioligand binding assays with membrane homogenates. It is therefore interesting to note that data presented in this study using isolated membranes are in good agreement with the literature, while affinities derived against intact cells differ significantly in some cases.

Similar differences in binding affinity between membrane homogenate and intact cell assays have been observed for the α1A-adrenoceptor (Williams et al., 1996). Affinity estimates for a range of antagonists were found to be typical of the ‘classical’ α1A-adrenoceptor when performed in membrane homogenates, but were more consistent with the α1L-adrenoceptor phenotype (defined by low affinity for prazosin and a range
of other adrenoceptor antagonists) when performed in intact cells (Williams et al., 1996). This is particularly interesting, since the α1L-adrenoceptor has only been defined pharmacologically, as the cDNA for a fourth α1-adrenoceptor has never been isolated. Convincing evidence exists that the α1L adrenoceptor is in fact the functional phenotype of the α1A-adrenoceptor gene product (Ford et al., 1997). If this is the case, it would seem that the binding affinities derived using whole cells provide a better indication of the ability of the antagonists to inhibit functional responses mediated by the α1A/α1L-adrenoceptor. It should be noted that both α1B- and α1D-adrenoceptors exhibit similar binding affinities in membranes and intact cells (Williams et al., 1996) and equivalent functional and binding affinity profiles (Ford et al., 1997), indicating that the affinity state pleiotropism observed for the α1A/α1L-adrenoceptor is specific to that subtype. It is also important to consider that the observed differences in binding (and functional) affinity profiles do not merely reflect 'frame-shifted' affinities between preparations, as 3 of the 8 antagonists investigated displayed similar affinities in membrane binding assays as in functional assays/whole cell binding studies (Williams et al., 1996; Ford et al., 1997). Therefore, whatever the molecular basis for this phenomenon, it is only apparent in the case of a specific sub-set of α-adrenoceptor antagonists.

It has been reported that the ionic composition of the assay buffer in which radioligand binding assays are performed, can significantly influence the apparent binding affinities observed (Pedder et al., 1991). It is therefore possible that the differences in binding affinity observed between intact cell and membrane assays are due to the use of low ionic strength buffer (e.g. 20 mM HEPES in this investigation) in membrane binding assays, compared with the more 'physiological' buffers usually employed in intact cell experiments (e.g. KHB in the present study). However, inclusion of NaCl (100 mM) and MgCl2 (10 mM) in the HEPES buffer had no significant influence on the affinity of darifenacin for CHO-m2 or CHO-m3 membranes. Of course, it cannot be ruled out that other components in KHB could influence the binding of antagonists to the muscarinic receptors, or that ligands other than darifenacin might be more sensitive to the ionic composition of their environment. However, data presented here suggest that the choice of buffer has little influence upon the equilibrium binding constants of the mACh receptor antagonists investigated.
Williams et al. (1996) performed binding experiments with membrane homogenates of α1-adrenoceptor-expressing CHO cells at a lower temperature (20 °C versus 37 °C) than intact cell experiments. The potential influence of temperature upon equilibrium binding assays has been highlighted (e.g. Pedder et al., 1991), so it is possible that this may have contributed to the variation in the observed affinity constants. However, both intact cell and membrane homogenate binding assays were performed at 37°C in the present study, eliminating the possibility that temperature may have influenced the observed binding affinities.

It therefore seems that intact cellular conditions may in some way modulate the binding of muscarinic antagonists to the M2 and M3 receptors. The influence of the membrane lipid environment upon GPCR binding characteristics has been documented. Berstein et al. (1989) reported a loss of mACh receptor subtype selectivity of pirenzepine upon receptor solubilisation that was restored after reconstitution of receptors into lipid vesicles. Rinken (1994) found that solubilisation of M1 to M4 receptors decreased the binding affinity of most antagonists, with most ligands also displaying considerably less subtype selectivity than seen in experiments with non-solubilised (membrane associated) receptors. A more recent study reported that the solubilised M2 receptor exists predominantly in the active (Rg) state, based upon the interaction of antagonists and the sulphydryl-specific alkylating agent N-ethylmaleimide with membrane bound and solubilised receptors (Sum et al., 2002). This may explain the lower affinities observed for "antagonists" in earlier studies using solubilised receptors, as antagonists which are in fact inverse agonists would be expected to bind with lower affinity to the active state (Rg) of the receptor. These studies also indicate an important role for the membrane lipid bilayer in constraining GPCRs in the inactive conformation. The specificity of such interactions is suggested by the differential effects upon ligand binding affinities of reconstituting muscarinic receptors into vesicles of differing lipid composition (Berstein et al., 1989). However, unless the membrane preparation process employed in the present study sufficiently disrupts lipid/protein interactions or depletes the membranes of critical lipid components, it would seem unlikely that such interactions could explain the differences in binding affinity observed here.

In second messenger functional studies, darifenacin was again the most M3 selective antagonist, inhibiting M3 receptor-mediated [3H]-inositol phosphate accumulation
with significantly higher (32.4 fold) affinity than its antagonism of M2 receptor-mediated inhibition of forskolin-stimulated cyclic AMP accumulation. Oxybutynin was moderately M3 selective (3.4 fold; intermediate between its selectivities in membranes and intact cell binding assays), while atropine and tolterodine were slightly, but not statistically significantly M2 selective. Absolute functional affinity estimates tended to be intermediate between binding affinity estimates using membranes and intact cells at each receptor subtype. This would suggest that in contrast to the scenario with the α1-adrenoceptor, estimates of binding affinity in recombinant cell lines, even using intact cells, do not fully reflect the ability of muscarinic antagonists to inhibit second messenger responses. Data presented here highlight the requirement for thorough functional characterization of ligands, particularly in the drug discovery process, or else the potential efficacy of candidate compounds might easily be over- or under-estimated based upon binding studies alone. Equally, consideration of the assay system(s) used is of obvious importance when comparing affinity estimates for a given compound and ascribing "subtype-selectivity", particularly to muscarinic ligands.

Few previous studies have investigated the functional activities of darifenacin, tolterodine and oxybutynin against recombinantly expressed mACh receptors as has been done here. Comparison of our affinity estimates with previously published values from native tissues, even those where the response is reportedly mediated by a single mACh receptor subtype, are complicated by the apparent tissue-dependent functional selectivity exhibited by these muscarinic antagonists (e.g. Wallis & Napier, 1999; see section 1.3.6.4 and Chapter 4 for further discussion). For instance, Newgreen & Naylor (1996) reported that darifenacin displayed functional affinities of 8.66 and 7.0 in guinea pig urinary bladder and submandibular salivary gland, even though both functional responses (bladder contraction and 86Rb efflux in salivary gland) are believed to be predominantly or exclusively mediated by the M3 receptor. Functional affinity estimates derived in recombinant cells can therefore only give us limited information regarding the pharmacology of these muscarinic antagonists and it is clear that an examination of their actions in a more physiological preparation will be required to understand the nature of their functional selectivity.

One of the obvious shortcomings of the CHO cell models used thus far is that they over-express homogeneous populations of a single mACh receptor subtype. Many
clinically relevant tissues and cells express multiple mACh receptor subtypes. For instance, a wide variety of smooth muscle cell types (including bladder detrusor smooth muscle, the primary site of action of anti-muscarinic OAB therapies) co-express M₂ and M₃ mACh receptor subtypes (Eglen et al., 1994; 1996a). Although these two subtypes preferentially couple to distinct G protein subpopulations (Gᵢₒ and Gₚq11, respectively), “cross-talk” between the two signalling pathways has been well documented at several points downstream of G protein activation (Eglen et al., 1994; Selbie & Hill, 1998). For instance, M₂ receptors can influence M₃-mediated phosphoinositide turnover through Gₚq-derived βγ-subunit activation of phospholipase C isozymes (Rhee, 2001).

We therefore decided to examine the functional affinities of atropine, darifenacin, tolterodine and oxybutynin in a CHO cell line co-expressing M₂ and M₃ receptors (CHO-m₂m₃; Homigold et al., 2003), in order to determine whether the expression of an M₂ receptor population influenced the ability of these antagonists to inhibit muscarinic agonist-mediated phosphoinositide turnover. Initial competition [³H]-NMS binding experiments designed to determine the relative proportions of M₂ and M₃ receptors expressed in the CHO-m₂m₃ cell line, yielded conflicting results. Darifenacin and methoctramine both recognised two distinct muscarinic receptor populations (i.e. competition binding curves were fitted significantly better to a two-site model), with affinities that correlate well with previously determined (both in this study and in others: e.g. Eglen et al., 1996a) values for these ligands at the M₂ and M₃ mACh receptors. However, while data with methoctramine indicate that approx. 80 % of the total mACh receptor population is of the M₃ subtype (in good agreement with the initial characterisation of the CHO-m₂m₃ (B2) clone which indicated a 27:73 M₂:M₃ ratio; Homigold et al., 2003), analysis of the darifenacin competition binding curves suggests that only 40 % of the total muscarinic receptor complement is of the M₃ subtype. It is not apparent why the estimates with darifenacin and methoctramine should differ by such a margin, but it is possible that the relatively low selectivity of darifenacin (14.8 fold), observed in intact cell binding assays (in CHO-m₂ and -m₃ cells), may have led to inaccuracies in the fitting of the double logistic model. However, the affinity estimates for the two sites recognised by darifenacin in CHO-m₂m₃ cells indicate greater selectivity (almost 100 fold), with the high affinity population (presumably M₃ receptors) displaying a pKᵢ value of 9.10. This is almost
10-fold higher than the apparent affinity of darifenacin for the M₃ receptor population expressed in singly expressing CHO-m3 cells (pKᵢ = 8.14) and is more in line with darifenacin's estimated M₃ affinity in membrane homogenates (pKᵢ = 9.33). Whether this apparent difference in M₃ receptor affinity in intact cells reflects a clonal difference between CHO-m3 and CHO-m2m3 cell lines or whether the co-expression of M₂ and M₃ receptors has in some way altered the affinity of the “M₃” population for darifenacin is not clear from these binding studies.

The mean EC₅₀ for methacholine-stimulated [²H]-inositol phosphate accumulation in CHO-m2m3 cells (0.54 μM) was significantly lower than that observed in CHO-m3 cells (1.26 μM). This may be due to the higher M₃ receptor expression level in the co-expressing cell line: total mACh receptor expression = 3.84 pmol mg⁻¹ protein; therefore approx. 2.8 – 3.0 pmol M₃ receptor mg⁻¹ protein (based upon 73-80 % M₃) in CHO-m2m3 cells versus 1.75 pmol mg⁻¹ protein in CHO-m3 cells. However, previous work in these cell lines found methacholine to be more potent at stimulating Ins(1,4,5)P₃ accumulation in CHO-m2m3 cells than in CHO-m3 cells, even though estimation of the total mACh receptor expression level (2.16 pmol mg⁻¹ protein) was lower than that estimated in the present study (Hornigold et al., 2003). Also, following pertussis toxin treatment to inactivate members of the Gᵢₒ family of G proteins, the Ins(1,4,5)P₃ response in CHO-m2m3 cells closely resembled that of CHO-m3 cells (Hornigold et al., 2003). It would therefore seem that signalling through Gᵢₒ proteins, presumably activated via the M₂ receptor, enhances the potency of the agonist-mediated Ins(1,4,5)P₃ response in CHO-m2m3 cells, so it follows that M₂ receptor activation would be expected to be involved in the [²H]-inositol phosphate response in these cells too.

In functional experiments in CHO-m2m3 cells, however, darifenacin inhibited methacholine-stimulated [²H]-inositol phosphate accumulation with almost identical affinity to that observed in CHO-m3 cells. Atropine and oxybutynin also antagonised agonist-mediated phosphoinositide turnover with similar affinities in the two cell lines. Only tolterodine displayed any difference in functional affinity, exhibiting significantly higher affinity in CHO-m2m3 cells than in CHO-m3 cells (p < 0.05). Although the affinity of tolterodine may suggest a role for the M₂ receptor population in the accumulation of [²H]-inositol phosphates in CHO-m2m3 cells, the apparent lack of discrimination between CHO-m2m3 and CHO-m3 responses by the more
clearly M₂/M₃ selective oxybutynin and darifenacin would argue against this. Indeed, Griffin et al. (2003) reported that the pharmacological antagonism of oxotremorine-M-stimulated [³H]-inositol phosphate accumulation displayed similar properties in CHO cells co-expressing M₂ and M₃ receptors as in those expressing M₃ receptors alone.

Interestingly, the Hill slopes for each of the antagonists used in the present study tended to be slightly greater than unity, perhaps indicative of a cooperative interaction in the inhibition of phosphoinositide turnover. Although seemingly steeper than the corresponding slopes in CHO-m3 (and CHO-m2) cells, only in the case of atropine was the mean slope factor statistically significantly greater than unity. It is therefore not possible categorically to invoke a direct role for the M₂ receptor in the [³H]-inositol phosphate response of CHO-m2m3 cells based upon these data.

Previous work in a number of different smooth muscle types co-expressing M₂ and M₃ receptors has implicated roles for M₂ receptors in muscarinic agonist-mediated contraction, either via inhibition of the relaxation to agents that increase intracellular cyclic AMP and/or the potentiation of M₃ receptor-mediated contractions, perhaps by effects on calcium-dependent ion channels (see Ehlert, 2003 for review). Mathematical modelling studies suggest that the competitive antagonism of a muscarinic response interactively mediated by two distinct receptor subtypes (e.g. M₂ and M₃ receptors in this case) will tend to resemble the pharmacological profile of the directly acting receptor (M₃) and not the conditionally acting subtype (M₂) (Sawyer & Ehlert, 1999; Ehlert, 2003). The fact that the pharmacological profile of the phosphoinositide response in CHO-m2m3 cells is mostly indistinguishable from that observed in CHO-m3 cells does not therefore exclude the possibility that the M₂ receptor may play a role in this response.

This study has demonstrated that the equilibrium binding affinity, and as a result the apparent subtype selectivity profile, of mACh receptor antagonists may be dependent upon the receptor milieu and the conditions under which binding assays are performed. In addition, binding affinities of muscarinic antagonists were not necessarily predictive of the affinity with which functional (second messenger) responses were inhibited and more importantly, differences between binding and functional selectivity profiles were not merely due to a ‘frame-shifting’ between assays as all ligands were not uniformly affected. Co-expression of an M₂ receptor
population with an M₃ receptor complement appeared to have little effect upon the capability of mACh receptor antagonists to inhibit muscarinic agonist-stimulated PLC activity. However, as this cell line does not truly reflect the relative proportions of M₂ and M₃ receptors expressed in a variety of smooth muscles (see Eglen et al., 1996), a role for the predominantly Gₒ₅-coupled M₃ receptor in PLC signalling in vivo cannot be ruled out.
Chapter 4: An investigation of the functional affinities of mACh receptor antagonists in guinea-pig urinary bladder and salivary glands.

4.1 Introduction

Overactive bladder (OAB) is a common and under-reported disorder, with recent estimates indicating that approx. 17% of men and women over the age of 40 suffer one or more of the symptoms of frequency, urgency and urge incontinence (Milsom et al., 2001). The prevalence of OAB symptoms are known to increase with advancing age, yet only a minority of sufferers were found to be receiving treatment for their symptoms (Milsom et al., 2001). This is largely due to the often debilitating side-effect profiles of OAB treatments, in particular muscarinic receptor antagonists.

The cholinergic nervous system is the major pathway by which bladder contraction is initiated in humans (Andersson, 1993). Molecular techniques have identified five muscarinic acetylcholine (mACh) receptor subtypes (m1-m5), while pharmacological data can distinguish only four subtypes, denoted as M1-M4 (Caulfield and Birdsall, 1998). Both M2 and M3 mACh receptors have been identified in bladder (detrusor) smooth muscle at the level of mRNA (Yamaguchi et al., 1996) and protein, with quantitative immunoprecipitation demonstrating a 75-90% predominance of the M2 subtype in all species studied (Wang et al., 1995). However, pharmacological characterization of the mACh receptors mediating bladder contraction indicate the predominant involvement of the minority M3 receptor population in a variety of species including rat (Wang et al., 1995; Longhurst et al., 1995), guinea-pig (Wang et al., 1995; Ikeda et al., 1999), and human (Chess-Williams et al., 2001; Fetscher et al., 2002).

The most widely prescribed treatments for OAB are mACh receptor antagonists with high affinity for the M3 receptor subtype. These include the non-subtype selective tolterodine and oxybutynin, which has, however, been reported to exhibit selectivity for the M3 subtype over other muscarinic receptors (Nilvebrant et al., 1997a). The clinical utility of mACh receptor antagonists has been limited by lack of selectivity, which leads to classical anti-muscarinic side-effects, such as dry mouth, tachycardia and blurred vision. Dry mouth is experienced by at least 50% of patients taking
oxybutynin and is the most common reason why at least 25% of patients discontinue medication (Yarker et al., 1995). This has led to the search for mACh receptor antagonists with not only greater selectivity for the M3 receptor, but specifically for mACh receptor agonist-mediated bladder contraction, as several anti-muscarinic side-effects are likely to result from antagonism of M3 receptors expressed elsewhere in the periphery (e.g. the salivary glands and gastrointestinal tract).

The mACh receptor antagonist darifenacin displays both high affinity (pKi = 9.12) and selectivity for the human M3 receptor (Wallis and Napier, 1999). Intriguingly, darifenacin has been reported to inhibit urinary bladder contraction at concentrations lower than those required to influence salivary secretion in vivo in both rat (Wallis and Napier, 1999) and dog (Gupta et al., 2002) despite both responses being predominantly or exclusively M3-mediated. In contrast, atropine inhibited both responses equipotently, while oxybutynin has been shown to be either non-selective (Newgreen and Naylor, 1996; Ikeda et al., 2002), or slightly selective for the salivation response (Nilvebrant et al., 1997a; Kobayashi et al., 2004).

Similar tissue-specific selectivity had earlier been reported between different smooth muscle types for other muscarinic receptor antagonists, including zamifenacin (Watson et al., 1995) and p-fluorohexahydrosiladifenidol (p-FHHSID) (Eglen et al., 1990). However, tissue-dependent selectivity may not only be a property of subtype-selective antagonists. Thus, tolterodine has been reported to display a greater functional affinity for urinary bladder than salivary gland, despite failing to exhibit selectivity between mACh receptor subtypes (Nilvebrant et al., 1997a). It has been proposed that such ‘functional selectivity’ could lead to a superior clinical side-effect profile in the management of OAB and may at least in part explain why tolterodine is equally as effective as oxybutynin for improving the symptoms of OAB, yet is better tolerated by patients (Malone-Lee et al., 2001).

The main aim of this study was to establish whether a range of subtype-selective and non-selective mACh receptor antagonists display in vitro selectivity for M3-mediated responses between urinary bladder and salivary gland, within the same species, at the level of second messenger generation. Darifenacin, along with the current OAB therapies oxybutynin and tolterodine and the classical non-selective muscarinic antagonist atropine, were investigated for their ability to inhibit mACh receptor
agonist-mediated phosphoinositide turnover in guinea-pig urinary bladder and submandibular salivary gland.

While it is generally accepted that acetylcholine-induced phosphoinositide hydrolysis and contraction are mediated almost exclusively by the $M_3$ receptor in bladder (Harriss et al., 1995; Chess-Williams et al., 2001), some controversy remains over the potential involvement of 'non-$M_3$' mACh receptors in cholinergic responses of salivary glands (Laniyonu et al., 1990; Watson et al., 1996). The mACh receptor subtype expression profiles of the major salivary glands (submandibular, parotid and sublingual) of the guinea-pig tissues were therefore investigated to clarify which muscarinic receptors might be involved in mediating cholinergic control of salivation. A range of the most subtype-selective muscarinic antagonists were utilized in competition radioligand binding assays against $[^3H]NMS$ to define which mACh receptor subtypes are expressed in guinea-pig urinary bladder, submandibular, parotid and sublingual salivary glands.
4.2 Results

4.2.1 Investigation of the functional selectivity of mACh receptor antagonists in guinea pig tissues

4.2.1.1 Characterisation of functional (second messenger) assays in guinea-pig urinary bladder and submandibular salivary gland

In cross-chopped, myo-[\textsuperscript{3}H]-inositol-loaded guinea-pig bladder, carbachol (CCh) caused a time- and concentration-dependent accumulation of [\textsuperscript{3}H]-IP\textsubscript{x} in the presence of LiCl (5 mM). Figure 4.1 (a) illustrates the time-course of the response to CCh (300 μM), which was linear up to 30 min. This time-point was chosen for all subsequent experiments. Figure 4.1 (b) shows a mean concentration-response curve for the accumulation of [\textsuperscript{3}H]-IP\textsubscript{x}; analysis of each curve yielded a mean pEC\textsubscript{50} value of 5.11 ± 0.08 (n = 9). The mean Hill slope of the concentration-response curves to CCh did not differ significantly from unity.

Figure 4.2 (a) illustrates the time-course of the Ins(1,4,5)P\textsubscript{3} response to CCh (300 μM) in submandibular gland slices. In the presence of a physiological concentration of extracellular Ca\textsuperscript{2+} (1.3 mM), the response increased linearly up to 60 s, after which a plateau was reached and maintained throughout the 10 min time-course investigated. In the nominal absence of extracellular calcium (KHB - Ca\textsuperscript{2+} + 100 μM EGTA), the response follows a similar pattern, but with a diminished peak and lower plateau phase (Figure 4.2 (a)). Figure 4.2 (b) displays the reversibility of the Ins(1,4,5)P\textsubscript{3} response to 5 min (the time-point chosen for all subsequent experiments in submandibular gland slices) stimulation with CCh (300 μM), upon the addition of atropine (10 μM). The reversal was fitted to a single exponential decay, with a half-life of 12.6 sec (Figure 4.2 (b)).

In guinea pig submandibular gland slices, carbachol produced a concentration-dependent increase in Ins(1,4,5)P\textsubscript{3}, as shown in Figure 4.3. The pEC\textsubscript{50} value for this response, calculated from a number of similar experiments, was 4.67 ± 0.09; n = 9. The mean slope factors associated with these concentration-response curves did not differ significantly from unity. Since differences in agonist potency are accounted for by the functional equivalent of the Cheng-Prusoff relationship (Craig, 1993),
Figure 4.1 Characterisation of the \(^{[3]}\text{H}\)-inositol phosphate response in guinea-pig bladder slices

(a) Time-course of \(^{[3]}\text{H}\)-IP\(_\text{x}\) response to CCh (1 mM) and (b) concentration-response curve of total \(^{[3]}\text{H}\)-IP\(_\text{x}\) accumulation to CCh in guinea-pig bladder slices at 30 min. Data are expressed as d.p.m. \(^{[3]}\text{H}\)-IP\(_\text{x}\) mg\(^{-1}\) protein or mean percent of maximum response. Results are expressed as means ± s.e.m., n ≥ 3.
Figure 4.2 Characterisation of Ins(1,4,5)P₃ response in guinea-pig submandibular gland slices
(a) Time-course of Ins(1,4,5)P₃ response to CCh (300 μM) in the presence (closed symbols) and absence (open symbols) of extracellular Ca²⁺ ([Ca²⁺]₀) and
(b) reversal of CCh (300 μM)-stimulated Ins(1,4,5)P₃ response upon addition of atropine (10 μM) at t = 0. Data are expressed as pmol Ins(1,4,5)P₃ mg⁻¹ protein or mean percent of maximum response. Results are expressed as means ± s.e.m., n ≥ 3.
Figure 4.3
Concentration-response curve of Ins(1,4,5)P₃ response to CCh in guinea-pig submandibular gland slices. Data are expressed as mean percent of maximum response. Results are expressed as means ± s.e.m., n ≥ 3.
comparison of pharmacological values derived from the two tissue responses is valid, despite the slight differences in CCh potency.

4.2.1.2 Comparison of the functional affinities of mACh receptor antagonists for the inhibition of phosphoinositol turnover in guinea-pig urinary bladder and submandibular salivary gland

Figure 4.4 displays inhibition curves for each of the mACh receptor antagonists investigated (atropine, darifenacin, oxybutynin and tolterodine), expressed as a percentage of the maximal response (i.e. that evoked by 50 μM CCh in the absence of antagonist) in both cross-chopped guinea-pig bladder and submandibular gland. Mean functional affinity estimates (pKₐ), Hill slope values (nₒ) and relative tissue-selectivities for each antagonist are summarized in Table 4.1. Oxybutynin (9.3 fold; p < 0.01), darifenacin (7.9 fold; p < 0.05) and tolterodine (7.4 fold; p < 0.05) each displayed significant selectivity for inhibition of the functional response in the bladder over that seen in the submandibular gland. In contrast, atropine displayed similar affinities for inhibition of phosphoinositol responses seen in each tissue (Figure 4.4 (a); Table 4.1). This provides further support for the validity of the comparison of pharmacological values derived from the two different tissue responses.

Slope factors associated with antagonist inhibition curves in both bladder and submandibular gland generally approximated unity (Table 4.1). The only exception to this was for the atropine-mediated inhibition of [³H]-IP₃, accumulation in bladder, which yielded a Hill slope that was moderately, but significantly steeper than unity (1.24; p < 0.05).

4.2.2 Determination of mACh receptor subtype expression profiles in guinea-pig tissues

4.2.2.1 Radioligand binding studies in membrane homogenates of guinea-pig urinary bladder and salivary glands

Figure 4.5 (a – d) shows representative [³H]-NMS saturation binding curves in membrane homogenates of guinea pig bladder, submandibular, parotid and sublingual salivary glands. Mean expression level (Bₘₐₓ), which defines the total mACh receptor
Figure 4.4
Inhibition of CCh (50 µM) -mediated phosphoinositide responses in guinea-pig bladder (open symbols) and submandibular gland (closed symbols) slices by atropine (a), darifenacin (b), oxybutynin (c) and tolterodine (d). Data are expressed as percent of maximum response (in the absence of antagonist). Results are expressed as means ± s.e.m., n ≥ 3.
Figure 4.4 (cont.)
Inhibition of CCh (50 μM) -mediated phosphoinositide responses in guinea-pig bladder (open symbols) and submandibular gland (closed symbols) slices by atropine (a), darifenacin (b), oxybutynin (c) and tolterodine (d). Data are expressed as percent of maximum response (in the absence of antagonist). Results are expressed as means ± s.e.m., n ≥ 3.
Table 4.1 Functional affinity (pK_\text{A}) and Hill slope estimates for mAC_h receptor antagonists in guinea-pig bladder and submandibular salivary gland. Data are expressed as mean (s.e.m.) values from n \geq 3 experiments. * indicates value significant difference between bladder and submandibular gland (p < 0.05; Student’s t test).

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<td>8.04 (0.11)</td>
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Figure 4.5

$[^3H]$-NMS saturation binding curves for membrane homogenates of guinea-pig urinary bladder (a); submandibular salivary gland (b); parotid salivary gland (c); and sublingual salivary gland (d) Data are expressed as specific binding in fmol receptor expression mg$^{-1}$ protein and are representative curves from 3 or more experiments.
Figure 4.5 (cont)
$[^3]H$-NMS saturation binding curves for membrane homogenates of
guinea-pig urinary bladder (a); submandibular salivary gland (b);
parotid salivary gland (c); and sublingual salivary gland (d). Data are
expressed as specific binding in fmol receptor expression mg$^{-1}$
protein and are representative curves from 3 or more experiments.
population expressed in each tissue, and binding affinity constant \( (K_d) \) estimates for each tissue homogenate are given in Table 4.2. Values for guinea-pig cerebral cortex are also included in Table 4.2.

In order to determine the mACh receptor subtypes expressed in these tissues, a range of subtype-selective mACh receptor antagonists were utilized in competition radioligand binding assays against \([H]^-NMS. The compounds tested were as follows: atropine (non-selective), pirenzepine (M₁-selective), 4-DAMP (M₁/M₂-selective), methoctramine (M₂-selective), darifenacin (M₂-selective) and MT-7 (a highly M₁-selective toxin; see Adem & Karlsson, 1997). Figures 4.6 – 4.9 illustrate the resulting mean inhibition curves generated for the six antagonists at membranes prepared from guinea-pig bladder (Figure 4.6), submandibular gland (Figure 4.7), parotid gland (Figure 4.8) and sublingual gland (Figure 4.9). Affinity binding constant (pKᵢ) values were calculated from a number of experiments and are summarized, along with the corresponding slope factors, in Table 4.3.

The binding profile in membranes prepared from bladder tissue was consistent with a mixed M₂/M₃ mACh receptor population. Thus, the displacement curve for the M₂-selective antagonist methoctramine was best fitted by 2-site analysis. The high affinity site, accounting for approx. 84% of the total mACh receptor population, was consistent with the reported affinity of methoctramine at the M₂ receptor, while its affinity at the remaining 16% of bladder mACh receptors was consistent with the reported affinity of methoctramine at the M₁ receptor (Eglen et al., 1996a). The low affinity of pirenzepine and the absence of any displacement by the highly M₁-selective toxin MT-7 was inconsistent with a detectable M₁ mACh receptor population in the guinea-pig bladder (Table 4.3; Figure 4.6).

In submandibular gland membranes, pirenzepine displayed low affinity and MT-7 caused no significant displacement of \([H]^-NMS binding (Tables 4.3 and 4.5; Figure 4.7). Similar results were also obtained in both parotid and sublingual gland membranes (Tables 4.3 and 4.5; Figures 4.8 and 4.9), indicating that none of the guinea-pig salivary glands investigated here express a detectable M₁ mACh receptor population. The high affinity observed for 4-DAMP in the salivary gland membranes is consistent with an M₁ and/or M₃ receptor population, while the relatively high affinity of darifenacin for the mACh receptor populations of the salivary glands is most consistent with an M₂ population (Table 4.3).
Table 4.2 Receptor densities ($B_{max}$) and [$^3$H]-NMS binding affinity constants ($K_d$) for the mACh receptor populations in guinea-pig tissues. Data are expressed as mean (s.e.m.) values from $n \geq 3$ experiments.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>$B_{max}$ (fmol mg protein$^{-1}$)</th>
<th>$K_d$ (pM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bladder</td>
<td>335 (23)</td>
<td>79 (27)</td>
</tr>
<tr>
<td>Submandibular gland</td>
<td>120 (22)</td>
<td>81 (20)</td>
</tr>
<tr>
<td>Parotid gland</td>
<td>258 (9)</td>
<td>35 (16)</td>
</tr>
<tr>
<td>Sublingual gland</td>
<td>144 (23)</td>
<td>51 (15)</td>
</tr>
<tr>
<td>Cerebral cortex</td>
<td>614 (12)</td>
<td>58 (11)</td>
</tr>
</tbody>
</table>
Figure 4.6

[3H]-NMS competition binding curves for guinea-pig urinary bladder membranes for the muscarinic acetylcholine receptor antagonists: atropine, pirenzepine, 4-DAMP, methoctramine, darifenacin and MT-7. Data are expressed as mean percent of control specific binding ± s.e.m., n ≥ 3.
Figure 4.7
[\textsuperscript{3}H]-NMS competition binding curves for guinea-pig submandibular salivary gland membranes for the muscarinic acetylcholine receptor antagonists: atropine, pirenzepine, 4-DAMP, methoctramine, darifenacin and MT-7. Data are expressed as mean percent of control specific binding ± s.e.m., n ≥ 3.
Figure 4.8
[\(^3\)H]-NMS competition binding curves for guinea-pig parotid salivary gland membranes for the muscarinic acetylcholine receptor antagonists: atropine, pirenzepine, 4-DAMP, methoctramine, darifenacin and MT-7. Data are expressed as mean percent of control specific binding ± s.e.m., n ≥ 3.
Sublingual gland

- Atropine
- Pirenzepine
- 4-DAMP
- Methoctramine
- Darifenacin
- MT-7

Figure 4.9
$[^3H]$-NMS competition binding curves for guinea-pig sublingual salivary gland membranes for the muscarinic acetylcholine receptor antagonists: atropine, pirenzepine, 4-DAMP, methoctramine, darifenacin and MT-7. Data are expressed as mean percent of control specific binding ± s.e.m., n ≥ 3.
Table 4.3 Binding affinity (pK₅) and Hill slope values for mACh receptor antagonists in guinea-pig bladder and salivary gland membrane homogenates. Data are expressed as mean (s.e.m.) values from n ≥ 3 experiments. * indicates value significantly different from unity (p < 0.05; F-test).

<table>
<thead>
<tr>
<th></th>
<th>Bladder</th>
<th>Submandibular gland</th>
<th>Parotid gland</th>
<th>Sublingual gland</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pK₅</td>
<td>Hill slope</td>
<td>pK₅</td>
<td>Hill slope</td>
</tr>
<tr>
<td>Atropine</td>
<td>9.26 (0.09)</td>
<td>1.02 (0.05)</td>
<td>9.23 (0.09)</td>
<td>1.16 (0.07)</td>
</tr>
<tr>
<td>Pirenzepine</td>
<td>6.84 (0.03)</td>
<td>1.05 (0.03)</td>
<td>6.92 (0.09)</td>
<td>0.94 (0.07)</td>
</tr>
<tr>
<td>4-DAMP</td>
<td>8.79 (0.06)</td>
<td>1.04 (0.04)</td>
<td>9.20 (0.04)</td>
<td>*</td>
</tr>
<tr>
<td>Methoctramine</td>
<td>pKᵢᵣ</td>
<td>N/A</td>
<td>6.57 (0.01)</td>
<td>*</td>
</tr>
<tr>
<td></td>
<td>pKᵥ</td>
<td></td>
<td>6.76 (0.12)</td>
<td></td>
</tr>
<tr>
<td>Darifenacin</td>
<td>7.99 (0.03)</td>
<td>0.92 (0.03)</td>
<td>8.54 (0.05)</td>
<td>*</td>
</tr>
</tbody>
</table>
Figure 4.10 shows the data from control experiments, designed to confirm the selectivity of the muscarinic toxin MT-7. While MT-7 caused no significant displacement of $[^3H]$-NMS binding to guinea-pig bladder or salivary glands, even at a concentration (100 nM) more than 100 fold in excess of its reported affinity for the $M_1$ receptor (see Adem and Karlsson, 1997), Figure 4.10 illustrates that MT-7 is able to displace $[^3H]$-NMS from both the human $M_1$ receptor ((a), CHO-m1 membranes) and guinea-pig $M_1$ receptor ((d), guinea-pig cerebral cortex membranes) with high affinity. Table 4.5 summarizes the effect of MT-7 (100 nM) on the maximum specific $[^3H]$-NMS binding to all of the guinea-pig tissue homogenates investigated, in addition to membrane homogenates of CHO-m1 cells and canine submandibular glands. It is interesting to note that MT-7 binds to a significant population of $M_1$ receptors in the submandibular glands of dog (64.3 %), indicating that the lack of a significant $M_1$ mACh receptor population in the equivalent gland of the guinea-pig may be species-specific.

MT-7 displaced the specific $[^3H]$-NMS binding component by 94 ± 1% and 46 ± 3% in CHO-m1 and guinea-pig cerebral cortex membranes, respectively (Figure 4.10 (a) and (b); Table 4.5), indicating that the cortex expresses both $M_1$ and non-$M_1$ mACh receptors. Table 4.4 summarizes the binding affinities (pK$_i$) and Hill slope estimates for MT-7 (in addition to atropine and pirenzepine) in CHO-m1 and guinea-pig cerebral cortex membranes. Pirenzepine competition binding curves for CHO-m1 membranes were fitted well with a one-site model displaying a high affinity (pK$_i$ = 8.15) consistent with published affinity estimates for pirenzepine at the $M_1$ receptor (Eglen et al., 1996a). In agreement with the data obtained for MT-7, pirenzepine recognized two distinct sites in guinea-pig cerebral cortex (Table 4.4). The higher affinity site (pK$_i$ = 8.47), constituting 51.8 ± 5.0 % of the total mACh receptor population, is most consistent with an $M_1$ population (based upon published affinity estimates for pirenzepine), while the low affinity site (pK$_i$ = 7.07) approximates the reported affinity for pirenzepine at $M_3$, $M_4$ or $M_5$ receptors (Eglen et al., 1996a).

In contrast to the high affinity displacement observed in CHO-m1 membrane homogenates, MT-7 (up to a concentration of 100 nM) failed to significantly displace $[^3H]$-NMS from membrane homogenates derived from CHO-m2 or CHO-m3 cell lines (Figure 4.10 (b) and (c)). This is in line with the reported affinity of MT-7 for non-$M_1$ mACh receptors, which is in excess of 1 µM (Adem & Karlsson, 1997) and
Figure 4.10
[\textsuperscript{3}H]-NMS competition binding curves for the muscarinic acetylcholine receptor antagonists: atropine, pirenzepine and MT-7 at membranes prepared from CHO-m1 cells (a) and guinea-pig cerebral cortex (d). Data are expressed as mean percent of control specific binding ± s.e.m., n ≥ 3. Representative [\textsuperscript{3}H]-NMS competition binding data for MT-7 at membranes prepared from CHO-m2 (b) and CHO-m3 (c) cells. Data are from a single experiment performed in duplicate. Results are expressed as d.p.m. [\textsuperscript{3}H]-NMS bound.
Figure 4.10 (cont.)
[^H]-NMS competition binding curves for the muscarinic acetylcholine receptor antagonists: atropine, pirenzepine and MT-7 at membranes prepared from CHO-m1 cells (a) and guinea-pig cerebral cortex (d). Data are expressed as mean percent of control specific binding ± s.e.m., n ≥ 3. Representative[^H]-NMS competition binding data for MT-7 at membranes prepared from CHO-m2 (b) and CHO-m3 (c) cells. Data are from a single experiment performed in duplicate. Results are expressed as d.p.m.[^H]-NMS bound.
Table 4.4 Binding affinity (pK$_a$) and Hill slope values for mACH receptor antagonists in CHO-m1 membranes and guinea-pig cerebral cortex membrane homogenates. Data are expressed as mean (s.e.m.) values from n ≥ 3 experiments. * indicates value significantly different from unity (p < 0.05; F-test).

<table>
<thead>
<tr>
<th>Atropine</th>
<th>CHO-m1</th>
<th>Guinea-pig Cerebral Cortex</th>
</tr>
</thead>
<tbody>
<tr>
<td>pK$_a$</td>
<td>Hill slope</td>
<td>pK$_a$</td>
</tr>
<tr>
<td>9.13 (0.01)</td>
<td>0.91 (0.02)</td>
<td>9.50 (0.03)</td>
</tr>
<tr>
<td>Pirenzepine</td>
<td>8.15 (0.01)</td>
<td>0.91 (0.02)</td>
</tr>
<tr>
<td>MT-7</td>
<td>9.48 (0.01)</td>
<td>*1.86 (0.07)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 4.5 Effect of MT-7 (100 nM) on the specific[^H]-NMS binding to guinea-pig tissues, canine submandibular gland and CHO-m1 membrane homogenates. Data are expressed as mean (s.e.m.) values from n ≥ 3 experiments. * indicates value significantly different from control (p < 0.05; Student’s t test).

<table>
<thead>
<tr>
<th>Tissue</th>
<th>% control specific binding</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bladder (GP)</td>
<td>102.6 (3.9)</td>
</tr>
<tr>
<td>Submandibular (GP)</td>
<td>104.8 (5.2)</td>
</tr>
<tr>
<td>Parotid (GP)</td>
<td>98.3 (8.8)</td>
</tr>
<tr>
<td>Sublingual (GP)</td>
<td>102.3 (3.2)</td>
</tr>
<tr>
<td>Cerebral Cortex (GP)</td>
<td>54.2 (0.3) *</td>
</tr>
<tr>
<td>Canine Submandibular</td>
<td>35.7 (1.5) *</td>
</tr>
<tr>
<td>CHO-m1</td>
<td>6.43 (0.07) *</td>
</tr>
</tbody>
</table>
highlights the highly selective nature of this toxin in the concentration range utilized in this study.

Figure 4.11 shows $[^H]$-NMS competition binding curves for 4 compounds considered to be the most $M_3/M_5$ selective mACh receptor antagonists available (darifenacin, oxybutynin, pF-HHSiD and AF-DX 384) in membrane homogenates of CHO-m3 cells (a) and guinea-pig submandibular salivary gland (b). Table 4.6 summarizes the binding affinities (pK$_A$) for each ligand at CHO-m3 and submandibular gland membranes. Published affinity estimates for these antagonists at the $M_3$ and $M_5$ mACh receptors are included in Table 4.6 for reference. Affinity estimates in submandibular glands correlate well with those estimated for the $M_3$ receptor and are not consistent with published affinity estimates for these compounds at the $M_5$ mACh receptor (Eglen et al., 1996a; Caulfield & Birdsall, 1998). The present study therefore provides no evidence for the expression of a significant $M_5$ mACh receptor population in the submandibular gland of the guinea-pig.

Overall, the competition radioligand binding data provide no evidence that any of the three major salivary glands (submandibular, parotid and sublingual) of the guinea-pig express multiple mACh receptor subtypes. None of the mean Hill slopes of the competition binding curves of any of the subtype-selective antagonists were significantly less than unity (as might be expected if more than one subtype were expressed) (Table 4.3). Indeed, in many cases, particularly for MT-7 and methoctramine, but also for 4-DAMP and darifenacin, slope factors were significantly greater than unity (see section 4.3). Overall, the data are most consistent with a homogeneous population of $M_3$ mACh receptors expressed in guinea-pig salivary glands.

4.2.2.2 Functional (second messenger) studies to investigate mACh receptor subtypes mediating carbachol-stimulated phosphoinositide turnover in guinea-pig salivary glands

Figure 4.12 (a) shows the mean concentration-dependent accumulation of $[^H]$-IP$_A$ to CCh in CHO-m1 cells. The mean EC$_{50}$ value derived from these experiments was $5.78 \pm 0.07 \mu M$ (n = 3). Figure 4.12 (b) illustrates the concentration-dependent inhibition, by MT-7, of the accumulation of $[^H]$-IP$_A$ in CHO-m1 cells to CCh (10
Figure 4.11
$[^{3}H]$-NMS competition binding curves for the muscarinic acetylcholine receptor antagonists darifenacin, oxybutynin, $p$-fluorohexahydrosiladifenidol ($p$-FHHSiD) and AF-DX 384 for membranes prepared from CHO-m3 cells (a) and guinea-pig submandibular gland (b). Data are expressed as mean percent control specific binding ± s.e.m., n ≥ 3.
Table 4.6 Binding affinity (pKₐ) values for mACh receptor antagonists in CHO-m3 membranes and guinea-pig submandibular gland membrane homogenates. Data are expressed as mean (s.e.m.) values from n = 3 experiments.

<table>
<thead>
<tr>
<th></th>
<th>CHO-m3</th>
<th>Submandibular gland</th>
<th>M₃ (published)ᵃ</th>
<th>M₅ (published)ᵇ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Darifenacin</td>
<td>9.13 (0.04)</td>
<td>9.05 (0.02)</td>
<td>8.9</td>
<td>8.1</td>
</tr>
<tr>
<td>Oxybutynin</td>
<td>8.99 (0.04)</td>
<td>8.54 (0.03)</td>
<td>8.7</td>
<td>7.6</td>
</tr>
<tr>
<td>pF-HHSd</td>
<td>7.62 (0.07)</td>
<td>7.57 (0.03)</td>
<td>7.5</td>
<td>6.7</td>
</tr>
<tr>
<td>AF-DX 384</td>
<td>7.31 (0.06)</td>
<td>7.02 (0.03)</td>
<td>7.2</td>
<td>6.3</td>
</tr>
</tbody>
</table>

ᵃ M₃ and M₅ mACh receptor binding affinities adapted from Caulfield & Birksall (1998) and Eglen et al. (1996a).
Figure 4.12

(a) Concentration-response curve for total $[^{3}H]$-IP$_{a}$ accumulation in response to CCh in CHO-m1 cells. (b) Inhibition of CCh (3 μM)–mediated $[^{3}H]$-IP$_{a}$ accumulation in CHO-m1 cells by MT-7. (c) Effect of MT-7 upon CCh (3 μM)–mediated $[^{3}H]$-IP$_{a}$ accumulation in CHO-m3 cells. Data are expressed as percent of maximum response. Results are expressed as means ± s.e.m., n ≥ 3.
μM). The Kᵦ value derived from 3 experiments was 1.14 ± 0.33 nM. In contrast, MT-7 up to a concentration of 100 nM (a maximally effective concentration at the M₄ receptor) had no effect on the accumulation of [³H]-IP₃ in CHO-m3 cells, in response to carbachol (10 μM) (Figure 4.12 (c)). The selectivity of MT-7 for phosphoinositide turnover mediated by M₁ versus M₃ mACh receptors is therefore confirmed by these data.

In order to examine the effects of MT-7 on phosphoinositide turnover in guinea-pig salivary glands, the Ins(1,4,5)P₃ response to CCh in sublingual gland slices was first characterized. Figure 4.13 illustrates the time-course of Ins(1,4,5)P₃ generation to CCh (300 μM) (a) and the concentration-dependency of this response (b). As seen in the submandibular gland, the response appears biphasic, with an initial linear increase which peaks slightly earlier in the case of the sublingual gland (Figures 4.2 (a) and 4.13 (a)). Also, prolonged stimulation with agonist leads to a sustained rise in Ins(1,4,5)P₃ generation, in contrast to the plateau observed in the submandibular gland (Figures 4.2 (a) and 4.13 (a)). The magnitude of the response is also greater in the sublingual gland, generating ~ 100 pmol mg⁻¹ protein by the 10 min time-point (compared with approx. 30 pmol mg⁻¹ protein in the submandibular gland). However, it should be noted that the basal Ins(1,4,5)P₃ levels in the sublingual gland (12.5 ± 3.3 pmol mg⁻¹ protein) are also significantly higher than those in submandibular gland (4.0 ± 1.0 pmol mg⁻¹ protein). The 5 min time-point was selected for all subsequent experiments. The mean CCh EC₅₀ value (generated at 5 min), calculated from 4 separate concentration-response curves, was 15.7 ± 3.5 μM.

Figure 4.14 illustrates the effect of pre-incubation (15 min) with MT-7 (100 nM) upon CCh (50 μM) – mediated Ins(1,4,5)P₃ generation in both submandibular and sublingual gland slices. Ins(1,4,5)P₃ responses in the absence and presence of MT-7 were 41.1 ± 3.4 and 33.8 ± 7.3 pmol mg⁻¹ protein in submandibular gland slices (n = 3) and 101.1 ± 8.5 and 80.1 ± 9.2 pmol mg⁻¹ protein in sublingual gland slices (n = 3), respectively. Although slightly lower responses were observed following pre-incubation of MT-7, these were not significantly different, in either tissue preparation, from control values. Varying MT-7 pre-incubation time up to 60 min had no apparent effect on agonist-mediated Ins(1,4,5)P₃ responses (data not shown), indicating that equilibration of the toxin with the preparation was not limiting its effectiveness. These
Figure 4.13
(a) Time-course of Ins(1,4,5)P$_3$ response to CCh (300 μM) in guinea-pig sublingual gland slices. (b) Concentration-response curve of Ins(1,4,5)P$_3$ generation in response to CCh in guinea-pig sublingual gland slices. Data are expressed as pmol Ins(1,4,5)P$_3$ mg$^{-1}$ protein or percent of maximum response. Results are expressed as means ± s.e.m., n ≥ 3.
Figure 4.14
Effect of MT-7 (100 nM) on CCh (50 μM)-stimulated Ins(1,4,5)P₃ generation in guinea-pig salivary glands. Data are expressed as pmol Ins(1,4,5)P₃ mg⁻¹ protein. Results are expressed as means ± s.e.m., n ≥3.
data therefore support the conclusion from radioligand binding studies, that guinea-pig salivary glands (at least sublingual and submandibular glands) do not express a significant M₁ mACh receptor population that could be involved in the CCh-mediated phosphoinositide turnover measured in these systems.
4.3 Discussion

Muscarinic receptor antagonists are a mainstay in the pharmacological management of overactive bladder (OAB) (Wyndaele, 2001). However, a lack of receptor subtype selectivity can result in a significant side-effect profile (e.g. dry mouth), which is associated with a high degree of patient non-compliance (Wallis and Napier, 1999). This has prompted a search for anti-muscarinic compounds with greater selectivity. The observation that some, but not all, mACh receptor antagonists display in vivo functional selectivity for bladder versus salivary gland (see section 1.3.6.4) represents a significant step towards more selective OAB therapies.

4.3.1 Characterisation of functional assays in guinea-pig bladder and salivary gland slices.

The main aim of this study was to investigate the potential for mACh receptor antagonists to exhibit tissue-dependent ‘functional selectivity’ at the level of second messenger generation. Although it would have been ideal to use identical methodologies in both urinary bladder and submandibular salivary gland, this was not technically possible. In order to sufficiently label the phospholipid pools of the tissue slices with \[^{3}H\]-myo-inositol, a 24 hour labelling incubation in minimum essential medium-\(\alpha\) (MEM-\(\alpha\)) was required. While the urinary bladder slices remained healthy and viable after this treatment, slices of the submandibular gland were unable to maintain viability over this time period. However, following an initial equilibration period in Krebs-Henseleit buffer (KHB), submandibular (and sublingual) gland slices displayed a robust \(\text{Ins}(1,4,5)\text{P}_3\) response to the muscarinic agonist carbachol. Unfortunately, bladder smooth muscle slices showed no measurable \(\text{Ins}(1,4,5)\text{P}_3\) response over the time-course investigated, despite exhibiting a robust carbachol-stimulated accumulation of \(\[^{3}H\]-\text{inositol phosphates (under lithium block) following chronic labelling with \([^{3}H]\)-myo-inositol. Slightly different assays in each tissue preparation (\(\text{Ins}(1,4,5)\text{P}_3\) responses in salivary gland slices and \(\[^{3}H\]-\text{inositol phosphate accumulation in bladder slices) were therefore adopted, though since both are indices of the mACh receptor-mediated stimulation of phospholipase C (PLC)
activity, comparison of antagonist functional affinity values derived from inhibition of these two responses should be valid.

Characterisation of the time-course of the Ins(1,4,5)P₃ response in submandibular gland slices yielded a slightly different temporal pattern than may have been expected from studies of M₃ receptor-mediated Ins(1,4,5)P₃ responses in CHO cells (Tobin et al., 1992). In CHO-m3 cells, a rapid, but transient peak is observed at around 15 sec, followed by a reduced, but sustained accumulation that appears to be resistant to desensitisation (Tobin et al., 1992). A similar early linear phase of Ins(1,4,5)P₃ accumulation was observed in both submandibular and sublingual glands, although the peak in the submandibular gland slices occurred later than in CHO cells (60 sec). The second phase was maintained at only slightly lower than maximal levels in the submandibular gland, but continued to rise beyond the initial peak in the sublingual gland preparation. In the absence of extracellular calcium in the submandibular gland slices, both peak and plateau responses were decreased but the overall pattern of the response was maintained. This indicates that both the first and second phases are contributed to by both calcium-independent and calcium-dependent mechanisms. This implicates a calcium-dependent phospholipase-C component, occurring secondarily to Gₛ₉₁-stimulated PLC activation, as has been proposed to occur in the activation of several PLC isozymes (see Rhee & Bae, 1997; Rebecchi & Pentyala, 2000). From this basic characterisation of the time-course in the submandibular (and sublingual) gland, it was decided that pharmacological analysis of the inhibition of Ins(1,4,5)P₃ generation would be conducted against 5 min agonist stimulation, during the plateau phase, where the response was consistent and a good signal-to-noise ratio was obtainable. At this time-point, it was also demonstrated that addition of the muscarinic receptor antagonist atropine (10 μM) caused a rapid reversal of carbachol-stimulated Ins(1,4,5)P₃ accumulation, indicating that the measured Ins(1,4,5)P₃ response is the result of Ins(1,4,5)P₃ degradation matching the synthetic rate.

In contrast to the clearly biphasic time-course of the Ins(1,4,5)P₃ response in submandibular and sublingual gland slices, [³H]-inositol phosphate accumulation in guinea-pig urinary bladder slices occurred linearly over a time-course up to 30 min, in line with previous reports (e.g. Noronha-Blob et al., 1989). A 30 min time-point was therefore selected for all subsequent experiments. Analysis of a number of concentration-response curves in each tissue preparation indicated that carbachol was
approx. 3-fold more potent in stimulating \[^{3}H\]-inositol phosphate accumulation in bladder slices (EC\textsubscript{50} = 8.9 \mu M) than in mediating Ins(1,4,5)P\textsubscript{3} generation in submandibular gland slices (EC\textsubscript{50} = 25.3 \mu M). Carbachol displayed an intermediate potency (EC\textsubscript{50} = 15.7 \mu M) for Ins(1,4,5)P\textsubscript{3} generation in sublingual gland slices. It is likely that the greater potency exhibited in the bladder \[^{3}H\]-inositol phosphate response reflects the positive feedback effect of raised intracellular Ca\textsuperscript{2+} upon phospholipase C activity over the longer (30 min vs 5 min) time-course of agonist exposure. However, comparison of the inhibition of the responses in each tissue by muscarinic antagonists is valid, as differences in agonist potency are taken into account in the functional equivalent of the Cheng-Prusoff correction (Craig, 1993).

4.3.2 Functional selectivity of mACh receptor antagonists for inhibition of phospholipase C (PLC) activity in guinea-pig bladder versus salivary gland slices.

Using indices of mACh receptor-stimulated phospholipase C activity we have been able to show that darifenacin, oxybutynin and tolterodine each display higher affinity for the inhibition of responses in urinary bladder slices compared to submandibular gland slices. Data presented here therefore support the notion that certain mACh receptor antagonists show differential inter-tissue effects on cellular responses reputedly mediated by the same mACh receptor subtype in the same species. In the case of darifenacin, oxybutynin and tolterodine, this tissue-specific selectivity is manifested \textit{in vitro} at the level of inhibition of agonist-induced second messenger turnover, in line with earlier reports of the \textit{in vivo} selectivity of these compounds (Nilvebrant et al., 1997a; Wallis and Napier, 1999; Gupta et al., 2002).

The observation that oxybutynin displays selectivity (9.3 fold) for bladder versus salivary gland responses is at odds with previous reports in the literature. Newgreen and Naylor (1996) found that oxybutynin was non-selective \textit{in vitro} in the guinea-pig (by comparing inhibition of bladder contractions and \[^{86}\text{Rb}\] efflux from submandibular glands). Ikeda et al. (2002) also found that oxybutynin inhibited bladder contraction and salivation with similar potency \textit{in vivo}, in anaesthetized rat. The same study concluded that oxybutynin was non-selective between urinary bladder and submandibular salivary glands for the inhibition of calcium mobilization (Ikeda et al.,
2002), but this was based upon data obtained in two different species (guinea-pig bladder versus mouse submandibular glands) so the validity of the comparison is questionable. However, the same group later reported that oxybutynin was moderately selective for the inhibition of agonist-mediated calcium mobilization in submandibular gland relative to the urinary bladder in Cynomolgus monkey cells (Kobayashi et al., 2004).

In agreement with the latter finding, Nilvebrant et al. (1997a) found oxybutynin to exhibit selectivity for inhibition of salivation over bladder contraction in anaesthetized cat. Oxybutynin is subject to metabolism in vivo, producing both active and inactive metabolites (Waldeck et al., 1997), and has also been reported to have significant 'non-muscarinic' effects, such as blockade of Ca\(^{2+}\) channels (Wada et al., 1995). Thus, the selectivity observed in vitro may be reduced or lost in vivo and effects on Ca\(^{2+}\) channels may complicate the interpretation of calcium mobilization experiments. Alternatively, the selectivity profile of oxybutynin may differ between animal species and/or be contingent upon the choice of methodology.

The bladder selectivity demonstrated by darifenacin and tolterodine is generally in agreement with previous studies. Thus, Gupta et al. (2002) reported that darifenacin displays a 10 fold selectivity for inhibition of pelvic nerve-stimulated bladder contractions versus salivary secretion in dog, while tolterodine was 4-5 fold selective in the same study (Gupta et al., 2002). In guinea-pig, comparison of the inhibition of carbachol-stimulated \(^{86}\)Rb efflux from submandibular salivary glands with the inhibition of acetylcholine-mediated bladder contractions indicates that darifenacin (6 fold) and tolterodine (2 fold) are both selective for bladder responses (Newgreen & Naylor, 1996). In vivo data also support the observation that tolterodine displays selectivity for the urinary bladder versus the salivary glands (Nilvebrant et al., 1997a; Gillberg et al., 1998). However, the work of Kobayashi et al. (2004) indicates that in Cynomolgus monkey cells, tolterodine and darifenacin display similar selectivity profiles as oxybutynin; i.e. are moderately selective for the inhibition of carbachol-induced calcium mobilization in submandibular gland cells over bladder cells. It should be emphasized that in the vast majority of studies, including the present one, atropine fails to discriminate bladder and salivary gland responses. This cogently argues against an affinity 'frame-shift' between preparations and strongly supports the conclusion that observed tissue selectivities are particular to a subset of mACh
receptor antagonists. However, Kobayashi et al. (2004) reported that atropine displayed a similar selectivity for submandibular gland relative to bladder as darifenacin, oxybutynin and tolterodine. It is therefore possible that an intrinsic difference between the two tissues, or in the preparation of the cells might be responsible for the salivary gland selectivity of atropine and other compounds investigated by Kobayashi et al. (2004).

4.3.3 Is there more than one mACh receptor subtype involved in the phosphoinositide response of the submandibular gland?

One obvious possible explanation for tissue-specific differences in the pharmacology of antagonists would be the involvement of multiple receptors in the functional response under investigation. We therefore sought clarification of whether the presence of ‘non-M3’ (in particular M2) muscarinic receptors could be involved in the phosphoinositide response in submandibular gland, using competition radioligand binding assays with a range of the most selective muscarinic ligands available.

In agreement with a number of earlier studies (see Eglen et al., 1996a), the binding profile in bladder membranes was consistent with a mixed M2 and M3 population. The low affinity of pirenzepine and the absence of any displacement with MT-7 was inconsistent with the expression of a significant M1 receptor population, while the intermediate affinities of darifenacin and 4-DAMP pointed to the expression of a mixed receptor population including an M3 receptor component. Methoctramine recognized two distinct classes of binding site with different affinities in the bladder and comparison of the affinities calculated for these sites with the reported affinities of methoctramine for the muscarinic receptor subtypes (Caulfield & Birdsall, 1998) indicated that a mixed M2 and M3 population was present. The estimated ratio of 84:16 (M₂:Μ₃) is in line with literature values which estimate that most species (including guinea-pig) other than rat express a majority 75-80 % M2 mACh receptor population (Eglen et al., 1996a).

As in membrane homogenates of urinary bladder, the highly M1-selective toxin MT-7 failed to cause any significant displacement from membranes prepared from any of the three major salivary glands of the guinea-pig. Pirenzepine and methoctramine displayed similarly low affinities in each gland, indicating that the salivary glands do
not express significant levels of M₁ or M₂ mACh receptors. Since both pirenzepine and methoctramine display moderately high affinities for the M₄ subtype, these data would also suggest that this subtype is not abundantly expressed in these glands. The high affinities exhibited by 4-DAMP and darifenacin, in conjunction with the lack of effect of MT-7 and the low affinity of pirenzepine, are indicative of a homogeneous M₃ mACh receptor population in guinea-pig parotid, sublingual and submandibular salivary glands.

It is also interesting to note that none of the mean slope factors associated with the competition binding curves at any of the salivary gland homogenates were significantly less than unity. This suggests that none of the subtype-selective antagonists detected multiple binding sites with different affinities, providing further evidence that these glands express homogeneous mACh receptor populations. However, in some cases (see Table 4.3) mean slope factor estimates were significantly greater than unity. This was particularly apparent in the case of methoctramine and may be explained by methoctramine’s reported allosteric, non-competitive interaction with muscarinic receptors (Eglen et al., 1988b). However, the reasons for darifenacin and 4-DAMP exhibiting steep slope factors are less clear. The majority of previous studies indicate that 4-DAMP displays reversible, competitive interactions with muscarinic receptors in radioligand binding assays in recombinant systems (e.g. Kukkonen et al., 1998) and a variety of tissues, including salivary glands (e.g. Dai et al., 1991; Culp et al., 1996; Watson et al., 1996), as well as in functional studies (e.g. Longhurst et al., 1995; Hegde et al., 1997; Meloy et al., 2001). Limited evidence of non-competitive behaviour by 4-DAMP includes the observation of Schild slopes of less than unity in perfused rabbit ear artery (Choo et al., 1986), but only against the agonist McN-A-343, which itself interacts allosterically with muscarinic receptors (Birdsall et al., 1983). Brunner et al., (1991) found that 4-DAMP antagonized acetylcholine-mediated responses in bovine coronary artery with a Schild slope of greater than unity, but in radioligand binding assays 4-DAMP behaved as a simple competitive antagonist (Brunner et al., 1991). The majority of evidence therefore indicates that 4-DAMP is a competitive antagonist at muscarinic receptors, so it seems unlikely that the steep Hill slopes observed in this study result from interactions with allosteric sites on the muscarinic receptor population expressed in guinea-pig salivary glands. However (as discussed in Chapter 1: Introduction), some
evidence, albeit circumstantial, exists that darifenacin may bind to the muscarinic receptors in a more complex manner, at least when functional assays are employed in native tissues (e.g. Hegde et al., 1997). It is therefore possible that the steep slopes consistently observed for darifenacin in the salivary gland membrane homogenates, may be due to interaction with an allostERIC site on the muscarinic receptor. However, without any direct evidence, this remains highly speculative.

The absence of a detectable M1 receptor population in both submandibular and sublingual glands was confirmed by the lack of effect of MT-7 (at 100 nM) on carbachol-stimulated phosphoinositide responses in cross-chopped slices of these glands. In contrast, MT-7 potently inhibited muscarinic agonist-stimulated accumulation of [3H]-inositol phosphates in CHO-m1 cells (but not CHO-m3 cells), confirming its ability to selectively antagonize functional responses mediated by the M1 mACh receptor (Olianas et al., 2000). MT-7 also caused significant inhibition of specific [3H]-NMS binding to membranes prepared from CHO-m1 cells (but not CHO-m2 or CHO-m3 cells), canine submandibular gland and guinea-pig cortex, confirming that this toxin binds selectively to human, canine and guinea-pig M1 mACh receptors with high affinity.

Data obtained with MT-7 and pirenzepine in guinea-pig cerebral cortex membrane homogenates are in good agreement regarding the proportion of M1 mACh receptors expressed in this region. While MT-7 displaced 46% of the specific [3H]-NMS bound to cortical membranes, the high affinity site recognized by pirenzepine, corresponding to its reported affinity for the M1 receptor, comprised 51.8% of the total number of binding sites. Based on the affinity of pirenzepine for the remaining sites, it seems likely that the majority of the non-M1 muscarinic receptors expressed in guinea-pig cerebral cortex are of the M4 subtype. This is in agreement with the results of subtype-selective immunoprecipitation of receptor protein in rat brain (Tice, et al., 1996). The other point of interest to arise from the MT-7 competition binding curves is that in both CHO-m1 and cerebral cortex membrane homogenates, the mean Hill slopes are significantly greater than unity (see Table 4.4). This is likely to result from the allostERIC nature of MT-7’s interaction with the M1 muscarinic receptor, where it has been demonstrated to markedly decrease the rate of atropine-induced dissociation of [3H]-NMS and non-competitively inhibit agonist-mediated functional responses (Olianas et al., 2000).
The ability of MT-7 to displace 64.3% of the specific \[^{3}H\]-NMS binding to canine submandibular gland membranes is in agreement with a recent study utilizing the same method (competition radioligand binding) and a similar range of antagonists in the submandibular gland of the dog (Clarke et al., 2003a). This study found that approx. 50% of the total mACh receptor population of the canine submandibular gland was of the M\(_1\) subtype (based on experiments with MT-7), while the remaining population was most consistent with an M\(_3\) receptor profile (Clarke et al., 2003a). Our finding that MT-7 is without effect in any of the major salivary glands of the guinea-pig, when using the same experimental design, strongly suggests that there are striking differences in muscarinic receptor subtype expression between species. This may explain the apparent lack of consensus in the literature regarding muscarinic receptor sub-populations expressed in the salivary glands. Previous work in this area has utilized the major salivary glands of a variety of different species including rat, rabbit, mouse and guinea-pig, with often conflicting results (see section 1.4.1).

The mACh receptor expression profile of the submandibular gland in particular appears to be lacking in consensus, but careful analysis of the literature does point to species-specific trends, which may explain the overall lack of agreement amongst the published body of work. Studies using radioligand binding (Martos et al., 1987; Moriya et al., 1999) and second messenger turnover assays (Laniyonu et al., 1990) in rat submandibular gland were most consistent with a homogeneous population of M\(_3\) receptors. In contrast, studies in rabbit submandibular gland have found M\(_1\) and M\(_3\) receptors to be expressed in approximately equal proportions, using quantitative immunoprecipitation (Dorje et al., 1991), while pirenzepine was found to exhibit a relatively high affinity for the inhibition of carbachol-evoked K\(^+\)-release from isolated submandibular glands of the rabbit (Tobin, 1995). In the latter study, 4-DAMP also displayed high affinity, implicating roles for both M\(_1\) and M\(_3\) muscarinic receptor subtypes in carbachol-mediated responses of the rabbit submandibular gland (Tobin, 1995), in agreement with the quantitative immunoprecipitation work of Dorje et al., (1991). Competition radioligand binding studies in mouse (Watson et al., 1996) and dog (Clarke et al., 2003a) also indicate that the submandibular gland co-expresses muscarinic receptors of the subtypes M\(_1\) and M\(_3\).

The findings of this study that guinea-pig submandibular glands express a homogeneous or near-homogeneous M\(_3\) receptor population is therefore most
consistent with the observations of a number of groups studying rat submandibular glands and suggests that the muscarinic receptor expression profiles of rat and guinea-pig may differ from those of rabbit, mouse and dog, respectively. It is not clear why relatively closely related species such as mouse and rat should express different complements of mACH receptors in the same tissue, but it could reflect differences in the cell types contained within the glands. M1 receptors are preferentially expressed on mucous secretory cells, while M3 receptors are largely confined to serous cells (Buckley & Burnstock, 1986). The submandibular gland is generally considered to express a mixed population of these two cell types (Pinkstaff, 1980), but it is conceivable that the relative proportions may vary between species, giving rise to variations in the apparent involvement of M1 and M3 receptors in salivary gland responses.

Data obtained in this study for the guinea-pig parotid gland are generally in agreement with the majority of previous studies (in a variety of different species), which have found that the predominant muscarinic receptor expressed in the parotid gland is of the M3 subtype (e.g. Dai et al., 1991; see section 1.4.1 for further discussion). This is likely to reflect the serous secretory cell population believed to predominate in the parotid gland (Pinkstaff, 1980).

Both radioligand binding and functional data in the sublingual gland of the guinea-pig were most consistent with a homogeneous M3 receptor population. This may seem counter-intuitive as the sublingual glands are thought to comprise of both mucous and serous secretory cells and would therefore be expected to express significant populations of both M1 and M3 receptors (Pinkstaff, 1980). Several studies in rat sublingual glands have detected both M1 and M3 mACH receptors by means of radioligand binding (Martos et al., 1987), Northern blot and immunoprecipitation (Watson & Culp, 1994). The latter group also demonstrated a functional role for both M1 and M3 muscarinic receptors in the regulation of secretion from rat sublingual glands, reporting that the M1-selective antagonist m1-toxin was only able to partially (40 %) decrease the maximal secretory response to the muscarinic agonist arecaidine propargyl ester (APE) (Culp et al., 1996). However, little work has been published relating to the muscarinic receptor subtype expression in any of the guinea pig’s major salivary glands and given the apparent species-dependent differences, a lack of
consistency with published data (in other albeit closely related species) is perhaps not surprising.

If the salivary gland mACh receptor expression profiles of the commonly studied laboratory animal species are so diverse, their suitability as model systems for the study of the muscarinic receptor-mediated regulation of salivary secretion in humans, a species only distantly related to rodents, rabbits and guinea-pigs, must inevitably be questioned. Unfortunately, studies in human salivary glands are scarce. Martos et al., (1985) presented data consistent with a single class of muscarinic receptors being expressed in human submandibular glands, but a more thorough investigation using the more selective muscarinic ligands now available is eagerly awaited.

The effects of muscarinic antagonists on the 'quality' of saliva produced also require more detailed investigation, as little is understood about the relative effects of anti-muscarinic therapy on fluid and exocrine (proteinaceous) secretion from the salivary gland system. Although the muscarinic receptor-mediated activation of phospholipase C and subsequent intracellular calcium mobilization is considered to be the major pathway by which fluid secretion from all three major salivary glands is regulated, exocrine secretion by the parotid and submandibular glands is largely under the control of the β-adrenoceptor/cyclic AMP system (Baum, 1993). It is only in the sublingual gland that the cholinergic system is believed to predominate in the control of exocrine secretion (Baum, 1993), so it is interesting to note that it is in this gland (in the rat at least) that the apparent functional redundancy of PLC-linked mACh receptors (i.e. co-expression of M₁ and M₃ subtypes) is most prominent. It is possible that the greater reliance on the cholinergic system for the overall control of salivary secretion from the sublingual gland necessitates a greater degree of redundancy in the activation of the PLC pathway downstream of muscarinic receptor activation.

Consideration of the complexity of the salivary gland system highlights the need for caution in interpreting data related to specific signalling pathways (e.g. phosphoinositide turnover) in single salivary glands (e.g. submandibular) and relating these to the in vivo regulation of salivary output. However, the aim of this study was to define the influence of clinically relevant mACh receptor antagonists on specific
intracellular signalling pathways that are potentially compromised in patients undertaking anti-muscarinic therapy for overactive bladder. In the case of darifenacin and tolterodine at least, the in vivo selectivity for urinary bladder versus salivary gland responses was already well established (Nilvebrant et al., 1997a; Wallis and Napier, 1999; Gupta et al., 2002). Data obtained in this investigation indicate that functional selectivity is also observed in the inhibition of muscarinic agonist-mediated phosphoinositide hydrolysis (the major pathway implicated in the control of secretion and contraction), providing an in vitro correlate of previously published in vivo selectivity.

Although both radioligand binding and functional data suggest that the carbachol-stimulated phosphoinositide turnover assayed in guinea-pig submandibular gland slices is unlikely to occur through the activation of any significant M₃ muscarinic receptor population, the potential expression of the M₅ subtype also had to be addressed. Competition radioligand binding experiments were performed in membrane homogenates prepared from guinea-pig submandibular salivary glands, using the most M₅/M₃ selective ligands available. Affinities for all four compounds studied (darifenacin, oxybutynin, pF-HHSiD and AF-DX 384) did not significantly differ from published affinity estimates at the human M₃ receptor (Eglen et al., 1996a; Caulfield & Birdsall, 1998) and also generally correlated well with affinities derived for CHO-m3 membranes, in experiments performed alongside the submandibular gland binding assays. Overall, there was no evidence of a significant, detectable M₅ receptor population in the submandibular gland of the guinea-pig.

Some evidence for the presence of an M₅ receptor population in the submandibular gland has been inferred from previous radioligand binding studies (Flynn et al., 1997), while microphysiometry studies in rat submandibular gland generated an affinity profile, based on a wide range of subtype-selective mACh receptor antagonists, which correlated best with cloned human M₃ and M₅ receptors (Meloy et al., 2001). Data from mice, lacking the gene for the M₅ mACh receptor, suggest a role for this muscarinic receptor subtype in salivation only at longer time-periods (15-60 min) following intravenous administration of the muscarinic partial agonist pilocarpine (Yeomans et al., 2001; Takeuchi et al., 2002). Whether this accurately defines the functional role of the M₅ receptor in muscarinic agonist-induced salivation, or
whether compensatory changes occur in the M<sub>3</sub> receptor signalling pathway is unclear.

Once again, data presented here in guinea-pig tissue hint at species-dependent differences in muscarinic receptor expression, as the aforementioned studies were performed in rat and mouse. However, a role for the M<sub>3</sub> receptor in guinea-pig salivation cannot be ruled out as the reported M<sub>3</sub>/M<sub>2</sub> selectivity of the mACh receptor ligands used in this study is 10-fold at best (Eglen et al., 1996a; Caulfield & Birdsall, 1998) and so a small M<sub>3</sub> receptor population is unlikely to be discriminated by these agents. It is also possible that the other major salivary glands express more significant levels of M<sub>2</sub> receptor and could therefore be responsible for the effects seen in M<sub>3</sub> 'knockout' mice, but little evidence relating to this is available. Data from in vivo studies, such as those in 'knockout' mice, once again highlight the difference between investigating the mACh receptor expression profile of a particular salivary gland, which can provide specific information about the signalling pathways involved in the regulation of secretion by that gland, and measuring salivary output, which is likely to be the result of the concerted actions of a number of different glands.

4.3.4 What mechanism(s) could underlie the functional selectivity of certain mACh receptor antagonists?

So if agonist-stimulated phosphoinositide turnover in both bladder and submandibular gland is mediated via activation of M<sub>3</sub> mACh receptors, the question remains as to why some, but not all mACh receptor antagonists inhibit these responses with different affinities? In the absence of any evidence for genetic heterogeneity of the M<sub>3</sub> receptor, the influence of the environment in which the receptor is presented upon M<sub>3</sub> receptor pharmacology must be assessed. Thus, it has been proposed that GPCRs may express 'phenotypic' profiles specific to the host cell environment, such that the same gene can exhibit distinct pharmacologies when expressed in different cells (Kenakin, 2003). While a definitive role for the majority M<sub>2</sub> receptor population expressed in the urinary bladder remains elusive, it is tempting to speculate on the involvement of this population in the functional selectivity of some mACh receptor antagonists. Although some evidence has been presented for a direct role in contraction for the M<sub>2</sub> receptor (e.g. Hegde et al., 1997; see section 1.3.4.4), it is unlikely that this could explain
Darifenacin’s higher functional affinity in the bladder, due to the compound’s low affinity for the inhibition of M3 receptor-mediated functional responses (as reported in the present study and in previous investigations; e.g. Wallis & Napier, 1999).

However, several examples of the co-expression of distinct pairs of G protein-coupled receptors giving rise to a novel functional unit, exhibiting ligand binding and functional characteristics distinct from either constituent receptor, have been documented (see Angers et al., 2002). These are examples of ‘hetero-oligomerisation’, or ‘hetero-dimerisation’, assuming the formation of the simplest possible oligomer. For example, Jordan & Devi (1999) found that x and δ opioid receptors formed hetero-dimers when co-expressed in a variety of model cell lines. Selective agonists for each receptor subtype were able to bind cooperatively to hetero-dimers and induce synergistic functional responses (Jordan & Devi, 1999). Hetero-dimers also displayed unique binding characteristics that were virtually identical to those previously reported for the putative k-2 receptor (Jordan & Devi, 1999). Interestingly, cDNAs corresponding to alternative opioid receptors, such as the k-2, have yet to be identified. This raises the possibility that the molecular basis for the pharmacologically defined ‘k-2 subtype’ is in fact the endogenous expression of opioid receptor hetero-dimers, such as the k-δ dimer characterised by Jordan & Devi, (1999).

Another example of the influence of GPCR hetero-dimerisation on functional responses is in the interaction of dopamine D2 and somatostatin SSTR5 receptors (Rocheville et al., 2000). Membranes of cells co-expressing these two receptors displayed synergistic binding and functional responses to agonists specific to each receptor, while a dopamine receptor antagonist was able to induce a reduction in binding of a somatostatin receptor-selective ligand (Rocheville et al., 2000). AbdAlla et al. (2000) demonstrated hetero-dimerisation of angiotensin AT1 and bradykinin B2 receptors, resulting in the increased potency and efficacy of angiotensin II. These experiments were conducted at low receptor expression levels, providing evidence that hetero-dimerisation can occur, with profound functional consequences, even at endogenous receptor expression levels. Indeed, AbdAlla et al. (2000) provided evidence for hetero-dimerisation in native smooth muscle cells, using the smooth muscle-derived A10 cell line. Reduction in B2 receptor expression using antisense cDNA caused a concomitant decrease in angiotensin II-stimulated intracellular
calcium rises, suggesting the importance of the B2 receptor population in facilitating
the full response to angiotensin II (AbdAlla et al., 2000).

The first evidence of a role for GPCR hetero-dimerisation in the pathophysiology of a
clinical condition was provided by a later study from the same group. Abdalla et al.
(2001) reported that pre-eclamptic women exhibited a five-fold rise in the levels of
bradykinin B2 receptor in vascular smooth muscle, leading to a concomitant increase
in the level of B2-AT1 receptor dimers (Abdalla et al., 2001). This increase was
associated with enhanced angiotensin II-mediated Ca2+ mobilisation in platelets and is
likely to underlie the angiotensin II hypersensitivity observed in pre-eclampsia, the
cause of which had previously been unclear, as patients displayed unaltered levels of
AT1 receptor and angiotensin II itself (Abdalla et al., 2001).

But could muscarinic receptor subtypes, such as the M2 and M3 receptors, associate as
hetero-dimers and could this influence signalling through them? Wreggett & Wells
(1995) used purified M2 receptors from porcine atria to demonstrate oligomeric
complexes in biochemical studies. They also presented evidence from binding studies
of cooperative interactions within an oligomeric receptor complex, which could be
accounted for by a tetrameric assembly of M2 receptors (Wreggett & Wells, 1995).
Park et al. (2001) co-immunoprecipitated differentially tagged (FLAG and c-Myc) M2
receptors co-expressed in insect Sf9 cells, deducing from the efficiency of
immunoprecipitation that at least some of the receptors existed as trimers or higher
order oligomers (Park et al., 2001). However, with the high levels of receptor
expression associated with insect cell expression systems and the observation that the
majority of M3 muscarinic receptors are located intracellularly in Sf9 cells
(Vasudevan et al., 1995) the physiological relevance of these observations is
questionable. More convincing evidence for muscarinic receptor homo­
ligomerisation was provided by biochemical studies in COS-7 cells expressing the
rat M3 muscarinic receptor (Zeng & Wess, 1999). Similar results were obtained in
native rat brain membranes, indicating that M3 receptors are capable of forming
homo-dimers at endogenous levels of expression (Zeng & Wess, 1999). The
importance of these dimers in M3 receptor signalling was highlighted by the severe
reduction in agonist potency (> 10,000-fold) of double-cysteine mutant receptors that
were unable to form homodimers (Zeng & Wess, 1999). However, it should be noted
that in the same study, Zeng & Wess could not detect any M2/M3 co-
immunoprecipitation in cells co-expressing the two subtypes (Zeng & Wess, 1999). A recent yeast 2-hybrid study investigated the potential for N- and C- termini, extracellular loops 1 and 2, and the first and third intracellular loops of the human M₁, M₂ and M₃ receptors to interact with each other (Kang et al., 2003). No physical interactions were observed between any of the domains of any of the three muscarinic receptor subtypes (Kang et al., 2003). However, this does not preclude mACh receptor oligomerisation as interactions may occur through the hydrophobic transmembrane domains or may require the complete receptor in its natural conformation.

Maggio et al. (1993) provided evidence for both homo- and hetero-dimerisation of muscarinic receptors, first using chimeric M₃/α₂C adrenoceptor constructs in which the sixth and seventh trans-membrane domains were interchanged. While either chimera alone was unable to bind ligands or signal, co-expression of the two reciprocal combinations rescued function, suggesting that the two receptors physically associate to form appropriate binding sites (Maggio et al., 1993). M₂/M₃ receptor interactions were implied in a later study, using receptors split at the third intracellular loop to form either M₂- or M₃- 'trunc' (TM I-V) or 'tail' (TM VI-VII) fragments (Chiacchio et al., 2000). For instance, co-expression of wild-type M₂ receptors with M₃-tail (which does not itself bind muscarinic ligands) fragments yielded biphasic displacement curves for the M₁-selective antagonist pirenzepine, in radioligand binding studies, with binding affinities consistent with the M₂ receptor and an M₂ trunc/M₃-tail chimera (Chiacchio et al., 2000). This would suggest a physical association between the M₂ receptor and C-terminal tail of the M₃ receptor, forming a viable structure capable of antagonist binding. Interestingly, pirenzepine was able to clearly distinguish two sites in these co-expressing cells due to its significantly higher affinity for the M₂/M₃ chimera than for either wild-type M₂ or M₃ receptors (Chiacchio et al., 2000). This enhanced antagonist affinity in cells co-expressing M₂ and M₃ receptors clearly resembles the higher affinities seen for darifenacin, oxybutynin and tolterodine in bladder tissue containing M₂ and M₃ receptor populations. It is therefore tempting to speculate that hetero-dimerisation of these two receptor subtypes may be involved in the observed tissue-selectivities of certain compounds.
There are, however, numerous other possible mechanisms that could be implicated in tissue-specific pharmacology. Although the M3 receptor is encoded by a single, intronless gene (eliminating the possibility of splice variants with different tissue distribution patterns) (Bonner et al., 1987), the possibility remains that the M3 receptor could undergo differential post-transcriptional modification between two different cell/tissue types. For instance, the 5-HT2C receptor has been reported to undergo RNA editing events at up to five specific sites (Niswender et al., 1999). The degree of RNA editing was shown to influence the level of constitutive activity and the potency of serotonergic agonists (Niswender et al., 1999). A later study demonstrated that G-protein coupling was altered, with specific elimination of G13 coupling in the more extensively edited receptors (Price et al., 2001). Further effects on receptor-G-protein-effector coupling were seen when edited and un-edited 5-HT2C receptor-mediated activation of arachidonic acid release and phosphoinositide hydrolysis was investigated (Berg et al., 2001). Whereas the un-edited receptor displayed a reversal of relative efficacy order of the 5-HT agonists TFMPP and bufotenin for the two signalling pathways investigated (i.e. demonstrating agonist-directed trafficking of signalling), the edited forms of the receptor displayed no such difference in efficacy (Berg et al., 2001). Whether RNA editing is a common mechanism for increasing the diversity of GPCR signalling remains to be seen.

Perhaps as a consequence of the high level of sequence homology between the acetylcholine (orthosteric) binding site of the muscarinic receptor subtypes (Bonner et al., 1987), many of the more subtype-selective muscarinic ligands have been discovered to interact with allosteric sites on the muscarinic receptors, where perhaps sequences are less well conserved amongst mACh receptor subtypes. Examples of these include gallamine (Stockton et al., 1983), methoctramine (Eglen et al., 1988b), himbacine (Lee & El-Fakahany, 1990) (all M2-selective) and more recently the M1-selective muscarinic toxin 7 (MT-7; Olianas et al., 2000). The observation in a number of functional studies that darifenacin causes insurmountable antagonism of muscarinic agonist-mediated responses (e.g. Hegde et al., 1997; see section 1.3.6.3 for further discussion) has raised the question of whether darifenacin behaves as a true competitive antagonist at the orthosteric binding site or if its selectivity is due to interactions with alternative, allosteric sites on the muscarinic receptors. However, the use of [3H]-darifenacin, in addition to [3H]-NMS association binding experiments...
following pre-incubation with un-labelled darifenacin, in a recent study by Clarke et al. (2003b) provides strong evidence that darifenacin is a competitive and reversible antagonist at the M\textsubscript{3} receptor (at least at the cloned human M\textsubscript{3} receptor expressed in CHO cells). The insurmountable antagonism observed in experiments in native tissue may be due to insufficient equilibration, or may reflect differences in binding kinetics in tissue preparations relative to those seen in recombinant cell lines. However, since the reversibility and binding kinetics of darifenacin at the M\textsubscript{2} receptor have not yet been fully investigated, the possibility (although remote) remains that darifenacin may interact allosterically with the M\textsubscript{2} receptor and that the insurmountable component of its action is related to inhibition of M\textsubscript{2}-mediated contraction. In this respect it is interesting to note that even after inactivation of the M\textsubscript{3} receptor population by 4-DAMP mustard, darifenacin-mediated inhibition of the residual muscarinic receptor-induced contraction of rat bladder, presumably mediated by M\textsubscript{2} receptors (which were protected during 4-DAMP mustard treatment), was insurmountable (Hegde et al., 1997). The M\textsubscript{2} receptor has also been recognised as the muscarinic receptor that it most sensitive to allosteric ligands (Lee & El-Fakahany, 1991; Ellis et al., 1991) and it is therefore conceivable that compounds might interact with both the orthosteric and allosteric sites of this subtype, but act purely competitively at other subtypes. However, considering the low affinity of darifenacin’s interaction with the M\textsubscript{2} receptor (observed in both binding and functional assays), it is unlikely that this could account for the enhanced affinity observed for darifenacin in the M\textsubscript{2}-expressing bladder tissue.

The realisation in recent years that GPCRs are able to couple to their cognate G-proteins in the absence of agonist binding (i.e. are constitutively active) has opened up the possibility that tissue-specific differences in constitutive activity of the same receptor may have influences upon the pharmacology of that receptor. Indeed, constitutive activity has been observed for muscarinic receptors, both in recombinant cell systems and in native tissue (e.g. Jakubik et al., 1995; see section 1.5 for further discussion). This may be of particular relevance to antagonists that are in fact ‘inverse agonists’ (i.e. stabilise the inactive form of the receptor, decreasing constitutive activity), as has been documented for some muscarinic antagonists (review, see Kenakin, 2004). Cell- or tissue-specific factors such as receptor density and G-protein availability can influence not only agonist potency and efficacy, but also negative
efficacy of inverse agonists and even the propensity of GPCRs to interact with multiple G-proteins in a given cell (Kenakin, 2003). Certain antagonist ligands have been demonstrated to differentially antagonize two distinct signal transduction pathways activated through the same receptor (e.g. Pommier et al., 1999). It is therefore conceivable that certain ‘antagonists’ that display negative efficacy might selectively uncouple specific receptor-G-protein interactions. Tissue-dependent variations in receptor-G-protein coupling fidelity and efficiency could then conceivably underlie differences in apparent ‘antagonist’ affinities. It would therefore be useful to establish the inverse agonist nature of the mACh receptor ‘antagonists’ under investigation here and relate this to their actions in the native tissues.

Spatial organization of GPCRs and other signalling components into specific membrane domains or scaffolding networks has also been shown to influence signal transduction (Ostrom, 2002). A wide variety of proteins have been documented to form stable or transient associations with GPCRs, including scaffolding proteins (e.g. AKAP79/150, Homer, spinophilin), cytoskeletal proteins and receptor activity-modifying proteins (RAMPs) (Milligan & White, 1999; Brady & Limbird, 2002). The A-kinase anchoring protein (AKAP) family are known to bind not only protein kinase A (PKA) but also a variety of signalling proteins including protein kinase C (PKC) and various protein phosphatases, targeting these enzymes to sub-membrane sites in close proximity to their cellular targets (e.g. ion channels) (Colledge & Scott, 1999). AKAP150 has also been reported to associate with the C-terminus and third intracellular loop of the β2-adrenoceptor, facilitating cyclic AMP-dependent phosphorylation of the receptor by anchoring PKA in close proximity to the receptor (Fraser et al., 2000). More widespread roles for AKAPs in GPCR signalling await characterisation but along with the increasing number of proteins reported to associate with a variety of different GPCRs, it is anticipated that functionally significant protein-protein interactions will become increasingly well defined, perhaps providing novel targets for the development of cell-type specific therapeutics (Brady & Limbird, 2002).

One of the most striking examples of accessory proteins altering the pharmacology of their associated GPCR is that of the RAMPs. Co-expression of RAMP3 with the calcitonin receptor has been shown not only to influence agonist potency ranking orders, but also antagonist affinity estimates (Armour et al., 1999). Interaction
between RAMPs and the calcitonin receptor-like receptor (CRLR) determine the ligand binding of the receptor, as receptors associated with RAMP1 specifically bind CGRP while those interacting with RAMP2 or RAMP3 bind adrenomedullin (McLatchie et al., 1998). A wider role for this family of single transmembrane-spanning domain proteins was suggested by their ubiquitous expression (a significantly wider expression profile than that of the calcitonin and calcitonin-like receptors) and it was subsequently found that the majority (e.g. glucagon and parathyroid hormone receptors) of the class II peptide receptor family of GPCRs selectively interact with one or more members of the RAMP family (review, see Morfis et al., 2003). However, it would appear that this particular family of accessory proteins interacts exclusively with the class II GPCRs as similar studies have failed to observe any association between RAMPs and class I GPCRs (Morfis et al., 2003).

Accessory proteins interacting with G-proteins are also known to influence GPCR signalling by altering the kinetics of signal termination. The regulators of G-protein signalling (RGS proteins) are a large family of proteins that are known to bind directly to activated Gα-GTP, where they act as GTPase-activating proteins (GAPs) to terminate signalling through the associated G protein (Ross & Wilkie, 2000). Further diversity of RGS proteins is provided by the alternative splicing of certain subtypes, most notably RGS6 for which 36 different transcripts have been identified from the single RGS6 gene (Chatterjee et al., 2003). Although some examples of RGS proteins specifically regulating the activity of a certain class of Gα have been reported, most RGS proteins seem surprisingly promiscuous in their Gα binding (Hepler, 2003). It is becoming increasingly apparent that the receptors themselves may be responsible for conferring specificity upon RGS/Gα interactions, and that receptors, G proteins, RGS proteins and effectors may be held in multi-protein scaffolding complexes at the plasma membrane (Hepler, 2003). Since any combination of proteins interacting with a given GPCR is likely to be dependent upon the cell background, it is conceivable that the kinetics of termination of signalling may be regulated in a cell-type specific manner. Whether it is possible that cell background-specific signalling characteristics could be reflected in antagonist inhibition profiles is not yet clear.

The notion that certain antagonists can display tissue/cell background-dependent pharmacology, particularly in the inhibition of functional responses, may not be restricted to the muscarinic acetylcholine receptors. An interesting example is in the
functional characterisation of the \( \alpha_1 \)-adrenoceptors, for which a fourth subtype (\( \alpha_{1L} \)), in addition to the \( \alpha_{1A} \), \( \alpha_{1B} \) and \( \alpha_{1D} \) receptors, has been proposed on the basis of pharmacological, but not molecular studies. The \( \alpha_{1L} \)-adrenoceptor is well characterised in the lower urinary tract (e.g. Ford et al., 1996) but cDNA corresponding to this novel phenotype has not been isolated. However, it was found that when expressed in CHO cells, the functional inhibitory profile of a range of antagonists at the \( \alpha_{1A} \)-adrenoceptor closely resembled the pharmacological profile of the putative \( \alpha_{1L} \)-receptor (Ford et al., 1997). In contrast, the binding affinities of the same range of ligands in membrane homogenates prepared from the same cells were consistent with the classically defined \( \alpha_{1A} \)-adrenoceptor (Ford et al., 1997). However, affinity estimates were not merely 'frame-shifted' as 3 of the 8 antagonists acted as good internal controls, displaying similar affinities in both binding and functional assays. Also, binding and functional affinity profiles were indistinguishable for both \( \alpha_{1B} \)- and \( \alpha_{1D} \)-adrenoceptors similarly expressed in CHO cells, indicating that the altered pharmacological properties exhibited by the \( \alpha_{1A} \)-adrenoceptor were specific to that subtype (Ford et al., 1997).

Subsequent work by the same group has eliminated the potential involvement of alternative splicing of the \( \alpha_{1A} \)-adrenoceptor gene in the observed differences in pharmacology, as all four known splice variants displayed the \( \alpha_{1L} \) phenotype in functional assays (Daniels et al., 1999). However, intact cell binding studies, undertaken in more 'physiological' buffer conditions and at 37°C, yielded affinity estimates more consistent with the \( \alpha_{1L} \)-adrenoceptor phenotype (Williams et al., 1996). The intact cellular environment may therefore be required to generate the \( \alpha_{1L} \)-adrenoceptor phenotype and may explain the paucity of pure \( \alpha_{1A} \)-adrenoceptor profiles in human tissues. The \( \alpha_{1L} \) phenotype may therefore represent the generally observed (and more physiologically relevant) functional state of the \( \alpha_{1A} \)-adrenoceptor gene product, but the molecular basis for this 'switch' in profile remains unresolved.

One of the potential clinical implications of the pleiotropic behaviour of \( \alpha \)-adrenoceptors is particularly reminiscent of the functional selectivity of some muscarinic receptor antagonists. Many of the \( \alpha_1 \)-adrenoceptor antagonists currently prescribed for the treatment of benign prostatic hyperplasia (BPH) fail to display selectivity between the \( \alpha_{1A} \), \( \alpha_{1B} \) and \( \alpha_{1D} \) subtypes \textit{in vitro}, despite appearing to
selectively target the prostate gland and have little impact upon the cardiovascular system (Langer, 1999). Analysis of the α-adrenoceptor subtypes expressed in the prostate by a variety of means had previously led to the conclusion that the α1A-adrenoceptor is the predominant subtype expressed (e.g. Testa et al., 1993). Thorough analysis with α-adrenoceptor antagonists known to discriminate between the α1A and α1L phenotypes (including prazosin) indicated that the functional profile of the α-adrenoceptors expressed in rabbit (Leonardi et al., 1997) and human (Muramatsu et al., 1994) prostate was most consistent with the α1L phenotype.

Characterisation of the α-adrenoceptor subtypes expressed in the vasculature initially focused on the aorta, where the α1B (in rat (Kenny et al., 1995) and rabbit (Leonardi et al., 1997)), or α1D (in dog (Leonardi et al., 1997)) receptors have been shown to predominate. This has led to the notion that the prostate gland selectivity of certain α1A/α1L antagonists may result from the minor role of this subtype in the vascular system. However, studies of the α-adrenoceptor sub-populations involved in the contraction of resistance arteries suggests a profile more consistent with the α1L phenotype in the rat small mesenteric artery (Van der Graaf et al., 1996) and the canine subcutaneous resistance arteries (Argyle & McGrath, 2000). Since these small resistance arteries are likely to play a more pivotal role in the control of blood pressure than the large conduit arteries, it would seem that subtype selectivity alone is insufficient to explain the preservation of blood pressure control while effectively antagonizing the α-adrenoceptor responses of the prostate. Argyle & McGrath, (2000) have therefore proposed that the variable functional affinity of certain α-adrenoceptor antagonists may be due to a tissue-specific property of the receptors. The authors point to a study reporting that the affinity of a prazosin analogue for the native human α1A-adrenoceptors (expressed in single human prostate cells) is higher than for the cloned human α1A-adrenoceptor expressed in recombinant cells (Mackenzie et al., 2000), providing in vivo supporting evidence for the notion that the same receptor genotype can display differing phenotypes in a cell background-dependent manner.

The nature of the muscarinic receptors involved in the contraction of uterine smooth muscle is also the subject of some controversy. Although the radioligand binding profile of the guinea-pig uterus is consistent with a homogeneous M2 receptor population (Boxall et al., 1998), functional studies fail to agree on the potential roles
of $M_2$, $M_3$ and $M_4$ receptors in muscarinic agonist-mediated contraction (e.g. Leiber et al., 1990; Dorje et al., 1990; Dooods et al., 1993). Boxall et al. (1998) found that in contrast to their radioligand binding data, a range of $M_2$/$M_3$ selective antagonists inhibited contraction with affinities suggestive of a dual role for $M_2$ and $M_3$ receptors in this response. However, selective alkylation of the putative $M_3$ receptor population with phenoxybenzamine had little effect upon contraction, suggesting a purely $M_2$-mediated response (Boxall et al., 1998). Treatment with pertussis toxin, however, also had little effect upon the contractile response to carbachol, appearing to rule out an involvement of $G_{i/o}$ proteins in the $M_2$ receptor-mediated contraction (Boxall et al., 1998). It is possible that a small $M_3$ receptor population, not detected in the binding assays, could be acting synergistically with the majority $M_2$ population. However, previous radioligand binding studies support the notion that the guinea-pig uterus expresses a homogeneous $M_2$ receptor population (Eglen et al., 1989; Dooods et al., 1993). Overall, the pharmacological profile is not typical of the classically defined mACh receptor subtypes and suggests that functionally, the $M_2$ receptor population detected in radioligand binding studies behaves unlike that predicted for such a population.

Similar 'atypical' mACh receptor profiles have been reported in other smooth muscles. Both radioligand binding (Gomez et al., 1992) and functional (Kerr et al., 1995) studies in colonic smooth muscle have reported atypical affinities for muscarinic antagonists, including AF-DX 116. This prompted Mansfield et al. (2003) to undertake a thorough binding study in membranes prepared from human colon, utilising a wide range of the more subtype-selective muscarinic antagonists now available (e.g. methoctramine, darifenacin, AQ RA 741). No evidence of atypical pharmacology was found with methoctramine and darifenacin agreeing upon an approximate 85:15 ratio of $M_2$:$M_3$ receptors and the affinities for all compounds investigated were in line with those determined for $M_2$ and $M_3$ receptors expressed in recombinant cell lines (Mansfield et al., 2003). However, it should be noted that this study only investigated the binding affinities of these ligands and only in membrane homogenates. It is possible that in intact cells/tissues the functional activity of certain antagonists may indeed be 'atypical', as previously described (Kerr et al., 1995).

The term 'atypical' is also commonly used to describe endogenously expressed $\beta$-adrenoceptors that display pharmacology that differs from our current descriptions of
the cloned $\beta_1$, $\beta_2$ and $\beta_3$ receptors (Molenaar, 2003). These include the putative $\beta_4$-adrenoceptor, originally described in cardiac tissue (Kaumann, 1997) and now recognised as a 'low affinity' state of the $\beta_1$-adrenoceptor (Kaumann et al., 2001), and attempts to define the $\beta$-adrenoceptor(s) involved in sympathetic-mediated relaxation of various smooth muscle types (e.g. Yamazaki et al., 1998; Igawa et al., 1999). Yamazaki et al. (1998) examined the $\beta$-adrenoceptors involved in adrenergic-mediated relaxation of rat, rabbit and dog urinary bladder smooth muscle and found that although there were considerable species differences, $\beta$-adrenoceptor-linked relaxation could be explained in each case by the actions of one or more of the typical ($\beta_1$-$\beta_3$) $\beta$-adrenoceptors. In contrast, functional characterisation of the same response in human bladder detrusor indicated that neither $\beta_1$-nor $\beta_2$-adrenoceptor subtypes were involved (Igawa et al., 1999). Strong evidence was provided for a role for the $\beta_3$-adrenoceptor in detrusor relaxation, but the low slope obtained from Schild analysis of the inhibition of relaxation by the $\beta_3$-selective antagonist SR58894A implicated more than one subtype in this response (Igawa et al., 1999). The lack of effect of $\beta_1$- or $\beta_2$-selective antagonists led the authors to suggest a role for an 'atypical' $\beta$-adrenoceptor in addition to the $\beta_3$-mediated relaxation (Igawa et al., 1999).

Atypical $\beta$-adrenoceptors have also been implicated in $\beta$-adrenoceptor-mediated relaxation in the rat mesenteric artery (Sooch & Marshall, 1997). A recent study found that the non-$\beta_1$/non-$\beta_2$-adrenoceptor-mediated relaxation in the rat mesenteric artery does not involve $\beta_3$-adrenoceptors, but is mediated by an atypical receptor that resembles the low affinity state of the $\beta_1$-adrenoceptor in cardiac tissue in some respects (Kozlowska et al., 2003). However, cyanopindolol and CGP 12177 ('non-conventional' partial $\beta$-adrenoceptor agonists, central to the definition of the low affinity $\beta_1$-adrenoceptor) displayed lower potencies and the opposite rank order of potency in rat mesenteric artery to that reported at the low affinity $\beta_1$-adrenoceptor in cardiac tissue (Kozlowska et al., 2003). Similar observations have been reported in rat aorta (Brawley et al., 2000; Brahmadevara et al., 2003). It is therefore likely that an atypical $\beta$-adrenoceptor distinct from the cardiostimulant atypical $\beta$-adrenoceptor mediates vasorelaxation in these vessels. However, given the consistent failure to identify more than three $\beta$-adrenoceptor genes by molecular techniques, it is possible that this atypical receptor might represent an alternative 'state' of one of the three
conventional β-adrenoceptors (as is the case with the cardiotimulant atypical β-adrenoceptor). However, further complexity in β-adrenoceptor classification is indicated by apparent differences in the relative potencies of cardiotimulant, arrhythmogenic and positive inotropic and chronotropic effects of CGP 12177, indicating that multiple β1-adrenoceptor states may co-exist in cardiac tissue, each perhaps mediating qualitatively different effects (see Kaumann et al., 2001). The exact nature of these ‘states’ and the potential roles of different cellular factors in determining them await further study.

Observations reported in this study and by others regarding tissue-dependent pharmacologies of G protein-coupled receptor antagonists, do not therefore appear to be unique to the mACh receptor family. However, whereas for other ‘atypical’ receptor profiles (such as the α11-adrenoceptor and the ‘low affinity’ cardiotimulant β1-adrenoceptor state) a consistent, definitive affinity profile has been described, the various examples of apparent tissue-dependent muscarinic receptor behaviour are lacking such a unifying characterisation. However, data presented here suggest that the enhanced functional affinities of darifenacin, tolterodine and oxybutynin for the urinary bladder relative to the salivary glands, at least in guinea-pig, may represent tissue-specific profiles of responses mediated by the M3 muscarinic receptor. Defining the cellular factor(s) responsible for these differences in antagonist affinity estimates remains the challenge for future work in this area.
Chapter 5: An investigation of constitutive activity and inverse agonism at transiently expressed wild-type and N410Y mutant M₂ mACh receptors.

5.1 Introduction

A crucial development in the understanding of GPCR function was the identification of their ability to activate their cognate G-proteins in the absence of an agonist (Costa & Herz, 1989). This was accompanied by the observation that certain ligands (termed 'inverse agonists'), previously characterised as competitive antagonists, could reverse agonist-independent activity (Costa & Herz, 1989). Subsequent research has identified significant agonist-independent (or 'constitutive') activity by a wide variety of both endogenously and recombinantly expressed GPCRs (for review, see: de Ligt et al., 2000; Seifert & Wenzel-Seifert, 2002). In most cases, in conjunction with constitutive activity (and arguably as an essential pre-requisite in the definition of constitutive activity), ligands displaying the property of inverse agonism have also been identified (for review, see: Seifert & Wenzel-Seifert, 2002; Kenakin, 2004).

One of the most powerful tools utilised by researchers in this area has been the development of GPCRs harbouring specific mutations known to enhance the agonist-independent coupling of receptor and G-protein (so-called constitutively-active mutant (CAM) receptors) (Parnot et al., 2002; Cotecchia et al., 2003). Lefkowitz and colleagues first identified single point mutations in the C-terminus of the third intracellular loop of the α₁B-adrenoceptor that significantly enhanced agonist-independent inositol phosphate accumulation in COS-7 cells recombinantly expressing the mutant receptors (Cotecchia et al., 1990). Specific mutations within a number of different domains have since been found to increase the observed constitutive activity of many GPCRs (Pauwels & Wurch, 1998).

A number of studies have identified constitutively-activating mutations within the mACh receptors, with the majority of work focussed upon the predominantly Gq/11-coupled M₁, M₃ and M₅ mACh receptors (for more detailed discussion, see section 1.5.3.2). Systematic mutation of various regions of the M₅ mACh receptor identified a large number of residues within the sixth transmembrane domain (TM 6) that, when mutated, contributed to enhanced agonist-independent G-protein coupling (Spalding
et al., 1998). In particular, this study confirmed earlier work by the same group indicating that mutation of adjacent serine (Ser465) and threonine (Thr466) residues near the junction of TM 6 and the third extracellular loop (see Figure 5.1.1) conferred significant constitutive activity upon the mutant M₃ mACh receptor (Spalding et al., 1995). More recently, the effects of the homologous double mutation on all five mACh receptor subtypes was examined by Ellis and co-workers (Ford et al., 2002). The mutant receptors mostly displayed the characteristic properties of CAM GPCRs, including enhanced agonist affinity and potency, in addition to an elevated basal functional activity (proportional to the receptor expression level), which was sensitive to the inverse agonist atropine (Ford et al., 2002).

Huang and colleagues had earlier investigated both double (Ser388Thr389 to TyrPro) and single (Ser388 to Tyr and Thr389 to Pro) mutations in the M₁ mACh receptor subtype and found that mutation of Ser388 alone was sufficient to generate a CAM receptor (Huang et al., 1998b). In contrast, mutation of Thr389 appeared to influence receptor-G-protein coupling fidelity, introducing multiple affinity binding states in agonist competition binding experiments (Huang et al., 1999). Moreover, Spalding et al. (1997) reported that mutation of the homologous residue (Ser 465) in the M₅ subtype, particularly to large (Phe or Val) or basic (Arg or Lys) residues, generated receptors with significantly enhanced constitutive activity, relative to wild-type. Taken together, these data would suggest that the conserved serine residue at the boundary between TM 6 and the third extracellular loop is implicated in constraining the M₁ and M₃ mACh receptors in the inactive state.

In light of this, the homologous mutation of asparagine at position 514 to tyrosine (N514Y) was investigated in the human M₃ mACh receptor (Dowling, PhD thesis, 2004). When transiently expressed in HEK-293 cells, the N514YM₃ mACh receptor produced a significantly greater (approx. 4-fold) basal accumulation of [³H]-inositol phosphates than in cells expressing similar or higher levels of wild-type M₃ receptor (see Figure 5.1.2 (a), reproduced by kind permission from Dowling (PhD thesis, 2004). Atropine was found to concentration-dependently reduce the basal phosphoinositide turnover in both wild-type and N514YM₃ receptor-expressing cells (Figure 5.1.2 (b)). Agonists exhibited higher binding affinity and potency at N514YM₃ receptors than at wild-type M₃ receptors, while antagonists were found to display lower binding affinity for N514YM₃ receptors (Dowling, PhD thesis, 2004). The
<table>
<thead>
<tr>
<th>mACH R subtype</th>
<th>TM 6</th>
<th>3rd Extracellular loop</th>
</tr>
</thead>
<tbody>
<tr>
<td>$M_1$</td>
<td>Pro Tyr Asn Ile Met Val Leu Val</td>
<td>Ser Thr Phe Cys Lys Asp Cys Val Pro</td>
</tr>
<tr>
<td>$M_2$</td>
<td>Pro Tyr Asn Val Met Val Leu Ile</td>
<td>Asn Thr Phe Cys Ala Pro Cys Ile Pro</td>
</tr>
<tr>
<td>$M_3$</td>
<td>Pro Tyr Asn Ile Met Val Leu Val</td>
<td>Asn Thr Phe Cys Asp Ser Cys Ile Pro</td>
</tr>
<tr>
<td>$M_4$</td>
<td>Pro Tyr Asn Val Met Val Leu Val</td>
<td>Asn Thr Phe Cys Gln Ser Cys Ile Pro</td>
</tr>
<tr>
<td>$M_5$</td>
<td>Pro Tyr Asn Ile Met Val Leu Val</td>
<td>Ser Thr Phe Cys Asp Lys Cys Val Pro</td>
</tr>
</tbody>
</table>

*Figure 5.1.1* Sequence alignment for the five human mACH receptor subtypes. Mutations at the residues highlighted in bold are reported to confer constitutive activity upon receptors (see Spalding et al., 1995, 1997, 1998; Huang et al., 1998b, 1999; Ford et al., 2002).
Figure 5.1.2 (a) Basal [3H]-inositol phosphate accumulation and responses to methacholine and atropine in HEK-293 cells transiently expressing wild-type and N514Y CAM M3 receptor.

(b) Concentration dependent inhibition of basal [3H]-inositolphosphate accumulation by atropine in HEK-293 cells transiently expressing wild-type (red) and N514Y CAM M3 (blue) receptor.
N514Y M2 receptor therefore shows all of the characteristics of a constitutively-active mutant mACh receptor and supports the notion that the Ser/Asn residue at the junction between TM 6 and the third extracellular loop is crucial in maintaining the muscarinic receptors in the inactive state.

The primary aim of this investigation was, therefore, to perform the homologous mutation in the M2 mACh receptor (at position N410) and investigate its constitutive activity, relative to the wild-type M2 receptor. There are limited examples in the literature of constitutively-active M2 receptor mutants. Liu et al. (1996) reported that the insertion of one or more alanine residues at a specific locus in the N-terminus of TM 6 significantly enhanced the constitutive activity of the M2 receptor transiently expressed in COS-7 cells. Ford et al. (2002) reported moderate, atropine-sensitive constitutive activity of an M2 mACh receptor harbouring the double mutation at Asn410Thr411. However, in each of these studies, a thorough characterisation of the mutant receptors was not performed beyond the establishment of constitutive activity. It was the aim of this investigation to provide such a rigorous analysis of the N410YM2 mACh receptor mutant.

The availability of constitutively active mutant M2 and M3 mACh receptors will then allow a comparison of the inverse agonism of a range of muscarinic ligands previously classified as antagonists. Of particular interest will be the muscarinic antagonists used in the clinical management of overactive bladder (oxybutynin and tolterodine), as well as the potential therapeutic darifenacin. To our knowledge, the potential for these ligands to display inverse agonism at muscarinic receptors has yet to be investigated. A clear definition of the efficacy (if any) of these ligands might therefore provide valuable information relating to their in vivo actions.
5.2 Results

5.2.1 Sub-cloning of $M_2$ mACh receptor gene out of pCD and into pcDNA3.

5.2.1.1 Primer design

In an effort to establish similar transfection efficiency and expression levels of $M_2$ and $M_1$ receptor constructs, it was necessary to sub-clone the $M_2$ receptor gene out of the pCD vector and into pcDNA3. Specific primers were designed to allow the $M_2$ receptor gene to be amplified by polymerase chain reaction (PCR), from the template of the $M_2$ receptor gene in pCD.

**Sense - primer # 1**

\[ \text{5'} \quad \text{CCC CCC GAA TTC ATG AAT AAC TCA ACA AAC TCC TCT AAC AAT AGC CTG} \quad \text{3'} \]

**Antisense - primer # 2**

\[ \text{5'} \quad \text{CCC CCC CTC GAG TTA CCT TGT AGC GCC TAT GTT CTT ATA ATG ACA CAT GAG} \quad \text{3'} \]

The nucleotides shown in blue represent the specific sites recognised and cleaved by the restriction endonucleases *Eco* RI (primer # 1) and *Xho* I (primer # 2) respectively, while those displayed in red act as spacer nucleotides to improve the efficiency of restriction endonuclease digestion. The ATG sequence shown in green represents the start codon for the $M_2$ receptor gene.

5.2.1.2 Polymerase chain reaction and restriction endonuclease analysis

PCR amplification of the $M_2$ receptor gene in pCD using the above primers, followed by purification, resulted in a distinct product running at approx. 1400 bp upon analysis by gel electrophoresis (see Figure 5.1). Whereas in the negative control lane (resulting from reactions lacking the pCD template) no product is detected, both lanes 2 and 3 (duplicate sample PCR reactions) displayed distinct PCR products running at the predicted length of 1400-1500 bp (Figure 5.1). Figure 5.2(a) illustrates a vector map of pcDNA3, including the multiple cloning site region. Both pcDNA3 and the $M_2$ receptor gene PCR product were subjected to double restriction endonuclease
Figure 5.1
Ethidium bromide-stained 1.5 % agarose gel of purified PCR products.
Lane (1): negative control (missing only template DNA from reaction)
Lanes (2) and (3): Duplicate PCR reactions including M\textsubscript{2} receptor gene in pCD vector as template.
Figure 5.2
(a) Vector map of pcDNA3, including multiple cloning site region.
(b) Ethidium bromide-stained 1.5 % agarose gel of DNA double digested by Eco RI and Xho I:
Lane (1): un-digested pcDNA3
Lanes (2) and (3): double-digested pcDNA3
Lane (4): double-digested PCR product
digestion with Eco RI and Xho I, and the resulting products were analysed by gel electrophoresis. Figure 5.2 (b) shows this gel, with lane 1 containing undigested pcDNA3, lanes 2 and 3 representing double digested pcDNA3 and lane 4 containing the double digested PCR product. As can be seen by comparison with the markers of known size, all products ran at the expected lengths and the presence of single bands of digested (linearised) pcDNA3 suggests that digestion was successful.

5.2.1.3 DNA extraction and ligation

Each of the bands was then excised from the gel and the pure DNA was recovered as detailed in Chapter 2: Materials and Methods. The resulting DNA was quantified and used to carry out a number of ligation reactions (with vector:insert ratios of 1:2, 1:3 and 1:4) between the M2 receptor gene PCR product and pcDNA3. Following ligation, the products were transformed into NovaBlue Strain Singles competent cells as detailed in Chapter 2: Materials and Methods. 20-24 h later a number of isolated colonies were visible across the 6 plates and 16 were selected at random for further analysis. DNA was prepared (small scale) from each colony selected and double restriction digests were performed (with Eco RI and Xho I) in each case. The results of gel electrophoresis analysis of the double digested DNA preparations are shown in Figure 5.3 (a). A selection of 6 of the 16 clonal DNA preparations are shown in Figure 5.3 (a) (lanes 2-7), while lane 1 represents double digested pcDNA3.1 and lane 8 contains the double digested M2 receptor PCR product. It is clear that only the clones represented in lanes 5 and 6 possess an insert of approx. 1400 bp, that is excised upon digestion with Eco RI and Xho I, indicating successful ligation of the M2 receptor gene into pcDNA3 in these 2 clones (designated clones # 24 and # 25).

Clones # 24 and # 25 were therefore selected for further analysis. Following large scale DNA preparation of each clone, restriction analysis of the DNA, followed by gel electrophoresis, was performed and is shown in Figure 5.3 (b). The gel clearly demonstrates the presence of the M2 receptor gene insert in both clones, but not in pcDNA3 (shown un-digested and double-digested in lanes 1 and 2). The sequence of each clone was then verified by dideoxy nucleotide sequencing.
Figure 5.3
(a) Ethidium bromide-stained 1.5 % agarose gel of small-scale preparation of DNA isolated from a selection of bacterial colonies, transformed with the products of ligation reactions between M₁ receptor PCR products and pcDNA3. All DNA was double digested by Eco R1 and Xho I (2h) prior to gel analysis.
Lane (1): pcDNA3 alone.
Lanes (2) – (7): DNA isolated from selected bacterial clones (# 21-# 26).
Lane (8): M₁ receptor PCR product alone.
(b) Ethidium bromide-stained 1.5 % agarose gel of large-scale preparation of DNA of clones # 24 and # 25, along with appropriate controls (see below), subjected to double digestion by Eco R1 and Xho I.
Lane (1): un-digested pcDNA3.
Lane (2): double-digested pcDNA3.
Lanes (3) and (4): double-digested clones # 24 and # 25.
5.2.1.4 Transient expression in mammalian cell lines

Upon transient expression in HEK-293 cells (at levels up to 0.5 μg DNA well⁻¹ on 24 well plates), both clones exhibited very poor expression levels (< 200 fmol mg protein⁻¹; data not shown) when assayed by [³H]-NMS binding. Examination of the nucleotides immediately preceding the ATG start codon of the M₂ receptor gene sub-cloned into pcDNA3 revealed a less favourable context for the initiation of translation (Kozak, 1987, 1991). The presence of the sequence GCC A/GCC ATG G (‘Kozak sequence’) at the preferred initiation site has been shown to have significant effects (up to 10-fold) on the translation efficiency (and therefore expression levels) of a number of proteins in mammalian expression systems (Kozak, 1991). The M₂ receptor gene was therefore sub-cloned again, but with the following sense primer, incorporating the GCC ACC 'Kozak-like' sequence immediately 5' to the ATG start codon:

Sense - primer # 3

5' CCC CCC GAA TTC GCC ACC ATG AAT AAC TCA ACA AAC TCC TCT AAC A A TA G C C T G  3'
i.e. identical to primer # 1, except for the addition of the nucleotides represented in purple.

The process of sub-cloning the M₂ receptor gene from pCD into pcDNA3 was repeated as detailed above, but substituting primer # 3 (above) for primer # 1 in the initial PCR amplification step (data not shown).

After verifying the sequence of 2 clones of the M₂ receptor gene (+ Kozak-like sequence) in pcDNA3, HEK-293 and CHO-K1 cells were transiently transfected with a range of cDNA loads of M₂ receptor constructs with and without Kozak-like sequences, in order to compare the effects of this sequence in the 5' untranslated region of the M₂ receptor gene. The M₂ receptor expression levels 48 hours post-transfection are indicated in Figure 5.4 and Table 5.1. In HEK-293 cells (a), across a range of transfection levels, the presence of a Kozak-like sequence enhanced expression by approx. 3 fold, while for similar transfections in CHO-K1 cells (b), the effect of the more favourable nucleotide sequence was more pronounced, increasing expression by between 4 and 5 fold (Figure 5.4; Table 5.1).
Figure 5.4
Transient expression of wild-type M₂ mACh receptor, ± Kozak-like sequence in 5' UTR, in HEK-293 (a) and CHO-K1 (b) cells. Data are presented as mean specific binding in fmol receptor expression mg⁻¹ protein. Results are expressed as mean ± s.e.m, n ≥ 3.
Table 5.1 Transient expression of M2 wild-type receptor with and without a Kozak-like sequence in HEK-293 and CHO-K1 cells. Data are expressed as mean (s.e.m.) values from n ≥ 3 experiments.

<table>
<thead>
<tr>
<th>[DNA] (µg well⁻¹)</th>
<th>Expression in HEK-293 cells (pmol mg⁻¹ protein)</th>
<th>Expression in CHO-K1 cells (pmol mg⁻¹ protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>- Kozak</td>
<td>+ Kozak</td>
</tr>
<tr>
<td>0</td>
<td>0.041 (0.005)</td>
<td>0.041 (0.005)</td>
</tr>
<tr>
<td>0.25</td>
<td>0.136 (0.017)</td>
<td>0.499 (0.031)</td>
</tr>
<tr>
<td>0.50</td>
<td>0.206 (0.021)</td>
<td>0.624 (0.037)</td>
</tr>
<tr>
<td>1.00</td>
<td>0.297 (0.044)</td>
<td>0.913 (0.045)</td>
</tr>
</tbody>
</table>

Table 5.2 [³H]-NMS competition binding with agonist (methacholine) and antagonist (atropine) at membrane homogenates of HEK-293 cells transiently expressing wild-type or N410Y mutant M2 mACh receptor. Data are expressed as mean (s.e.m.) values from n ≥ 3 experiments. * indicates value is significantly different from unity (p < 0.05; F-test).

<table>
<thead>
<tr>
<th></th>
<th>HEK-M2 wild-type membranes</th>
<th>HEK-M2 mutant membranes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pKᵢ</td>
<td>Hill slope</td>
</tr>
<tr>
<td>atropine</td>
<td>8.70 (0.03)</td>
<td>1.03 (0.10)</td>
</tr>
<tr>
<td>methacholine</td>
<td>5.21 (0.14)</td>
<td>0.76 (0.07)</td>
</tr>
</tbody>
</table>
5.2.2 Generation of a constitutively active mutant (CAM) M\(_2\) mACh receptor

Previous work had identified the \(^{N410Y}M_3\) receptor mutant as displaying the characteristics of a constitutively active mutant receptor (see section 5.1), so the homologous mutation was created in the \(M_2\) mACh receptor \((^{N410Y}M_2)\). The following primers were designed to specifically incorporate the N410Y mutation into the \(M_2\) receptor gene sub-cloned into pcDNA3 (see section 5.2.1):

**Sense – primer A**

\[5' \text{GCC CCA TAC AAT GTC ATG GTC CTC ATT TAC ACC TTT TGT GCA CCT 3'}\]

**Antisense – primer B**

\[5' \text{AGG TGC ACA AAA GGT GTA AAT GAG GAC CAT GAC ATT GTA TGG GGC 3'}\]

The nucleotides marked in blue code for the substitution of tyrosine for asparagine at position 410 (via substitution of T for A, changing AAC to TAC) within the \(M_2\) receptor gene. Those indicated in red represent a silent mutation (G to C, changing GTG to GTC, both of which code for valine), leading to the incorporation of an additional restriction site for the enzyme Ava II into the mutated gene. This is demonstrated in Figure 5.5, which shows the restriction map for the wild-type (a) and mutant (b) \(M_2\) receptor genes and illustrates the additional cut site at 2162 bp of the mutant gene. The restriction fragments predicted to result from digestion of each construct by Ava II are also given in Figure 5.5, with the fragments differing between wild-type and mutant (and therefore allowing discrimination between the two genes upon subsequent electrophoretic analysis of the fragments) highlighted in red.

Mutagenesis was performed using the QuikChange™ site-directed mutagenesis kit (Stratagene) and following transformation of XL1-Blue supercompetent cells, cells were grown on LB-ampicillin plates. Alongside the test plates, mutagenesis control plates were set up and are shown in Figure 5.6 (a). The left-hand plate contains untransformed super-competent cells and exhibits no growth of colonies. This would be expected as the cells only obtain ampicillin resistance from the ampicillin resistance gene within pcDNA3, so those cells not transformed will fail to grow on LB-ampicillin plates. The right-hand plate indicates the efficiency of mutagenesis, with successful mutagenesis indicated by the growth of blue colonies (due to the successful
Figure 5.5
Restriction digest maps for wild-type (a) and N410Y mutant (b) M₂ receptor genes on pcDNA3, subjected to treatment with the restriction endonuclease AvaII. The resulting restriction fragment lengths in base pairs (bp) are also given and those present in the N410Y mutant gene but not in the wild-type gene are highlighted in red.
Figure 5.6
(a) Mutagenesis control plates: *Left plate* - LB-ampicillin agar plate of un-transformed XL1-Blue supercompetent cells; *Right plate* - LB-ampicillin agar plate containing IPTG (20 mM) and X-gal (80 μg/ml), showing growth of XL1-Blue supercompetent cells transformed with the products of pWhitescript control plasmid that has undergone site-directed mutagenesis to restore β-galactosidase activity. The proportion of blue (β-gal+) colonies relative to white (non-mutated, β-gal−) colonies indicates the mutagenesis efficiency of the process.
Figure 5.6 (cont.)
(b) Ethidium bromide-stained 1.5 % agarose gel of small-scale prepared DNA isolated from a selection of bacterial colonies, transformed with the products of mutagenesis reactions on the M2 receptor gene in pcDNA3. Unless indicated otherwise DNA was digested (2h) by Ava II, prior to gel electrophoresis analysis.
Lane (1): undigested M2 wild-type pcDNA3.
Lane (2): digested M2 wild-type pcDNA3.
Lanes (3) – (10): digested DNA from selected clones (# 1 – # 8).
(c) Ethidium bromide-stained 1.5 % agarose gel of large-scale prepared DNA of clones # 4 and # 6, digested by Ava II.
Lane (1): undigested M2 wild-type pcDNA3.
Lane (2): digested M2 wild-type pcDNA3.
Lanes (3) and (4): digested clones # 24 and # 25.
mutation of $\beta$-gal$^+$ to $\beta$-gal$^-$). Since the plate shown in Figure 5.6 (a) displays almost entirely blue colonies, with almost no white colonies, mutagenesis efficiency appears to be almost 100%.

A total of 16 colonies were picked from the test plates for small-scale DNA preparation. Samples of each DNA preparation, as well as M2 wild-type pcDNA3, were then subjected to digestion by Ava II and analysed by gel electrophoresis. Figure 5.6 (b) illustrates the resulting gel with un-digested (lane 1) and Ava II digested (lane 2) wild-type, followed by digested clones # 1 to # 8 (lanes 3-10). For digested wild-type, the top band represents the 2782 bp fragment predicted from the restriction map (see Figure 5.5 (a)), while the next band constitutes an un-resolved double band of 1866 and 1780 base pairs. In contrast, all 8 clones shown in Figure 5.6 (b) exhibit a lower band, likely to represent the predicted 774 bp fragment (see Figure 5.5 (b)). Also, the middle band for each clone appears more diffuse than the wild-type equivalent, consistent with the extra un-resolved band predicted to run at 2008 bp. From this evidence it would appear that all 8 clones shown in Figure 5.6 (b) successfully incorporated the mutation. The only inconsistency is the presence of the top band in all clones (presumed to be the 2782 bp fragment in wild-type), as the additional Ava II restriction site introduced by the mutagenesis would be expected to eliminate this fragment (cleaving 2782 base pairs into fragments of 2008 and 774 base pairs).

However, clones # 4 and # 6 were selected for large-scale DNA preparation and the analysis of these DNA preparations by restriction digest and gel electrophoresis is shown in Figure 5.6 (c). Clones # 4 and # 6 display similar middle and lower bands to those seen in Figure 5.6 (b) following digestion with Ava II, but now the top band visible in wild-type digested DNA (lane 2) is not present in the mutant clones (lanes 3 and 4). The presence of the N410Y mutation in clones # 4 and # 6 was confirmed by dideoxy nucleotide sequencing. The reason for the difference in apparent restriction fragments resulting from small- and large-scale prepared DNA is not clear, but possible explanations include non-specific activity of Ava II in the digestion of small-scale prepared DNA samples, or contamination of the samples/lanes with wild-type DNA.
5.2.3 Characterisation of wild-type and N410Y mutant M2 mACh receptors transiently expressed in HEK-293 cells

Initial characterisation of the wild-type and N410Y mutant M2 receptors was performed in HEK-293 cells, consistent with previous work characterising the N314Y M2 receptor CAM. Cells were transiently transfected with either wild type or N410Y mutant M2 receptor cDNA and cell membranes prepared 48 h post-transfection (see Chapter 2: Materials and Methods). Saturation radioligand binding assays using [3H]-NMS characterised the receptor expression levels of wild-type and mutant receptor as 1.23 ± 0.08 and 2.09 ± 0.17 pmol mg⁻¹ protein respectively (n = 3). Representative saturation binding curves are shown in Figure 5.7. The mean binding affinity (Kd) of [3H]-NMS was not significantly different between wild-type and N410Y mutant M2 receptors expressed in HEK-293 cell membranes (0.55 ± 0.15 and 0.59 ± 0.19 nM respectively; n = 3).

Competition radioligand binding assays were then performed with [3H]-NMS and the antagonist atropine and agonist methacholine. Competition binding curves representing the mean data from 3 separate experiments are shown in Figure 5.8 (a) (atropine) and (b) (methacholine) and the mean pKᵯ and Hill slope values derived from these experiments are given in Table 5.2. Atropine displayed similar affinity for both wild-type and mutant receptors and the mean slope factors of atropine competition binding curves at either wild-type or mutant receptor populations were not significantly different from unity (see Table 5.2). Methacholine displayed slightly higher affinity for the N410Y mutant receptor relative to wild-type M2 receptor but this was not statistically significant. The methacholine competition binding data were fitted with a Hill slope of significantly less than unity at the N410Y mutant M2 receptor (p < 0.05).

[^35]S-GTPyS binding in response to the muscarinic agonist methacholine was also measured in wild-type and N410Y mutant receptor-expressing HEK-293 cell membranes. Mean concentration-response curves are shown in Figure 5.9 and the mean pEC₅₀ and Hill slope values from 3 experiments are given in Table 5.3. Mean basal levels of [35S]-GTPyS binding from 3 separate experiments did not differ significantly between wild-type (1747 ± 203 c.p.m.) and N410Y mutant (1761 ± 133 c.p.m.) receptor expressing membranes, while the response to maximal methacholine
Figure 5.7

$[^3]H$-NMS saturation binding curves at membrane homogenates of HEK-293 cells transiently expressing $M_2$ wild-type (a) or N410Y mutant (b) receptor. Data are expressed as mean specific binding in fmol receptor expression mg$^{-1}$ protein and are representative curves of 3 or more experiments.
Figure 5.8

[^H]-NMS competition binding curves for atropine (a) and methacholine (MCh) (b) at membrane homogenates of HEK-293 cells transiently expressing M_2 wild-type (closed symbols) or N410Y mutant (open symbols) receptor. Data are presented as mean percent of specific binding in the absence of competitor. Results are expressed as mean ± s.e.m, n ≥ 3.
Figure 5.9
Agonist (MCh)-stimulated total [35S]-GTPγS binding at membrane homogenates of HEK-293 cells transiently expressing M2 wild-type (closed symbols) or N410Y mutant (open symbols) receptor. Data are presented as mean percent increase over basal ± s.e.m, n ≥ 3.
Table 5.3 Methacholine-stimulated $[^35]S$-GTPγS binding to membrane homogenates of HEK-293 and CHO-K1 cells expressing wild-type or N410Y mutant M2 mACh receptor. Data are expressed as mean (s.e.m.) values from n ≥ 3 experiments. ‡ indicates significant difference between wild-type and mutant ($p < 0.05$; Student’s $t$ test); * indicates value is significantly different from unity ($p < 0.05$; F-test).

<table>
<thead>
<tr>
<th></th>
<th>HEK-293 cells</th>
<th>CHO-K1 cells</th>
<th>CHO-m2 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wild-type</td>
<td>Mutant</td>
<td>Wild-type</td>
</tr>
<tr>
<td>$\mu$EC$_{50}$</td>
<td>6.22 (0.35)</td>
<td>6.91 (0.11)</td>
<td>6.93 (0.36)</td>
</tr>
<tr>
<td>Hill slope</td>
<td>0.37 (0.01) *</td>
<td>0.53 (0.04)  *‡</td>
<td>1.12 (0.29)</td>
</tr>
</tbody>
</table>

Table 5.4 Basal $[^3]H$-inositol phosphate accumulation in CHO-K1 cells transiently co-expressing wild-type or mutant m2 receptor and $G_{\alpha5}$. Data are expressed as mean (s.e.m.) values from n ≥ 3 experiments. ‡ indicates significant difference between wild-type and mutant ($p < 0.05$; Student’s $t$ test); * indicates value is significantly different from basal values ($p < 0.05$; Student’s $t$ test).

<table>
<thead>
<tr>
<th></th>
<th>wild-type M2</th>
<th>mutant M2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>untreated</td>
<td>Atropine (300 nM) pre-treated</td>
</tr>
<tr>
<td>Basal</td>
<td>6880 (1287)</td>
<td>6949 (629)</td>
</tr>
<tr>
<td>+ Atropine (1 μM)</td>
<td>4889 (432)</td>
<td>3998 (636) *</td>
</tr>
</tbody>
</table>
(1 mM) concentrations were also equivalent (2727 ± 354 c.p.m. and 2883 ± 148 c.p.m. for wild-type and mutant, respectively). Basal [\textsuperscript{35}S]-GTP\textsubscript{S} binding was unaffected by pre-incubation with atropine (10 \textmu M) (data not shown).

While comparison of the relative potency of methacholine at wild-type and mutant receptors indicates a trend towards a higher agonist potency at the mutant receptor, statistical analysis found this difference to be insignificant (see Table 5.3). The most striking difference between wild-type and mutant receptor [\textsuperscript{35}S]-GTP\textsubscript{S} binding is seen in the slopes of the concentration-response curves to methacholine (see Figure 5.9; Table 5.3). While both slopes are significantly shallower than unity (\(p < 0.05\)), the slope factor associated with the wild-type methacholine response is significantly less than that at the N410Y mutant (\(p < 0.05\)).

Attempts to investigate further the functional characteristics of the wild-type and N410Y mutant M\textsubscript{2} mACh receptors expressed in HEK-293 cells by examining the inhibition of forskolin-stimulated cyclic AMP accumulation in response to M\textsubscript{2} receptor activation proved unsuccessful. Although significant accumulations of cyclic AMP were observed upon forskolin (3 or 10 \textmu M) stimulation (in the presence of 300 \textmu M IBMX) of HEK-293 cells transiently expressing either wild-type or mutant M\textsubscript{2} receptors, stimulation with a range of concentrations of methacholine (1 nM - 1 mM) failed to produce a significant inhibition of the forskolin-stimulated cyclic AMP response (in wild-type receptor-expressing cells: basal = 6.2 ± 0.3 pmol mg\textsuperscript{-1} protein; forskolin (10 \textmu M) = 504.5 ± 30.3 pmol mg\textsuperscript{-1} protein; Forskolin + MCh (1 mM) = 685.4 ± 0.7 pmol mg\textsuperscript{-1} protein (\(n = 2\)). Basal levels of cyclic AMP were not significantly different between wild-type and mutant-expressing cells and both basal and forskolin-mediated responses were unaffected by atropine (10 \textmu M) pre-treatment (data not shown).

5.2.4 Characterisation of wild-type and N410Y mutant M\textsubscript{2} mACh receptors transiently expressed in CHO-K1 cells

Comparison of the relative transient expression levels of wild-type M\textsubscript{2} mACh receptor between HEK-293 and CHO-K1 cells (see Figure 5.4) indicated that for a given cDNA load, higher expression levels were achieved in CHO-K1 cells. This was
Further investigated, using a range of wild-type M₂ and M₃ receptor cDNA transfections in both cell types and a measurement of the receptor expression level with a single high concentration (3-4 nM) of [³H]-NMS (Figure 5.10). As previously established for the wild-type M₃ receptor, concentration-dependent expression is achieved up to 1 µg cDNA well⁻¹ in HEK-293 cells (see Figure 5.10). A similar pattern is observed in CHO-K₁ cells, but with a slightly lower expression for a given cDNA load. In contrast, the M₂ receptor is expressed significantly better in CHO-K₁ cells. For instance, in the representative experiment illustrated in Figure 5.10, cell surface M₂ receptor expression in HEK-293 cells fails to reach 1 pmol mg⁻¹ protein even with 1 µg cDNA per well, while transfection of CHO-K₁ cells with the same quantity of cDNA yields in excess of 5 pmol mg⁻¹ protein.

Despite achieving higher levels of transient expression in CHO-K₁ cells, attempts to measure M₂ receptor-mediated inhibition of forskolin-stimulated cyclic AMP accumulation produced similar findings to those in HEK-293 cells (in wild-type receptor-expressing cells: basal = 3.2 ± 0.5 pmol mg⁻¹ protein; forskolin (10 µM) = 318.5 ± 25.9 pmol mg⁻¹ protein; Forskolin + MCh (1 mM) = 395.0 ± 15.6 pmol mg⁻¹ protein (n = 2)). However, it was possible to characterise the [⁸⁸S]-GTPyS binding profile in membranes of CHO-K₁ cells transiently expressing wild-type or N410Y mutant M₂ mACH receptors. In contrast to data obtained in HEK-293 cells (see section 5.2.3), N410Y mutant-expressing CHO-K₁ cell membranes bound significantly higher levels of [⁸⁸S]-GTPyS in the absence of agonist (6788 ± 284 c.p.m.) than membranes prepared from cells expressing wild-type receptors (5328 ± 337 c.p.m.) (p < 0.05), despite expressing significantly lower levels of mutant receptor relative to wild-type (0.77 ± 0.03 versus 3.10 ± 0.10 pmol mg⁻¹ protein respectively; n = 2, data not shown). However, pre-treatment with atropine (10 µM) had no significant effect on basal [⁸⁸S]-GTPyS binding (data not shown).

Maximal responses to methacholine were not statistically significantly different between membranes prepared from N410Y mutant-expressing CHO-K₁ cells (9744 ± 592 c.p.m.) compared with wild-type-expressing cells (8098 ± 440 c.p.m.). However, methacholine was significantly more potent at mutant relative to wild-type receptors for the activation of [⁸⁸S]-GTPyS binding in CHO-K₁ cells (p < 0.05) (see Figure 5.11 (a); Table 5.3). The mean Hill slope associated with methacholine-stimulated [⁸⁸S]-
Figure 5.10
Comparison of expression levels of transiently expressed wild-type and mutant M₂ and M₃ mACh receptors in HEK-293 (a) and CHO-K1 (b) cells. Data are presented as mean (± s.e.m.) specific binding in fmol receptor expression mg⁻¹ protein and are taken from a representative experiment, performed in duplicate.
Figure 5.11
(a) MCh-stimulated total \(^{35}\)S-GTP\(\gamma\)S binding at membrane homogenates of CHO-K1 cells transiently expressing \(M_4\) wild-type (closed symbols) or N410Y mutant (open symbols) receptor.
(b) MCh-stimulated total \(^{35}\)S-GTP\(\gamma\)S binding at membrane homogenates of CHO cells stably expressing wild-type \(M_2\) mACh receptors (CHO-slm2).
Data are presented as mean % increase over basal ± s.e.m, n = 3.
GTP\(_\gamma\)S binding at the wild-type M\(_2\) receptor did not differ significantly from unity, while that associated with the mutant receptor response was significantly less than one (\(p < 0.05\)) (Table 5.3).

Table 5.4 also includes mean pEC\(_{50}\) and Hill slope estimates for methacholine at membranes prepared from the stable CHO-m2 cell line and the mean concentration response curve from 3 separate experiments with membranes prepared from these cells is shown in Figure 5.11 (b). The mean Hill slope associated with the concentration response curves at CHO-m2 membranes was significantly less than unity (\(p < 0.05\)) and although not statistically significant, methacholine appeared slightly less potent at the M\(_2\) receptor stably expressed in the CHO-m2 cell line than at the transiently expressed wild-type receptor in CHO-K1 cells (see Table 5.3).

5.2.5 Use of the chimeric G-protein G\(_{\alpha q5}\) to investigate the constitutive activity of wild-type and N410Y mutant M\(_2\) receptors transiently expressed in CHO-K1 cells.

5.2.5.1 Expression of G\(_{\alpha q5}\) and coupling of the M\(_2\) mACh receptor to phosphoinositide hydrolysis.

G\(_{\alpha q5}\) consists of the human Ga\(_q\) protein with the C-terminal 5 amino acids (EYNLV) replaced by the corresponding sequence of the human Ga\(_{\alpha 2}\) protein (DCGLF) (Conkin et al., 1993). G\(_{\alpha q5}\) has previously been shown to couple a variety of predominantly Ga\(_{\alpha}\)-linked GPCRs, including the M\(_2\) mACh receptor (Liu et al., 1995), to phospholipase C-\(\beta\) activation and phosphoinositide turnover in recombinant cell systems. Transient co-transfection of cDNA coding for G\(_{\alpha q5}\) and either wild-type or N410Y mutant M\(_2\) receptor into CHO-K1 cells therefore allowed the measurement of [\(^{3}H\)]-inositol phosphate accumulation (under Li\(^+\) block) as an index of M\(_2\) receptor activation.

Figure 5.12 (a) displays the mean concentration response curves for methacholine at CHO-K1 cells co-expressing G\(_{\alpha q5}\) and either wild-type or mutant M\(_2\) receptor. The mean expression levels of M\(_2\) wild-type and N410Y mutant receptors over the 3 experiments were 2.74 ± 0.22 and 1.95 ± 0.09 pmol mg\(^{-1}\) protein, respectively. It can be seen from Figure 5.12 (a) that methacholine stimulated phosphoinositide turnover
Figure 5.12
(a) MCh-stimulated [3H]-inositol phosphate accumulation in un-transfected CHO-K1 cells (dashed line) or CHO-K1 cells transiently co-expressing G_{q5} and either wild-type (closed symbols) or N410Y mutant (open symbols) M_{2} receptor. Data are expressed as mean percent of [3H]-inositol phosphate accumulation over basal ± s.e.m, n = 3.
(b) Western blot showing expression of G_{q5}, relative to endogenous G_{q} expression in CHO-K1 cells transiently expressing wild-type M_{2} receptor or co-expressing G_{q5} and either wild-type or N410Y mutant M_{2} receptor (top panel). γ-Tubulin immunoreactivity for each sample is included as a protein-loading control (bottom panel). Data shown are representative of 3 similar experiments.
by approx. 4-fold over basal in either wild-type or mutant M<sub>2</sub> receptor-expressing CHO-K1 cells. In contrast, un-transfected CHO-K1 cells exhibit no significant [³H]-inositol phosphate response to methacholine.

In agonist concentration-response assays, basal [³H]-inositol phosphate accumulation did not significantly differ between wild-type (10770 ± 2816 d.p.m. mg⁻¹ protein) and mutant (14033 ± 2740 d.p.m. mg⁻¹ protein) transfected cells. The maximal responses of wild-type and mutant receptor populations to methacholine were also similar (53015 ± 6283 and 52730 ± 5123 d.p.m. mg⁻¹ protein, respectively). However, methacholine was significantly more potent (> 10-fold) at the N410Y mutant receptor (mean pEC<sub>50</sub> = 7.58 ± 0.09) than at the wild-type M<sub>2</sub> receptor (mean pEC<sub>50</sub> = 6.44 ± 0.11). While the mean slope factor associated with methacholine concentration-response curves at wild-type-expressing CHO-K1 cells was not significantly different from unity (0.90 ± 0.18), the mean Hill slope value calculated for the mutant receptor response of 0.65 ± 0.08 was significantly less than one (p < 0.05).

In order to confirm the expression of G<sub>q5</sub> in co-transfected cells, western blot analysis was performed using a Ga<sub>q</sub>-specific antibody (IQB; Mullaney et al., 1993), as this binds to a specific sequence of amino acids between residues 119-134 of Ga<sub>q</sub> and would therefore probe for both endogenous Ga<sub>q</sub> and transiently expressed G<sub>q5</sub>. A representative blot is shown in Figure 5.12 (b), indicating similar levels of over-expression of G<sub>q5</sub> in cells co-expressing either wild-type (lanes 3 and 4) or N410Y mutant (lanes 5 and 6) M<sub>2</sub> receptor with G<sub>q5</sub>. The relatively weak bands detected in CHO-K1 cells transfected with M<sub>2</sub> receptor only (lanes 1 and 2), suggest low expression of endogenous Ga<sub>q</sub> in CHO-K1 cells. The lower panel of Figure 5.12 (b) shows the same samples probed for γ-tubulin and confirms equal protein loading between lanes.

5.2.5.2 Constitutive activity and inverse agonism at the wild-type and N410Y mutant M<sub>2</sub> mACh receptor

To facilitate the investigation of the constitutive activity of the N410Y<sub>M2</sub> receptor mutant, relative to the wild-type M<sub>2</sub> receptor, receptors were over-expressed with G<sub>q5</sub> in CHO-K1 cells and the agonist-independent [³H]-inositol phosphate accumulation
was measured. Figure 5.13 and Table 5.4 summarise the mean basal [$^3$H]-inositol phosphate accumulation from a number of experiments in CHO-K1 cells co-expressing G_{q5} and either wild-type or mutant receptor, and also the effect of pre-incubation (15 min) with atropine (10 μM) upon the agonist-independent response.

It has been reported that pre-incubation of constitutively active receptors with inverse agonist can protect the receptor from rapid degradation/downregulation and thereby preserve the population of constitutively active receptors at the plasma membrane (Milligan & Bond, 1997). For this reason, experiments were performed on either untreated cells or those exposed to the putative muscarinic inverse agonist atropine (300 nM) for 24 h prior to the experiment. Preliminary experiments determined that binding of atropine to the M$_2$ receptor at this concentration was fully reversible with extensive washing of the cells prior to performing the assay. This approach was therefore adopted for all subsequent experiments (as detailed in Chapter 2: Materials and Methods).

The mean basal activity of the $^{N410Y}$M$_2$ receptor-expressing cells was approx. two-fold higher than that of the cells expressing wild-type M$_2$ mACh receptors, though this difference only achieved statistical significance in experiments performed following atropine (300 nM; 24 h) pre-treatment (Table 5.4). It should be noted that in all experiments, receptor expression levels were monitored and transfections were performed such that $^{N410Y}$M$_2$ receptor levels never exceeded those of the wild-type M$_2$ receptor. Indeed, in most cases, the wild-type receptor was expressed at significantly higher levels than the mutant M$_2$ receptor (e.g. relative expression levels for atropine-treated wild-type and mutant receptors were 2.66 ± 0.35 and 1.80 ± 0.28 pmol mg$^{-1}$ protein, respectively; n ≥3). A higher receptor density cannot therefore account for the enhanced agonist-independent activity observed for $^{N410Y}$M$_2$ receptor expressing cells. The effects of chronic atropine treatment upon receptor expression levels will be addressed in section 5.2.6.

In both untreated and atropine pre-treated cells expressing the $^{N410Y}$M$_2$ receptor, acute exposure to atropine prior to incubation with Li$^+$ (see Chapter 2: Materials and Methods for details of timings) significantly reduced constitutive [$^3$H]-inositol phosphate accumulation (p < 0.05) (Figure 5.13; Table 5.4). Basal activity of wild-type-expressing cells was reduced slightly by acute atropine treatment, although this was only statistically significant in chronically atropine-treated cells (Figure 5.13;
Figure 5.13
Basal $[^3\text{H}]$-inositol phosphate accumulation in the absence and presence of atropine (1 μM) in CHO-K1 cells (with or without 24 h pre-treatment with 300 nM atropine) transiently co-expressing $G_{q5}$ and either wild-type or N410Y mutant M$_3$ receptor. Data are expressed as mean $[^3\text{H}]$-inositol phosphate accumulation in d.p.m. mg$^{-1}$ protein. Results are expressed as mean ± s.e.m, n ≥ 3. ‡ indicates significant difference between wild-type and mutant ($p < 0.05$), * indicates significant difference from basal values ($p < 0.05$).
Table 5.4). In these experiments, atropine therefore appeared to be acting as an inverse agonist, reducing the spontaneous muscarinic receptor-mediated activity.

A number of other mACh receptor 'antagonists' were assayed for inverse agonist activity in this way, at both wild-type and N410Y mutant M<sub>2</sub> receptors. Maximal concentrations of each ligand were selected (approx. 100-1000 x binding affinity at the M<sub>2</sub> receptor) and the mean effects on agonist-independent [³H]-inositol phosphate accumulation are summarised in Figure 5.14 (a) (wild-type), Figure 5.15 (a) (mutant) and Table 5.5. In each case, the basal [³H]-inositol phosphate accumulation in the presence of the ligand is calculated as a percentage of that in the absence of ligand, after subtraction of the Li⁺-independent accumulation (i.e. the [³H]-inositol phosphate accumulation over the same time-course, but in the absence of Li⁺). In mutant receptor-expressing cells, all 'antagonists' tested significantly reduced the basal [³H]-inositol phosphate accumulation (p < 0.05). In wild-type receptor-expressing cells, all 'antagonists' displayed a trend towards lowering basal activity, but this only achieved statistical significance for atropine, oxybutynin, tolterodine, pirenzepine, methoctramine and 4-DAMP (p < 0.05) (see Table 5.5 and Figures 5.14 (a) and 5.15 (a)).

The concentration-dependent nature of the atropine-mediated reduction in basal [³H]-inositol phosphate accumulation was investigated in both wild-type and N410Y mutant receptor-expressing cells. The resulting concentration response curves from a number of similar experiments are illustrated in Figure 5.14 (a) (wild-type) and Figure 5.15 (a) (mutant). The mean pEC<sub>50</sub> values for atropine at wild-type and mutant M<sub>2</sub> receptors were 9.04 ± 0.17 and 8.42 ± 0.20. Atropine therefore more potently reduces constitutive [³H]-inositol phosphate accumulation in cells expressing wild-type M<sub>2</sub> receptors than N410Y mutant M<sub>2</sub> receptors (p < 0.05).

5.2.6 Investigation of the effects of chronic inverse agonist treatment on the expression of transiently transfected wild-type and N410Y mutant M<sub>2</sub> receptors.

Initial observations when monitoring the relative receptor expression levels in [³H]-inositol phosphate accumulation assays suggested that chronic treatment with inverse agonist might up-regulate the receptor expression level, particularly of the N410Y mutant M<sub>2</sub> receptor. As this was consistent with previous work with the N514Y M<sub>3</sub> CAM
Figure 5.14
(a) Inhibition of basal $[^{3}H]$-inositol phosphate accumulation, in CHO-K1 cells transiently co-expressing $G_{q5}$ and $M_2$ wild-type receptor, by a range of mACh receptor antagonists.
(b) Concentration-dependent inhibition of basal $[^{3}H]$-inositol phosphate accumulation, in CHO-K1 cells transiently co-expressing $G_{q5}$ and $M_2$ wild-type receptor, by atropine. Data are presented as % of basal $[^{3}H]$-inositol phosphate accumulation. Results are expressed as mean ± s.e.m, $n \geq 3$. * indicates significant difference from control ($p < 0.05$).
Figure 5.15
(a) Inhibition of basal [3H]-inositol phosphate accumulation, in CHO-K1 cells transiently co-expressing G_{q15} and N410Y mutant M_2 receptor, by a range of mACH receptor antagonists.
(b) Concentration-dependent inhibition of basal [3H]-inositol phosphate accumulation, in CHO-K1 cells transiently co-expressing G_{q15} and N410Y mutant M_2 receptor, by atropine.
Data are presented as % of basal [3H]-inositol phosphate accumulation. Results are expressed as mean ± s.e.m, n ≥ 3. * indicates significant difference from control (p < 0.05).
Table 5.5 Receptor up-regulation in response to chronic (24h) exposure to mACh receptor ligands and inhibition of constitutive [³H]-inositol phosphate accumulation in CHO-K1 cells transiently co-expressing m2 wild-type or m2 mutant receptor and Gqα5. Data are expressed as mean (s.e.m.) values from n ≥ 3 experiments. * indicates significant differences from control (p < 0.05; one-way ANOVA, Dunnett’s post test).

<table>
<thead>
<tr>
<th></th>
<th>wild-type M₂</th>
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<th>mutant M₂</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Inhibition of basal [³H]-IP₃ (%)</td>
<td>Receptor up-regulation (% above control)</td>
<td>Inhibition of basal [³H]-IP₃ (%)</td>
<td>Receptor up-regulation (% above control)</td>
</tr>
<tr>
<td>Atropine</td>
<td>58.52 (6.06) *</td>
<td>37.74 (6.94) *</td>
<td>68.31 (2.73) *</td>
<td>60.36 (7.36) *</td>
</tr>
<tr>
<td>Darifenacrin</td>
<td>30.90 (9.91)</td>
<td>36.20 (4.09) *</td>
<td>50.08 (8.18) *</td>
<td>59.53 (6.85) *</td>
</tr>
<tr>
<td>Oxybutynin</td>
<td>60.37 (2.79) *</td>
<td>-10.43 (4.23)</td>
<td>74.82 (9.52)</td>
<td>31.38 (7.76)</td>
</tr>
<tr>
<td>Tolterodine</td>
<td>65.64 (8.87) *</td>
<td>22.59 (6.57)</td>
<td>50.22 (10.54) *</td>
<td>26.90 (2.66)</td>
</tr>
<tr>
<td>Pirenzepine</td>
<td>61.61 (13.73) *</td>
<td>30.77 (6.05) *</td>
<td>68.77 (10.84) *</td>
<td>31.08 (6.56) *</td>
</tr>
<tr>
<td>Methoctramine</td>
<td>48.78 (5.66) *</td>
<td>24.31 (6.21) *</td>
<td>52.31 (8.46) *</td>
<td>4.69 (6.97)</td>
</tr>
<tr>
<td>4-DAMP</td>
<td>64.51 (8.94) *</td>
<td>13.35 (5.19)</td>
<td>43.77 (9.27) *</td>
<td>20.63 (2.76)</td>
</tr>
<tr>
<td>NMS</td>
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<td>0.69 (3.05)</td>
<td>ND</td>
<td>-6.95 (1.60)</td>
</tr>
<tr>
<td>QNB</td>
<td>41.77 (6.29)</td>
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<td>66.27 (9.25) *</td>
<td>-40.58 (8.45) *</td>
</tr>
<tr>
<td>CCh</td>
<td>ND</td>
<td>-57.28 (4.54) *</td>
<td>ND</td>
<td>-62.41 (0.89) *</td>
</tr>
<tr>
<td>DMSO</td>
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<td>2.72 (2.60)</td>
<td>ND</td>
<td>0.37 (4.24)</td>
</tr>
</tbody>
</table>
and other CAMs (see Milligan & Bond, 1997) the effects of chronic atropine treatment upon transiently expressed receptor levels were investigated further in the CHO cell model. Figure 5.16 illustrates the time-dependent up-regulation of mean receptor number from 3 experiments for wild-type (a) and N410Y mutant (b) receptors, up to 24 h.

The effects of maximal concentrations of a range of other ligands were also investigated for their ability to up-regulate M₃ mACh receptor expression. 24 h treatments were utilised to maximise the potential for up-regulation. Since the assay is dependent upon the complete removal of ligand binding prior to the addition of [²H]-NMS, lower concentrations of ligand were also tested, as the removal of test ligand during the washing phase might not be complete for high concentrations of certain ligands. The data shown in Figures 5.17 (wild-type) and 5.18 (a) (mutant), as well as in Table 5.5, represent the maximal effects observed for those ligands, as a percentage change relative to the expression level in vehicle-treated cells. At the wild-type M₃ receptor, only atropine, darifenacin, pirenzepine and methoctramine were found to significantly enhance receptor expression levels (p < 0.05), although other ligands (such as tolterodine) produced moderate, but statistically insignificant, increases (Figure 5.17; Table 5.5). At the N410Y mutant M₃ receptor atropine, darifenacin and pirenzepine were the only ligands found to statistically significantly increase receptor expression (p < 0.05) (Figure 5.18 (a); Table 5.5). It is notable that the maximal effects of atropine and darifenacin, as a percentage of control expression, were less pronounced at wild-type relative to mutant expressing cells (< 40 % versus approx. 60 %; Table 5.5).

In both wild-type and N410Y mutant M₃ receptor-expressing cells, 24 h treatment with the muscarinic agonist carbachol (100 μM) produced a significant decrease in the receptor expression, relative to vehicle-treated cells (p < 0.05) (Figures 5.17 and 5.18 (a); Table 5.5). This is consistent with the widely reported effects of chronic agonist treatment on cell surface GPCR expression levels (Koenig & Edwardson, 1994). Carbachol was used in these experiments to ensure consistency of agonist activation throughout the 24 h time-course of incubation, due to the significantly lower susceptibility of carbachol to any acetylcholine-esterase activity which might be present in the media bathing the cells. The non-selective muscarinic antagonist QNB (10 nM) also significantly reduced the M₃ mACh receptor density detected by [²H]-
Figure 5.16
Time-course of up-regulation of wild-type (a) and N410Y mutant (b) M₂ receptor expression, in CHO-K1 cells transiently co-expressing receptor and Gᵣ₅, by chronic treatment with atropine (300 nM). Data are expressed as mean percent of control expression. Results are expressed as mean ± s.e.m, n ≥ 3.
Figure 5.17
Effect of chronic (24 h) treatment of CHO-K1 cells transiently co-expressing G_{q5} and M_{2} wild-type receptor with a range of mACH receptor ligands on M_{2} receptor expression. Data are expressed as mean percent change in specific binding relative to control cells. Results are expressed as mean ± s.e.m, n ≥ 3. * indicates significant difference from control (p < 0.05).
Figure 5.18
(a) Effect of chronic (24 h) treatment of CHO-K1 cells transiently expressing G_{q5} and M_{2} mutant receptor with a range of mACh receptor ligands on M_{2} receptor expression. Data are expressed as mean percent change in specific binding relative to control cells. Results are expressed as mean ± s.e.m, n ≥ 3. * indicates significant difference from control (p < 0.05).

(b) Concentration-dependent up-regulation of N410Y mutant M_{2} receptor expression, in CHO-K1 cells transiently co-expressing G_{q5} and M_{2} mutant receptor, by atropine. Data are presented as mean percent increase in specific binding relative to control cells. Results are expressed as mean ± s.e.m, n ≥ 3.
NMS in both wild-type and N410Y mutant receptor-expressing cells \( (p < 0.05) \) (Figures 5.17 and 5.18 (a); Table 5.5). However, since QNB did not behave as an agonist in the \(^3\text{H}\)-inositol phosphate experiments, it is likely that the observed reduction in \(^3\text{H}\)-NMS binding is due to a lack of reversibility of QNB binding, resulting from its highly lipophilic nature (Lee & El Fakahany, 1985).

The concentration-dependency of the effect of atropine upon \(^{N410Y}M_2\) mutant receptor expression levels was also investigated and is illustrated in Figure 5.18 (b). The mean pEC\(_{50}\) value calculated from 4 separate experiments was 7.72 ± 0.18. Unfortunately, the smaller maximal effect of atropine upon wild-type \(M_2\) receptor expression precluded an analysis of the concentration-dependency of this response.

### 5.2.7 Constitutive activity and inverse agonism at the CAM \(^{N514V}M_3\) receptor.

The effects of a range of concentrations of the mACh receptor antagonists atropine, darifenacin, oxybutynin and tolterodine on the basal, constitutive accumulation of \(^3\text{H}\)-inositol phosphates were measured in HEK-293 cells transiently expressing the CAM \(^{N514V}M_3\) receptor. The mean expression level of CAM \(^{N514V}M_3\) receptor was 1.81 ± 0.10 pmol mg\(^{-1}\) protein (n = 5; data not shown), while the endogenously expressed mACh receptor population in HEK-293 cells was calculated to be 52.9 ± 1.3 fmol mg\(^{-1}\) protein (n = 3). The mean \(^3\text{H}\)-inositol phosphate accumulation in the presence of Li\(^{+}\) (10 mM) in cells expressing CAM \(^{N514V}M_3\) receptor was 61546 ± 5063 d.p.m. mg\(^{-1}\) protein (n = 5), while in the absence of Li\(^{+}\), the mean accumulation was 10440 ± 245 d.p.m. mg\(^{-1}\) protein (n = 4). Atropine (8497 ± 1848 d.p.m. mg\(^{-1}\) protein), darifenacin (8703 ± 1350), oxybutynin (9526 ± 2026) and tolterodine (8408 ± 601) each reduced the mean \(^3\text{H}\)-inositol phosphate accumulation in the presence of Li\(^{+}\) to similar levels (n ≥ 3 in each case), that were also not significantly different from the \(^3\text{H}\)-inositol phosphate levels measured in HEK-293 cells in the absence of Li\(^{+}\) (see above). All four ligands therefore acted as full inverse agonists in this system.

Analysis of the concentration-dependent inhibition of constitutive \(^3\text{H}\)-inositol phosphate accumulation in a number of experiments allowed the generation of concentration-response curves for each ligand (see Figure 5.19). Mean percent basal \(^3\text{H}\)-inositol phosphate accumulation was calculated by first subtracting the accumulation in the absence of Li\(^{+}\) from that in the presence of Li\(^{+}\). From a number of
Figure 5.19
Inhibition of basal [³H]-inositol phosphate accumulation in HEK-293 cells transiently expressing N⁵¹⁴⁵M₄ mutant receptor by the mACh receptor antagonists atropine, darifenacin, oxybutynin and tolterodine. Data are expressed as % mean basal [³H]-inositol phosphate accumulation. Results are expressed as mean ± s.e.m, n ≥ 3.
individual concentration-response curves, mean pEC$_{50}$ and Hill slope values for each ligand were derived and these are given in Table 5.6.

5.2.8 Comparison of the effects of overnight (24 h) agonist and inverse agonist treatment upon the expression of wild-type and constitutively active mutant M$_2$ and M$_3$ mACh receptors

Figure 5.20 summarises the comparison of the relative effects of 24 h agonist (carbachol; 100 μM) and inverse agonist (atropine; 300 nM) treatment upon the expression of M$_2$ and M$_3$ wild-type and constitutively active mutant receptors. Previous experiments with chronic agonist-inverse agonist treatments were performed on cells co-expressing M$_2$ receptors and the chimeric G-protein Gq65, as this is consistent with experiments investigating the constitutive activity of the M$_2$ receptor. However, the data shown in Figure 5.20 relate to similar experiments performed in cells expressing the wild-type or N410Y mutant M$_2$ receptors alone, in the absence of Gq65. A similar pattern of up-regulation in response to atropine and down-regulation following carbachol treatment is seen in both wild-type and mutant M$_2$ receptor-expressing cells, mirroring that seen in cells co-expressing receptor and Gq65. However, based upon the three experiments represented in Figure 5.20, there is an indication that the up-regulation of both wild-type, and in particular mutant, receptors is less marked in cells not expressing Gq65; e.g. mean up-regulation in response to atropine at N410Y mutant receptor = 23.2 ± 5.3 %; while at N410Y mutant receptor (+ Gq65), mean up-regulation = 60.4 ± 7.4 %.

In contrast, M$_3$ wild-type and N514Y mutant receptors expressed in CHO-K1 cells behave quite differently. While wild-type M$_3$ receptor expression is affected similarly to the M$_2$ receptor, the N514YM$_3$ mutant displays a much more pronounced up-regulation (268 ± 52 %) following 24 h incubation with atropine (300 nM) and also exhibits an enhanced expression (104.7 ± 23.2 %) upon carbachol (100 μM) treatment (Figure 5.20). The magnitude of the up-regulation in response to atropine treatment is consistent with previous work on the N514YM$_3$ receptor in HEK-293 cells (see section 5.1).
Table 5.6 Inhibition of basal [3H]-inositol phosphate accumulation in HEK-293 cells transiently expressing $^{35}$S-labeled M$_3$ CAM receptor, by mACh receptor antagonists. Data are expressed as mean (s.e.m.) values from n ≥ 3 experiments.

<table>
<thead>
<tr>
<th></th>
<th>pEC$_{50}$</th>
<th>Hill slope</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atropine</td>
<td>8.40 (0.16)</td>
<td>1.02 (0.16)</td>
</tr>
<tr>
<td>Darifenacín</td>
<td>7.94 (0.14)</td>
<td>1.11 (0.12)</td>
</tr>
<tr>
<td>Oxybutynin</td>
<td>7.00 (0.10)</td>
<td>1.33 (0.24)</td>
</tr>
<tr>
<td>Tolterodine</td>
<td>7.50 (0.11)</td>
<td>1.13 (0.15)</td>
</tr>
</tbody>
</table>
Figure 5.20
Effect of chronic (24 h) treatment of CHO-K1 cells transiently expressing wild-type or mutant M₂ or M₃ receptor with inverse agonist (atropine; 300 nM) and agonist (CCh; 100 µM) upon receptor expression levels. Data are expressed as mean percent specific binding relative to control cells. Results are expressed as mean ± s.e.m, n ≥ 3.
5.3 Discussion

The main purpose of this study was to generate a constitutively active mutant M₂ mACh receptor to facilitate the pharmacological investigation of a variety of putative inverse agonists at the M₂ receptor. To this end, a single point mutant (N410Y) of the cloned human M₂ mACh receptor was created, as homologous mutations in both the M₁ (Huang et al., 1998b) and M₃ (Dowling, PhD thesis, 2004) receptor subtypes have produced receptor mutants that display substantially enhanced agonist-independent activity. Indeed, when transiently co-expressed with the chimeric G-protein Goq in CHO-K1 cells, the mutant N410YM₂ receptor displayed a significantly higher basal [³H]-inositol phosphate accumulation than the wild-type M₂ mACh receptor expressed at similar or higher levels. This enhanced basal activity was sensitive to concentration-dependent inhibition by a range of muscarinic ‘antagonists’ (including atropine), suggesting that they act as inverse agonists in this system.

5.3.1 Assessment of constitutive activity and inverse agonism at wild-type and N410Y mutant M₂ mACh receptors

Initial attempts to functionally characterise the N410Y mutant M₂ mACh receptor by assaying receptor-mediated inhibition of forskolin-stimulated cyclic AMP accumulation proved unsuccessful in a transiently transfected system. Significant inhibition of the forskolin response was not consistently observed even at high levels of mACh receptor stimulation. However, even when substantial levels of receptor expression (> 1 pmol mg⁻¹ protein) were obtained in CHO-K1 cells, this is likely to reflect a high level of expression in a minor complement of cells within the total cell population, as in our experience transfection efficiencies with lipofection reagents in model cell lines are generally less than 50%. Given that forskolin would be anticipated to generate a robust response in 100% of cells within the assay, it is conceivable that a modest inhibitory response in < 50% of those cells might be difficult to detect.

In order to overcome this problem, a strategy was devised to couple the predominantly Goq-coupled M₂ mACh receptor to the phospholipase-C pathway, by co-expression with a Goq/Gα₁₂ chimera (Goq; Conklin et al., 1993), allowing
measurement of responses only from those cells successfully transfected with receptor and chimeric G-protein. Transient co-transfection of Goq45 and either wild-type or N410Y mutant M2 mACh receptor cDNA into CHO-K1 cells and subsequent labelling with [3H]-myo-inositol, allowed the measurement of [3H]-inositol phosphate accumulation as an index of M2 receptor activation. This technique has previously been utilised to couple a number of predominantly Gq/11-coupled receptors to the phospholipase-C pathway, including the M2 mACh receptors (Liu et al., 1995), μ-, δ- and κ-opioid receptors (Joshi et al., 1999) and adenosine A1, dopamine D2 and α2-adrenergic receptors (Conklin et al., 1993). A significantly enhanced (approx. 2-fold) basal [3H]-inositol phosphate accumulation in cells expressing the N410Y mutant M2 receptor (relative to wild-type-expressing cells) confirmed the constitutively activating effect of the mutation. Higher basal [35S]-GTPγS binding was also observed in membranes prepared from CHO-K1 cells transiently expressing N410Y mutant M2 receptors than in those expressing the wild-type receptor. Constitutive activity is known to increase proportionately with receptor expression (de Ligt et al., 2000), but since care was taken in all of these experiments to ensure that expression levels of N410Y mutant receptors were lower than or equivalent to wild-type, the larger agonist-independent activity of the mutant receptor is unlikely to result from differences in receptor expression level.

The expression of Goq45 in these experiments was confirmed by immunoblot analysis of cells transiently co-transfected with Goq45 and either wild-type or mutant M2 receptor, with a Goq-specific antisera (IQB; Mullaney et al., 1993). Strong bands were detected at the predicted molecular weight of Goq/Goq45 (approx. 42 kDa), demonstrating similar expression of Goq45 whether co-transfected with wild-type or mutant M2 receptor. Interestingly, in un-transfected cells or those transfected with receptor alone, very little endogenous Goq immunoreactivity was detected, suggesting that the majority of that detected in Goq45-expressing cells represents the over-expressed chimeric G-protein. Such low levels of endogenous Goq in CHO-K1 cells may appear inconsistent with the robust phosphoinositide responses observed in CHO cell lines, but a previous report by Mullaney et al. (1993) found that Goq11 was expressed at approx. 2.5-fold higher levels than Goq in CHO cells. This would suggest that phospholipase-C activation in CHO cells might be predominantly
mediated via Go11, facilitating a robust phosphoinositide response despite the modest Goq expression.

The concentration-dependent inhibition of basal [3H]-inositol phosphate accumulation in N410Y mutant M2 receptor-expressing cells by atropine (acting as an inverse agonist) is characteristic of a constitutively active receptor. Atropine also reduced the basal activity of the wild-type receptor expressed at high levels, indicating that although less pronounced than for the N410Y mutant receptor, when over-expressed the wild-type M2 mACh receptor is capable of functionally activating G-proteins in the absence of agonist. It is likely that the over-expression of G-protein (in this case Goq) also served to amplify the constitutive activity of the M2 receptor in this system, as has been reported for other mACh receptor subtypes (Burstein et al., 1995, 1997). However, the endogenously expressed wild-type M2 mACh receptor has previously been reported to exhibit constitutive activity (Hilf & Jakobs, 1992; Hanf et al., 1993; Jakubik et al., 1995). The failure of atropine to significantly reduce the enhanced basal [35S]-GTPyS binding in N410Y mutant-expressing cell membrane homogenates might be due to the modest nature of the constitutive activity measured in this system, perhaps reflecting the difference between the extent of pre-coupling to endogenous Gi proteins versus highly over-expressed Goq protein. However, the higher level of constitutive activity displayed by the N410Y mutant, despite a lower receptor expression level and equivalent G-protein expression, supports the notion that the mutation of asparagine 410 to tyrosine in the M2 mACh receptor enhances the propensity of the receptor to adopt an active state(s) in the absence of agonist.

The localisation of endogenous Gi proteines and transiently expressed Goq-protein is also likely to differ, as differential segregation of Gq into caveolae and Gi into lipid rafts has been demonstrated in lung tissue and cultured epithelial and endothelial cells (Oh & Schnitzer, 2001). The potential importance of such specialised membrane compartments for GPCR signalling has been discussed (see Razani et al., 2002 and section 1.2.6), so it is conceivable that the availability of Goq to the M2 mACh receptor might be modulated not only by expression level but also by the relative localisation of receptor and G-protein. It might therefore have been interesting to compare the basal [35S]-GTPyS binding in membranes prepared from cells co-expressing mutant M2 receptor and Goq, as it might be anticipated that agonist-
independent G-protein coupling would be augmented by the presence of high levels of Goαq.

5.3.2 Agonist-mediated activation of wild-type and N410Y mutant M₂ mACh receptors

Other hallmark features of constitutively active mutant GPCRs were observed for the N410Y mutant M₂ receptor in this study. Methacholine concentration-dependently stimulated [³H]-inositol phosphate accumulation in cells co-expressing either wild-type or mutant M₂ receptors to equivalent levels (approx. 4-5-fold above basal), but with a significantly greater potency (> 10-fold) at the mutant receptor. Enhanced agonist potency at the N410Y mutant receptor was also observed in [³⁵S]-GTPγS binding assays in transiently transfected CHO-K1 cells. An increased sensitivity to agonist activation has been reported for many constitutively active mutants, including those for Gq/11-coupled muscarinic receptor subtypes (Spalding et al., 1998; Huang et al., 1998b, 1999; Ford et al., 2002) and other Gq/11-coupled receptors (e.g. α₃A-adrenoceptor (Wurch et al., 1999); M₄ mACh receptor (Ford et al., 2002)). It has been proposed that such mutations, to a certain degree, relieve the conformational constraints of the inactive state of the GPCR, thereby reducing the free energy required to transform the receptor into an active state (Kenakin, 2002). In this model, receptor-activating conformational changes induced by agonist binding would occur more readily in the mutant receptor than in the more constrained wild-type receptor, an effect that would be manifested in an enhanced sensitivity to agonist activation, such as that observed in this study.

Significantly higher maximal agonist-mediated responses have been observed for certain constitutively active mutants, including the (S465Y, T466P) mutation in the M₃ mACh receptor (Spalding et al., 1998). Although maximal agonist-stimulated levels of both [³⁵S]-GTPγS binding and [³H]-inositol phosphate accumulation were similar for wild-type and N410Y mutant M₂ receptors, the lower expression levels observed for the mutant M₂ receptor than for the wild-type receptor suggest that methacholine might display a greater efficacy at the mutant receptor population. This highlights the importance of monitoring receptor expression levels when relating the functional properties of multiple independent receptor populations. In light of this, it
is interesting to note that Huang et al. (1998b) compared maximal responses to a range of agonists in cells expressing 10-fold greater levels of (S388Y, T389P) mutant M₁ mACh receptors than the equivalent wild-type receptor populations, while Spalding et al. (1998) noted more modest effects of the homologous mutation in the M₅ subtype, when comparing evenly matched receptor populations. Further investigation of the effects of the N410Y mutation upon M₂ receptor agonist pharmacology is detailed in Chapter 6.

Effects upon ligand binding affinity are also commonly observed for constitutively active mutant receptors (Strange, 2002). Typically for constitutively active receptor mutants, the more extensively characterised examples in the mACh receptor family display significantly greater affinity for agonist ligands, when compared to wild-type receptors (Huang et al., 1998b, 1999; Ford et al., 2002). In the limited binding studies performed with transiently expressed N410Y M₂ receptor (albeit in HEK-293 cells) in this study, a trend towards higher affinity for the agonist methacholine was observed, but this was not statistically significant. Further examination of agonist binding affinities for the wild-type and N410Y mutant M₂ receptors, stably expressed in a CHO cell background, is included in Chapter 6.

5.3.3 The prevalence of negative efficacy in muscarinic ‘agonists’

As the availability of constitutively active receptor systems has increased over the last decade, a growing number of ligands, previously classified as competitive antagonists, have demonstrated negative efficacy at constitutively active GPCRs, leading to their re-classification as inverse agonists (Kenakin, 2004). Recent estimates suggest that approx. 85% of all known ‘agonist’ ligands are in fact inverse agonists, indicating that it is only a small minority of ligands that fail to demonstrate detectable efficacy at their cognate receptors (Kenakin, 2004). Studies performed with both constitutively active mutant and wild-type receptors (expressed recombinantly and endogenously) have found that the majority of muscarinic ‘agonists’ investigated, act as inverse agonists in systems exhibiting constitutive activity (Jakubik et al., 1995; Burstein et al., 1997; Huang et al., 1998b, 1999; Dowling, PhD thesis, 2004).
Numerous other studies have characterised atropine as an inverse agonist (e.g. Hilf & Jakobs, 1992; Burstein et al., 1997; Ricny et al., 2002) but relatively few studies have investigated other muscarinic antagonists. In particular, mACh receptor antagonists that are widely used in the clinic for the treatment of overactive bladder (OAB), such as oxybutynin, tolterodine, as well as darifenacin (a candidate OAB therapy), have not yet been assayed for negative efficacy. The availability of constitutively active M$_2$ and M$_3$ mACh receptor mutants allowed this to be addressed. At both M$_2$ and M$_3$ mutant receptors, oxybutynin, tolterodine and darifenacin significantly reduced agonist-independent activity to similar levels to atropine i.e. all behaved as full inverse agonists. Whether their mechanism of action in vivo results from an inhibition of basal muscarinic receptor activity or simply from a competitive inhibition of acetylcholine-mediated receptor activation remains to be resolved. The aetiology of OAB is generally poorly understood (see section 1.3.5), but given that OAB is characterised by a tonic over-activity of bladder smooth muscle contraction, and is often successfully managed by muscarinic ‘antagonists’, it is tempting to speculate that, at least in some cases, the clinical efficacy of agents such as oxybutynin and tolterodine might be related to their ability to reverse muscarinic receptor-mediated contractions, even in the absence of endogenous agonist. Negative efficacy is of particular interest in ligands used in long-term therapy, such as in OAB, due to the well-documented up-regulatory effects of inverse agonists (see Milligan & Bond, 1997). This could lead to the development of tolerance to anti-muscarinic therapy and ‘super-sensitivity’ to endogenous cholinergic signalling upon cessation of treatment. It is anticipated that in the development of future therapeutic agents, efficacy profiles will be carefully scrutinised in relation to both the potentially beneficial and deleterious consequences of negative efficacy.

The ability of certain other common muscarinic receptor antagonists to reduce constitutive activity at the N410Y mutant M$_2$ receptor was also investigated. Pirenzepine, methoctramine, 4-DAMP and QNB all significantly inhibited basal $[^{3}H]$inositol phosphate accumulation in cells co-expressing Go$_{q5}$ and the N410Y mutant M$_2$ receptor. This is in agreement with previous studies detailing the inverse agonist properties of pirenzepine (Daefler et al., 1999a), 4-DAMP (Burstein et al., 1997) and QNB (Jakubik et al., 1995; Burstein et al., 1997). Little evidence exists relating to methoctramine, but as with pirenzepine and 4-DAMP, Dowling (PhD thesis, 2004)
has demonstrated that methoctramine acts as a full inverse agonist at the N514Y CAM M₃ receptor.

Just as atropine was capable of exhibiting inverse agonism at the wild-type M₂ receptor when co-expressed with Goαq in CHO-K1 cells, most of the other ligands investigated also significantly reduced basal [³H]-inositol phosphate accumulation resulting from the agonist-independent activity of the wild-type M₂ receptor. Indeed, only darifenacin and QNB failed to significantly inhibit the wild-type basal activity. It has been proposed that in a system displaying moderate constitutive activity, even full inverse agonists might display relatively low values of negative intrinsic activity, since the maximal effect observed is entirely dependent upon the constitutive activity of the system (Kenakin, 2004). Partial inverse agonists might therefore appear as neutral antagonists in such a system. Given the relatively low level of constitutive activity in wild-type M₂ receptor-expressing cells, the inability of QNB and darifenacin to significantly reduce basal phosphoinositide turnover could be interpreted as an indication that they are not full inverse agonists (i.e. they do not possess a negative efficacy equivalent to that of atropine). A range of inverse agonists efficacies have been reported at the β-adrenoceptors (Chidiac et al., 1994), but as yet no evidence is available for this at the muscarinic receptors. In N410Y mutant M₂ receptor-expressing cells, both darifenacin and QNB display full inverse agonism, perhaps as a result of the greater sensitivity of this system (conferred by the higher levels of constitutive activity) to inverse agonist effects. In support of this, darifenacin also acts as a full inverse agonist at the N514Y M₃ receptor mutant (as did all antagonist ligands tested), which displays an even higher level of constitutive activity (3-4-fold greater than wild-type) than the N410Y mutant M₂ receptor (approx. 2-fold greater than wild-type). It is also notable that even after subtraction of the lithium-independent basal accumulation of [³H]-inositol phosphates, maximal responses to inverse agonist caused < 75 % inhibition of total constitutive activity at the N410Y mutant M₂ receptor and ≤65 % inhibition in M₂ wild-type receptor-expressing cells (compared with almost 100 % inhibition at the N514Y M₃ receptor mutant), suggesting that moderate constitutive activity might indeed be linked to relatively low estimates of negative intrinsic activity (Kenakin, 2004).

There are obvious parallels between this concept and that applied to the measurement of positive efficacy, where partial agonists may behave as full agonists in a
particularly well-coupled system. It is only in a less well-coupled receptor expression system (perhaps with lower receptor and/or G-protein expression levels) that partial agonism may be 'revealed' by a sub-maximal functional response (Kenakin, 1999, 2004). Thus, for the detection of putative partial inverse agonists, a delicate compromise might be required, balancing sufficient constitutive activity to provide a signal:noise ratio in which different levels of inverse agonism may be discerned, with only a moderately sensitive system such that ligands possessing intermediate levels of negative efficacy will not maximally inhibit the constitutive activity.

5.3.4 Potency of inverse agonists at the M2 and M3 receptors

Although not possible for all of the antagonists investigated, estimates of the 'potency' of atropine for the inhibition of basal [3H]-inositol phosphate accumulation in both wild-type and N410Y mutant M2 receptor-expressing CHO cells were obtained. Similar values (quoted as pIC50 values) were established for the ligands assayed at the N514Y M3 mutant receptor. Interestingly, atropine displayed similar 'potency' at M2 (pEC50 = 8.42) and M3 (pEC50 = 8.40) CAM receptors and these values were comparable to binding affinity estimates for atropine at these two receptors. The lack of subtype selectivity at the level of atropine 'potency' is consistent with the findings of Burstein et al. (1997), who found that the anticipated receptor subtype selectivity profiles for a range of muscarinic antagonists (including the non-selective atropine) were maintained for the inhibition of constitutive activity (induced by Gq5 over-expression) at the M1, M3 and M5 mACh receptors. However, without 'potency' estimates for inverse agonists investigated in the present study that would be anticipated to display some degree of subtype selectivity, it is not possible to confirm this for the M2 and M3 mACh receptor CAMs.

Atropine displayed significantly higher 'potency' for the inhibition of basal activity at the wild-type M2 mACh receptor (pEC50 = 9.04) compared with the N410Y mutant receptor (pEC50 = 8.42), and perhaps even compared with its binding affinity at the wild-type receptor (pKd = 8.70 in HEK-293 cells and 8.56 when stably expressed in CHO-K1 cells (see Chapter 6)). Very few studies have directly compared inverse agonist 'potencies' at wild-type and constitutively active mutant receptors, as in most cases investigators focus upon negative efficacy or simply do not investigate wild-
type and CAM receptors side by side. At the muscarinic receptors, Burstein et al. (1997) compared the ability of inverse agonists to inhibit constitutive activity and agonist-induced responses at the $M_1$, $M_3$ and $M_5$ mACh receptors and found no significant differences between affinities derived for the inhibition of these two responses. However, the observed affinity or 'potency' of atropine will depend upon the concentration of agonist present or upon the extent of G-protein over-expression, respectively. Since neither of these factors were accounted for (agonist concentration, at least, could be accounted for by correcting the observed IC$_{50}$ value using functional equivalent of the Cheng-Prusoff equation (Craig, 1993)), a quantitative comparison between the inhibition of agonist-mediated versus constitutive activity is difficult to justify.

Given that the agonist methacholine demonstrated an enhanced potency for the activation of the N410Y mutant $M_2$ mACh receptor, it would seem intuitive that an inverse agonist would exhibit a lower potency for the reversal of the constitutive activity in the mutant receptor population. According to the extended ternary complex model (see Figure 1.5), inverse agonists may exert their effects via a selectively higher affinity for the inactive ($R_i$) than for the active ($R_g$) receptor species, or by reducing the affinity of the ligand-bound receptor for its cognate G-protein (Samama et al., 1993; Strange, 2002). From this it is clear that any perturbation of the system in favour of $R_g$ and/or $R_gG$ is likely to result in a reduction in the apparent binding affinity of an inverse agonist for the receptor population. Indeed, constitutively activating mutations have been reported to reduce the affinity of inverse agonists for the receptor (Costa & Herz, 1989; Samama et al., 1994; Huang et al., 1998b; Wade et al., 2001) as have other factors anticipated to increase the proportion of $R_g$ relative to $R_i$, such as G-protein over-expression (Azzi et al., 2001). Since the N410Y mutant receptor exhibits higher constitutive activity than the wild-type $M_2$ receptor, it is likely that even though a difference in atropine binding affinity was not apparent in radioligand binding studies, the greater proportion of $R_g$ in the mutant receptor-expressing system might be reflected in the lower potency of atropine for reversing the basal activity in this system. The inability to detect a significant shift in inverse agonist binding affinity between wild-type and CAM receptors has been reported previously (Kjelsberg et al., 1992; Ren et al., 1993; Ford et al., 2002) and theoretical analysis suggests that it is only when a mutation strongly favours the formation of the
active state that a significant shift in inverse agonist affinity will be observed; e.g. if 50% of the total CAM receptor population is in the active state, while a negligible proportion of wild-type receptors are active in the absence of agonist, only a 2-fold difference in binding affinity would be predicted for inverse agonists between wild-type and CAM receptor populations (Wade et al., 2001; Strange 2002).

5.3.5 Up-regulation of constitutively active mACh receptors by overnight (24 h) treatment with inverse agonist

An interesting aspect of the work of Dowling (PhD thesis, 2004) on the CAM N514Y M3 receptor was the demonstration that chronic (12-24 h) incubation of HEK-293 cells transiently expressing the mutant receptor with atropine (and indeed other inverse agonists) led to a significant increase in the level of cell surface receptor expression (Figure 5.3.1 (a) reproduced by kind permission; Dowling, PhD thesis, 2004). This was shown to occur in a concentration-dependent manner for the three inverse agonists investigated (Figure 5.3.1 (b)), suggesting that ligand binding might stabilise the inherently unstable CAM and thereby reduce the constitutive down-regulation/degradation of the receptor. The first example of receptor up-regulation following inverse agonist treatment was for the CAM β2-adrenoceptor (Pei et al., 1994; MacEwan & Milligan, 1996). Since these observations, similar phenomena have been reported for a number of constitutively-active mutant GPCRs, including the β1-adrenoceptor (Mclean et al., 2002), α1B-adrenoceptor (Lee et al., 1997), α2A-adrenoceptor (Pauwels & Tardif, 2002) and μ-opioid receptor (Li et al., 2001). Inverse agonist-mediated receptor up-regulation has also been observed for wild-type receptors known to display a significant degree of constitutive activity (e.g. histamine H3 receptor; Smit et al., 1996).

As was observed with the N514Y CAM M3 receptor (Dowling, PhD thesis, 2004), it was noted that upon transient transfection of either HEK-293 or CHO-K1 cells with equivalent amounts of wild-type or N410Y mutant M2 receptor cDNA, the resulting expression levels of the mutant receptor were significantly lower than wild-type. In CHO-K1 cells co-expressing Goq5 and either wild-type or N410Y mutant M2 mACh receptors, time- and concentration-dependent up-regulation of cell surface receptor levels was observed upon chronic treatment with atropine. More substantial increases
Figure 5.3.1 (a) Time-course of effects of muscarinic antagonists upon $^{3}H$NMS
receptor (except where indicated) expression level ($B_{max}$) in HEK-293 cells.
(b) Concentration dependent increase in percent specific binding (relative to
vehicle-treated cells) following 24 h treatment of HEK-293 cells transiently
expressing $^{3}H$NMS receptor with muscarinic antagonists.
5.3.6 Potential mechanisms of inverse agonist-mediated up-regulation

It is important to distinguish between the potential mechanisms underlying the lower expression of constitutively active receptor mutants, as even when this process is reversed by inverse agonists, it does not necessarily follow that receptor up-regulation (or more accurately, the prevention of down-regulation) occurs as a result of the negative efficacy of the ligands in question. Constitutive down-regulation and desensitisation was first demonstrated for the \( \beta_2 \)-adrenoceptor CAM, with sustained treatment with the inverse agonists betaxolol and sotalol producing a protein synthesis-dependent up-regulation of CAM receptor (Pei et al., 1994; MacEwan & Milligan, 1996). Subsequent studies identified the \( \beta_2 \)-adrenoceptor CAM as being structurally unstable, rendering it more susceptible to denaturation (Gether et al., 1997). This has led to the hypothesis that constitutive down-regulation of CAMs might represent cellular responses to their misfolded or unstable structures and that ligand binding stabilises their configuration, preventing their accelerated degradation. The protracted time-course and dependence upon \textit{de novo} protein synthesis suggests that the process may also rely upon the stabilisation of newly synthesised receptors to replenish those lost from the cell surface. It follows from this theory that even the binding of an agonist or neutral antagonist might therefore lead to a similar stabilisation and up-regulation of receptor expression. Indeed, several lines of evidence suggest that this may be the case for certain constitutively active mutants, with inverse agonist, neutral antagonist and positive agonist ligands all being demonstrated to significantly up-regulate the cell surface expression of CAM receptors (Li et al., 2001; Pauwels & Tardif, 2002; Zeng et al., 2003). Additionally, a clear resolution between inverse agonism (assayed by the ability of a ligand to reduce agonist-independent signalling) and receptor up-regulation has been observed for certain mutants of the \( \beta_1 \)- and \( \beta_2 \)-adrenoceptors (Mclean et al., 2002) and \( \alpha_{1B} \)-adrenoceptor (Stevens et al., 2000), where mutants displaying robust constitutive activation, sensitive to inverse agonist ligands, have not exhibited a significant up-

in mutant receptor expression were seen, even from lower baseline expression levels, suggesting that the effect may be related to the constitutive activity of the receptor and/or to the inherent stability of the receptor protein.
regulation of receptor expression upon chronic exposure to the same ligands, and *vice versa*. The 5-HT inverse agonist mianserin has even been reported to induce down-regulation of constitutively-active 5-HT2c receptors in primary epithelial cells (Barker et al., 1994).

In contrast, the H2 histamine receptor, which naturally displays constitutive activity, is significantly up-regulated in CHO cells by inverse agonist ligands (cimetidine and ranitidine) but not by the neutral antagonist burimamide (Smit et al., 1996). Zaki et al. (2001) investigated this concept more thoroughly at the G_{0/1}-coupled δ-opioid receptor, where a significant correlation was observed between ligand efficacy in [35S]-GTPγS binding studies and the effect upon receptor expression levels following overnight (18 h) treatment with the same ligands; i.e. inverse agonists caused an up-regulation, while positive agonists caused a down-regulation of receptor expression. This 'efficacy-dependent' modulation of cell surface receptor expression at wild-type GPCRs (even those displaying moderate constitutive activity) perhaps provides evidence that the 'active' state adopted by a constitutively active mutant receptor might not necessarily correspond to that adopted by the wild-type receptor upon functional activation of its cognate G-protein. The situation is complicated further by the increasing appreciation that the 'active' state with regard to G-protein coupling and downstream signalling is not necessarily the same conformation that is recognised by the cellular components mediating the phosphorylation and internalisation of GPCRs (Thomas et al., 2000). These are clearly important considerations for the interpretation of data derived from CAMs in relation to GPCR activation/regulation.

Even in the case of the CAM β2-adrenoceptor, where agonist treatment has been demonstrated to significantly reduce receptor expression, it is notable that this down-regulatory response is less pronounced than for the wild-type receptor (MacEwan & Milligan, 1996). It has been proposed that two opposing influences might be occurring at the CAM β2-adrenoceptor: a classical agonist-induced down-regulation of receptor expression (similar to that observed at the wild-type receptor), versus a stabilisation of the inherently unstable CAM receptor by agonist binding (Milligan & Bond, 1997). Data from the present study suggests that a similar scenario might be occurring at the N514Y CAM M3 receptor, but that the net effect of these two opposing influences might be more in favour of an agonist-mediated stabilisation, since carbachol induced a significant up-regulation that was, however, less
pronounced than that effected by atropine. In each case, the effect of chronic agonist exposure to the wild-type receptor is similar (i.e. a down-regulation), but if the constitutively activating mutation in the M₃ receptor has a greater de-stabilising effect and/or agonist binding elicits a more pronounced stabilising influence than that seen at the β₂-adrenoceptor, a net stabilising effect would be predicted.

In contrast, the equivalent experiments performed at wild-type and CAM M₂ mACh receptors suggest different effects of the homologous mutation on the M₂ and M₃ receptors. Opposing effects of agonist (carbachol) and inverse agonist (atropine) at both wild-type and CAM M₂ receptors indicates that the N410Y mutation in the M₂ receptor might be associated with a less severe effect upon receptor stability/viability than that induced by the homologous mutation in the M₃ receptor. The more modest constitutive activity and less marked effects upon agonist potency and affinity at the CAM M₂ receptor than at its M₃ counterpart would seem to support this notion.

Data obtained with a range of muscarinic antagonists found to possess negative efficacy (based upon the inhibition of constitutive [³H]-inositol phosphate accumulation) suggest that not all muscarinic inverse agonists are equally capable of up-regulating either wild-type or CAM receptor. The overall pattern of up-regulation appears fairly consistent between wild-type and CAM, with atropine and darifenacin maximally up-regulating and pirenzepine intermediate in its ability to increase both wild-type and mutant M₂ receptor expression. What is perhaps more intriguing are those ligands, such as tolterodine, 4-DAMP and oxybutynin, which displayed comparable negative efficacy to atropine and darifenacin in [³H]-inositol phosphate assays, but which for the most part do not appear capable of up-regulating CAM M₂ mACh receptors. Several technical considerations could contribute to these differences, including the possibility that, like QNB, some ligands may be less reversible than atropine and darifenacin and hence the inability to wash ligand from the receptor following 24 h incubation could be obscuring any up-regulatory effects. However, there is no evidence to suggest that the kinetics or lipophilicity of these ligands differ significantly from atropine and darifenacin. Indeed, the experiences of ourselves and others with darifenacin suggest that it is highly lipophilic, a property which might have been anticipated to limit the ease with which the ligand was washed off the cells at the end of the 24 h incubation. However, direct comparisons have indicated that darifenacin, oxybutynin and tolterodine all display rapid association and
dissociation kinetics, at least at the M₃ mACh receptor subtype (Clarke et al., 2003b). It would therefore seem unlikely that the reversibility of binding of certain ligands to the M₂ receptor would be insufficient to allow accurate measurement of receptor expression levels. Another potential explanation is that certain ligands may not be stable throughout the duration of the 24 h incubation. However, there is no evidence to suggest that ligands such as tolterodine and oxybutynin are particularly unstable in solution, so it is not possible to easily relate the observed differences in 'efficacy' with compound viability.

There is a growing body of evidence suggesting that certain ligands are capable of crossing the plasma membrane, binding to intracellular pools of receptor and 'chaperoning' GPCRs from intracellular compartments to the cell surface (Morello et al., 2000). For instance, Petaja-Repo et al. (2002) have reported that cell-permeable δ-opioid receptor ligands were capable of stabilising newly synthesised receptors at the endoplasmic reticulum (ER), facilitating their maturation and ER-export. Crucially, it was found that the ability to pharmacologically chaperone otherwise misfolded and ER-retained receptor proteins was independent of signalling efficacy, since both agonists and antagonists were capable of promoting receptor expression (Petaja-Repo et al., 2002).

Of particular relevance to the study of the down-regulation of CAM receptors is the work of Li and co-workers, who have reported that a CAM μ-opioid receptor can be time- and concentration-dependently up-regulated by chronic treatment with the inverse agonist naloxone (Li et al., 2001). However, treatment with the cell-impermeable quaternary ammonium analog naloxone methiodide caused only approximately 50 % of the maximal up-regulation observed upon naloxone treatment, suggesting that naloxone promoted cell surface expression of CAM μ-opioid receptors by both intra- and extra-cellular mechanisms (Li et al., 2001). The authors proposed that naloxone up-regulates receptor expression by binding to both cell-surface and intracellular receptor protein and preventing its constitutive degradation (Li et al., 2001). Notably, mutant receptor expression was also enhanced by both neutral antagonist and, to a lesser extent, by agonist treatment (Li et al., 2001). Once again, this is consistent with a model in which two opposing processes (agonist-mediated down-regulation versus stabilisation of receptor structure upon agonist binding) are occurring upon agonist treatment.
The failure to observe significant receptor up-regulation with certain muscarinic inverse agonists in the present study might reflect their inability to penetrate the cell and traffic intracellular pools of receptor to the plasma membrane. Since commercially available 4-DAMP is actually the methiodide analog, it is particularly interesting that this was one of the ligands that failed to elicit an up-regulation of either wild-type or mutant M<sub>2</sub> receptor. Attempts to use the highly lipophilic, cell-permeable QNB to investigate this further proved unsuccessful, as the ligand appeared to remain bound to the receptors even after repeated washing. Although the failure of 4-DAMP methiodide to induce up-regulation is consistent with a 'chaperoning' mechanism of action, it is not clear why ligands such as oxybutynin and tolterodine (which are only weakly soluble in water, but are readily soluble in organic solvents) would be significantly less cell permeable than atropine and, in particular, pirenzepine, which are only slightly soluble in organic solvents and would therefore not be predicted to readily cross the plasma membrane.

Reconciling the range of apparent 'efficacies' of muscarinic antagonists for the up-regulation of both wild-type and CAM M<sub>2</sub> receptors, with the apparently consistent level of inverse agonism displayed in the [³H]-inositol phosphate assays would therefore seem difficult. However, it has recently been proposed that the different functions of GPCRs (e.g. internalisation, oligomerisation, phosphorylation, in addition to G-protein coupling) may not result from a single 'active' receptor conformation and may instead be initiated by their own ensemble of micro-states (Kenakin, 2002). The appeal of such an 'ensemble theory' is in its ability to explain not only how different receptor behaviours can occur independently of each other (e.g. internalisation without G-protein activation; Roettger et al., 1997), but also the concept of agonist trafficking of receptor signalling (Kenakin, 1995), with each output being represented by a distinct ensemble of microstates which may or may not be congruent with the complement of microstates representing other GPCR behaviours. While the theory is consistent with several lines of pharmacological evidence (Kenakin, 2002), further investigation is required to adequately test it.

In relation to the current data with the M<sub>2</sub> receptor, it is conceivable within the framework of the ensemble model that ligands possessing significant negative efficacy might not necessarily stabilise receptor conformations that coincide with those required to induce receptor up-regulation. However, it is equally possible that
The inhibition of constitutive \(^{[3]H}\)-inositol phosphate accumulation might not be sufficiently sensitive to detect different levels of negative efficacy, as discussed earlier, while the up-regulation of receptor number might be revealing such ligand-dependent differences in efficacy. In this regard, it is interesting to note that the 'potency' of atropine for the up-regulation of CAM M\(_2\) mACh receptor (pEC\(_{50}\) = 7.72) is approx. 10-fold weaker than its binding affinity (pK\(_i\) = 8.66). In contrast, atropine 'potency' for the inhibition of constitutive \(^{[3]H}\)-inositol phosphate accumulation was approximately equivalent to the binding affinity of atropine for the receptor. The less sensitive output might therefore be more conducive to discerning different levels of negative efficacy in muscarinic inverse agonists. The basis for the lower sensitivity of the up-regulatory response is not clear, but might reflect the requirement for atropine to penetrate the plasma membrane in order to chaperone intracellular receptors to the cell surface. Further insight might have been provided by similar 'potency' estimates for other ligands at both CAM and wild-type M\(_2\) receptors, but unfortunately the construction of concentration-response curves was not possible in these cases. However, similar findings were reported for the CAM M\(_3\) receptor (created by swapping the third intracellular loop with that of the CAM \(\beta_2\)-adrenoceptor) in receptor up-regulation assays, where a range of muscarinic antagonists (including atropine, NMS and QNB) displayed lower 'potencies' in up-regulating receptors than in reducing constitutive activity (Zeng et al., 2003). The 'potencies' of atropine, pirenzepine and 4-DAMP for up-regulating N514Y CAM M\(_3\) receptors expressed transiently in HEK-293 cells were also significantly lower than their binding affinities for the receptor (e.g. atropine pK\(_i\) = 8.85; pEC\(_{50}\) for up-regulation = 8.22).

It is apparent from the comparative data for CAM M\(_2\) and M\(_3\) receptors that the homologous asparagine to tyrosine mutation at the junction between TM 6 and the third extracellular loop appears to exert differential effects on the two receptor subtypes. This is particularly evident in the opposing effects of chronic agonist (carbachol) treatment upon CAM receptor expression: carbachol causes a significant (approx. 60 %) down-regulation of CAM M\(_2\) receptor expression levels, while CAM M\(_3\) receptor expression is substantially enhanced (by approx. 100 %) upon agonist exposure. This hints at potentially distinct mechanisms underlying the constitutive down-regulation of cell surface receptor expression of these two CAMs. It has been
proposed that in some instances, the low receptor expression levels observed for CAMs might not result from the instability of the mutant protein at the cell surface or from a misfolded, and hence ER-retained, receptor (Parnot et al., 2002). Instead, evidence has been provided that certain CAM receptors undergo a permanent cycle of constitutive internalisation and recycling, as might be predicted for a receptor protein that is permanently in its active state. Miserey-Lenkei et al. (2002) reported that cell surface expression of three distinct CAM angiotensin II AT\textsubscript{1A} receptors was significantly increased by inverse agonist (losartan) treatment. However, this effect was clearly different to that observed for many CAM GPCRs, as the authors observed a rapid re-distribution of receptors from intracellular sites (so-called ‘externalisation’) to the plasma membrane, within 2 h of losartan treatment, that was not dependent upon \textit{de novo} protein synthesis, could not be replicated by agonist treatment and did not involve receptor stabilisation, as the metabolism of the CAM receptors was found to be similar to that of the wild-type receptors (Miserey-Lenkei et al., 2002). It was suggested that this mechanism might predominate for (or indeed be limited to) GPCRs that are rapidly internalised (in 5-10 min) and slowly recycled (2-3 h) as it might be anticipated that an increase in the rate of internalisation of such receptors (either by a constitutively activating mutation or by agonist treatment) would result in an intracellular accumulation of receptors.

It is interesting to consider the ‘externalisation’ model in relation to the M\textsubscript{2} mACh receptor, which has been shown to internalise extensively and rapidly (within minutes) upon agonist exposure in a variety of cell backgrounds (Koenig & Edwardson, 1996). However, recovery of the M\textsubscript{2} receptor to the cell surface is uncharacteristically slow (taking several hours) for the muscarinic receptors (Koenig & Edwardson, 1996; Roseberry & Hosey, 1999). It is therefore tempting to speculate that a constitutively active M\textsubscript{2} receptor mutant might also be rapidly internalised and slowly recycled, leading to an intracellular accumulation of mutant receptors. Inverse agonist-mediated up-regulation might therefore represent a net ‘externalisation’ of tonically internalised receptor, occurring as a direct result of the ability of the inverse agonist to prevent internalisation of plasma membrane-associated receptors, by stabilising the receptor in its inactive state. This model is consistent with the inability of agonists to induce up-regulation of CAM M\textsubscript{2} receptors and also does not require ligands to penetrate the cell in order to re-distribute intracellular pools of receptor.
Although the time-course of the observed up-regulation of Mz receptors is not entirely consistent with that proposed in this model, the kinetics of internalisation and in particular recycling, might differ between the CHO cell background used in this study and the HEK-293 and H295 cell lines utilised by Miserey-Lenkei et al. (2002). In fact differences in the extent of intracellular localisation (attributed to differences in the trafficking kinetics between cell lines) were noted between the two different cell backgrounds investigated by Miserey-Lenkei et al. (2002).

Since the protein synthesis dependence of receptor up-regulation was not investigated in the present study it is not possible to eliminate a role for the de novo synthesis of receptors in this process. However, it would be anticipated that if CAM Mz receptor up-regulation proceeds by a distinct mechanism to that observed for the CAM M3 mACh receptor, a difference in the sensitivity of these two responses to protein synthesis inhibitors such as cycloheximide would be observed. Further characterisation of the extent of intracellular localisation of M2 and M3 mutant receptors would also be necessary to fully investigate whether the Mz receptor up-regulation in response to inverse agonist treatment occurs via an 'externalisation' of intracellular receptors.

3.3.7 Concluding remarks

In conclusion, this study details the generation of a constitutively active mutant (N410Y) Mz mACh receptor and its characterisation in transient expression systems. The N410Y mutant receptor displays several of the hallmark features of a constitutively active mutant, including enhanced basal G-protein coupling that is sensitive to a range of inverse agonists, enhanced agonist potency (and to a lesser degree affinity) and a lower cell surface expression level than the wild type receptor (despite being expressed from the same promoter in the same expression vector). While all muscarinic antagonists investigated (including clinically important compounds such as oxybutynin and tolterodine) behaved as apparently full inverse agonists in functional assays, different levels of 'efficacy' were observed for the same ligands in the up-regulation of both wild-type and CAM M2 receptors resulting from chronic treatment with inverse agonists. Differences were also noted in the effect of chronic agonist incubation between CAM M2 and M3 mACh receptors, suggesting
that the homologous asparagine to tyrosine mutations might be having different
effects upon the M₂ and M₃ mACh receptors.
Chapter 6: Characterisation of the pharmacology of CHO cell lines stably expressing wild-type and N410Y mutant M2 mACh receptors.

6.1 Introduction

The transient expression system used in the previous chapter to characterise the constitutive activity of the N410Y mutant M2 mACh receptor provides a rapid and straightforward means of comparing the properties of wild-type and mutant receptor proteins in the context of the chosen cell background. However, as was alluded to in the discussion of Chapter 5, transient expression systems have limitations, notably in the proportion of cells successfully transfected and expressing the protein of interest. Stable expression of the receptor protein is preferable where a homogeneous population of cells expressing the same complement of proteins is required. The initial aim of this investigation was, therefore, to generate CHO cell lines stably expressing either wild-type or N410Y mutant M2 receptors, to facilitate further study of the pharmacological properties of the constitutively active mutant M2 mACh receptor.

It is anticipated that the availability of these cell lines will allow a more thorough pharmacological characterisation of the N410Y mutant M2 receptor, relative to the wild-type receptor, including a detailed investigation of the effects of the mutation upon M2 receptor affinity for both agonists and inverse agonists. Having established the constitutively activating nature of the mutation by the coupling of highly-expressed populations of receptor to the phospholipase-C pathway (by co-expression with the chimeric G-protein Goq3), the propensity for the mutant receptor to detectably activate its cognate G-proteins in the absence of agonist will be investigated using more conventional techniques (measurement of intracellular cyclic AMP accumulation and [35S]-GTPyS binding). Crucially, this may permit determination of the extent of constitutive activity exhibited by both wild-type and N410Y mutant M2 mACh receptors when expressed at levels approximately equivalent to endogenously expressed muscarinic receptors (< 1 pmol mg\(^{-1}\) protein).

In a population of cells homogeneously expressing a significant population of M2 receptors, agonist-mediated inhibition of forskolin-stimulated cyclic AMP
accumulation would be expected to occur in all cells upon muscarinic agonist treatment, allowing the agonist-sensitivity of wild-type and N410Y mutant M₃ receptor mutants to be fully investigated. Another interesting possibility offered by the availability of such stable cell lines is to investigate the potential for wild-type and mutant M₃ receptors to couple to Gαᵢ in addition to the classical M₃ receptor coupling to Gαᵩ proteins. It has previously been reported that the M₂ mACh receptor (Michal et al., 2001), as well as the closely related M₄ receptor (Jones et al., 1991; Dittman et al., 1994; Migeon & Nathanson, 1994), is capable of stimulating cyclic AMP accumulation via a direct activation of Gαᵢ. Michal et al. (2001) found that stimulation of M₂ mACh receptors, expressed stably in CHO cells, could generate a biphasic cyclic AMP response, with low concentrations of agonist inhibiting forskolin-stimulated cyclic AMP accumulation and higher levels of stimulation enhancing the forskolin response. However, various factors including the duration of stimulation, agonist efficacy and receptor density were found to determine the extent of the stimulatory component of the cyclic AMP response (Michal et al., 2001) and other investigators have failed to demonstrate a detectable augmentation of forskolin-stimulated cyclic AMP accumulation in stable CHO-m₂ cell lines (Mistry et al., in press). However, following pertussis toxin (PTx) pre-treatment to inactivate the Gαᵩ population of G-proteins, agonist activation of M₂ mACh receptors (in the presence of forskolin) readily causes a concentration-dependent increase in cyclic AMP accumulation (Michal et al., 2001; Mistry et al., in press). A further aim of this study is therefore to compare the abilities of a range of muscarinic agonists to enhance and to inhibit forskolin-stimulated cyclic AMP accumulation, at both wild-type and CAM M₂ receptors, in PTx-treated and non PTx-treated cells, respectively.

This system will also allow an investigation of the effects of muscarinic partial agonists (such as pilocarpine and oxotremorine; McKinney et al., 1991; Richards & van Giersbergen, 1995) in cell lines expressing similar levels of wild-type and CAM M₂ receptors. One of the defining features of constitutively active receptors (Samama et al., 1993; Lefkowitz et al., 1993) is that in addition to displaying greater potency, agonists (in particular partial agonists) exhibit enhanced efficacy in functional assays at these mutant receptors relative to wild-type receptors. It would therefore be anticipated that muscarinic partial agonists might show enhanced efficacy (perhaps approaching that of ‘full’ agonists such as methacholine) in N410Y mutant receptor-
expressing cells, as has been reported for other CAM receptors (Samama et al., 1993; Groblewski et al., 1997).
6.2 Results

6.2.1 Generation of CHO cell lines stably expressing wild-type or N410Y mutant M2 mACh receptors

6.2.1.1 Primary \(^{3}H\)-NMS binding screen

To facilitate investigation of the constitutive activity and pharmacology of the N410Y mutant M2 mACh receptor, CHO-K1 cell lines stably expressing the wild-type and N410Y mutant receptors were created, as detailed in section 2.3.3. Figure 6.1 (a) illustrates the results of the primary \(^{3}H\)-NMS binding screen of all 192 clones (96 wild-type and 96 N410Y mutant receptor-expressing clones). In each case, the majority of clones bound less than 500 d.p.m. of \(^{3}H\)-NMS. Although an estimate of the number of cells of each clone present in the assay (i.e. the amount of protein in each well screened) was not possible due to the large number of clones being screened, it is likely that the majority of clones falling into the '< 500 d.p.m.' category represented those expressing very little or no mACh receptor. Those clones that bound between 500 and 1000 d.p.m. were also likely to express relatively low levels of mACh receptor, so only clones that bound greater than 1000 d.p.m. were considered for further analysis. 12 clones were selected from each of the wild-type and N410Y receptor-expressing groups, to represent a broad range of apparent expression levels, from the clones that bound the most radioligand (approx. 40-50,000 d.p.m. in each case) down to some that bound only 1000-1500 d.p.m.

6.2.1.2 Secondary \(^{3}H\)-NMS binding screen

Figure 6.1 (b) and (c) illustrates the data generated from the secondary \(^{3}H\)-NMS binding screen of wild-type (b) and N410Y mutant (c) receptor-expressing clones. More accurate estimates of receptor expression levels were obtained by incorporating a determination of protein for each clone. As can be seen from Figure 6.1 (c), only three N410Y mutant clones appeared to express greater than 200 fmol mg\(^{-1}\) protein. To maximise the potential for detecting constitutive activity, an expression level of approx. 500-1000 fmol mg\(^{-1}\) protein would be desirable. Clones # 64 (705 fmol mg\(^{-1}\) protein) and # 87 (719 fmol mg\(^{-1}\) protein) were therefore selected for functional screening. In addition, the very highly expressing clone # 96 (3398 fmol mg\(^{-1}\) protein) was further investigated. A number of wild-type clones of approximately matched
Figure 6.1
Primary and secondary [³H]-NMS binding screens of CHO-K1 cells stably transfected with either wild-type or N410Y mutant M₂ receptor cDNA.
(a) Frequency distribution of total mACh receptor expression levels in primary screen of all 96 wild-type and 96 N410Y mutant receptor-transfected clones. Data are expressed as number of clones binding an amount of [³H]-NMS (in d.p.m.) falling within the given ranges.
(b) and (c) mACh receptor expression levels of wild-type (a) and N410Y mutant (b) clones selected for secondary [³H]-NMS binding screen. Data are expressed as mean specific binding in fmol receptor expression mg⁻¹ protein.
expression levels were also selected for functional screening (clones # 59, # 80 and # 83).

6.2.1.3 Functional screening

In the functional screening of these clonal cell lines, the basal and forskolin (10 μM)-stimulated cyclic AMP accumulations over a 10 min time-course were measured, as well as the maximal inhibition of forskolin-stimulated cyclic AMP accumulation by methacholine (100 μM). From this preliminary experiment, wild-type clone # 59 appeared to give the most robust methacholine response (inhibiting > 50 % of forskolin-stimulated cAMP accumulation), while the other wild-type clones gave relatively poor responses to muscarinic agonist (< 50 % inhibition) (data not shown).

Of the N410Y mutant clones, clone # 87 displayed a greater inhibition of forskolin-stimulated cyclic AMP accumulation than either of the other two clones investigated (data not shown). Wild-type clone # 59 and N410Y mutant clone # 87 were therefore chosen for further investigation (and will be referred to as CHO-m2 WT and CHO-m2 MUT, respectively). Full details of their functional responses to forskolin and mACh receptor agonists will be given in sections 6.2.2.6 and 6.2.2.7.

6.2.2 Pharmacological characterisation of CHO cell lines stably expressing wild-type and N410Y mutant M2 mACh receptors (and wild-type and N514Y mutant M3 mACh receptors)

6.2.2.1 [3H]-NMS saturation binding analysis

The mACh receptor expression levels of the selected CHO cell clones, stably expressing wild-type (CHO-m2 WT # 59) and N410Y mutant (CHO-m2 MUT # 87) M2 mACh receptors, were further characterised by [3H]-NMS saturation binding assays performed in membrane homogenates. Figure 6.2 displays a representative data-set from 3 or more similar experiments and the mean receptor expression (Bmax) and [3H]-NMS binding affinity constant (Kd) estimates from these experiments are summarised in Table 6.1. [3H]-NMS bound to a single, homogeneous population of mACh receptors with high affinity. Although the mean binding affinity constant (Kd = 0.55 ± 0.11 nM) determined in N410Y mutant M2 receptor (CHO-m2 MUT)-expressing membranes was slightly greater than that in wild-type (CHO-m2 WT)-expressing membranes (Kd = 0.36 ± 0.02 nM), this was not a statistically significant
Figure 6.2
[3H]-NMS saturation binding curves in membrane homogenates of CHO-K1 cells stably expressing wild-type (a) or N410Y mutant (b) M2 mACh receptor (CHO-m2 WT and CHO-m2 MUT respectively). Data are expressed as mean specific binding in fmol receptor expression mg⁻¹ membrane protein and are representative curves of 3 or more experiments.
Table 6.1 [1H]-NMS binding affinity constant ($K_D$) and receptor densities ($B_{max}$) estimates in membrane homogenates of CHO-K1 cells stably expressing M2 or M3 wild-type (CHO-m2 WT, CHO-m3 WT) or constitutively active mutant (CHO-m2 MUT, CHO-m3 MUT) receptors. Data are expressed as mean (s.e.m.) values from $n \geq 3$ experiments.

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<th>$B_{max}$ (fmol mg$^{-1}$ protein)</th>
<th>$K_D$ (nM)</th>
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<tr>
<td>CHO-m2 WT</td>
<td>627 (86)</td>
<td>0.36 (0.02)</td>
</tr>
<tr>
<td>CHO-m2 MUT</td>
<td>247 (51)</td>
<td>0.55 (0.11)</td>
</tr>
<tr>
<td>CHO-m3 WT</td>
<td>349 (30)</td>
<td>0.23 (0.08)</td>
</tr>
<tr>
<td>CHO-m3 MUT</td>
<td>166 (33)</td>
<td>0.81 (0.25)</td>
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difference. Although not as well matched as might be preferred, the higher mACh receptor expression levels in the CHO-m2 WT cell line (approx. 2.5 fold; see Table 6.1) ensured that any bias in the system favours the wild-type cell line, such that any enhanced constitutive activity or agonist affinity/potency/efficacy at the mutant receptor would, if anything, be under-estimated rather than over-estimated.

Similar experiments were performed with CHO cell lines stably expressing wild-type (CHO-m3 WT) an N514Y mutant (CHO-m3 MUT) M3 mACh receptors, respectively, to allow a comparison between some of the effects of the homologous mutation in both M2 and M3 mACh receptors. Representative [3H]-NMS saturation binding curves in CHO-m3 WT and CHO-m3 MUT cell membrane homogenates are illustrated in Figure 6.3 and the mean receptor expression (Bmax) and [3H]-NMS binding affinity constant (Kd) estimates from these experiments are also summarised in Table 6.1. As with the M2 receptor, the apparently lower [3H]-NMS binding affinity in the CHO-m3 MUT cell membranes failed to achieve statistical significance (see Table 6.1). Once again, the mACh receptor expression level in the wild-type cell line was greater (approx. 2-fold) than that in the mutant cell line (Table 6.1).

6.2.2.2 Effect of passage upon the receptor expression (Bmax) and [3H]-NMS binding affinity constant (Kd) estimates in stable CHO cell lines expressing wild-type and constitutively active mutant M2 and M3 mACh receptors

The mean receptor expression (Bmax) and [3H]-NMS binding affinity constant (Kd) estimates given in Table 6.1 were determined in experiments on a number of batches of membrane homogenates, with each batch prepared from cells of a different number of passages (post stable transfection with M2 receptor cDNA). The mean data included in Table 6.1 were calculated only from those membrane batches used in subsequent competition radioligand binding and [35S]-GTPyS binding assays (detailed in Sections 6.2.2.3, 6.2.2.4 and 6.2.2.5). However, as can be seen in Table 6.1, the standard errors associated with some of the mean estimates were quite high. Consideration of the receptor expression (Bmax) and [3H]-NMS binding affinity estimates for each batch of membranes (i.e. at each passage number) provides some indication of the basis for this apparent variation (see Figure 6.4).
Figure 6.3

$[^3H]$-NMS saturation binding curves in membrane homogenates of CHO-K1 cells stably expressing wild-type (a) or N514Y mutant (b) $M_3$ mACh receptor (CHO-m3 WT and CHO-m2 CAM respectively). Data are expressed as mean specific binding in fmol receptor expression mg$^{-1}$ membrane protein and are representative curves of 3 or more experiments.
Figure 6.4
Effect of passage upon receptor expression level [(a) and (c)] and [3H]-NMS binding affinity constant (K_D) estimates [(b) and (d)] in membrane homogenates of CHO-m2 WT [(a) and (b)] and CHO-m2 MUT [(c) and (d)] stable cell lines. Data are expressed as mean specific binding in fmol receptor expression mg⁻¹ membrane protein and K_D (nM). Results are expressed as mean ± s.e.m, n ≥ 2.
Receptor expression in CHO-m2 WT membranes increased with passage, with a statistically significantly higher receptor expression observed at passage 33 (P33; 1060 ± 94 fmol mg⁻¹ membrane protein) relative to that at passage 13 (P13; 542 ± 8 fmol mg⁻¹ membrane protein) (p < 0.05) (Figure 6.4 (a)). There was no significant difference in [³H]-NMS binding affinity between P13 (0.35 ± 0.02 nM) and P33 (0.54 ± 0.13) (Figure 6.4 (b)). In contrast, receptor expression levels in CHO-m2 MUT cell membrane homogenates were slightly (but statistically insignificantly) lower at higher passage number (208 ± 5 (P12) versus 148 ± 26 (P26) fmol mg⁻¹ membrane protein) (Figure 6.4. (c)), while there was no significant difference in [³H]-NMS binding affinity between membranes derived from cells at passage 12 (0.37 ± 0.05 nM) and those at passage 26 (0.54 ± 0.22 nM) (Figure 6.4 (d)).

Figure 6.5 displays similar data for the CHO-m3 MUT cell line, over a smaller range of passages (P7 and P11). Remarkably, the mean receptor expression level was significantly reduced over the course of only 4 passages (from 266 ± 29 to 121 ± 12 fmol mg⁻¹ membrane protein between P7 and P11) (p < 0.05), while the [³H]-NMS binding affinity was significantly reduced from 0.19 ± 0.04 to 0.93 ± 0.17 nM (p < 0.05) (Figure 6.5 (a) and (b), respectively). However, the latter observation might owe something to the inaccuracies inherent in measuring K_d values from such a small population of receptors.

6.2.2.3 [³H]-NMS competition binding assays with mACh receptor ‘antagonists’ in membranes expressing wild-type and constitutively active mutant M2 and M3 mACh receptors

Based upon the extended ternary complex model, ligands that exhibit negative efficacy at GPCRs (i.e. are inverse agonists) would be predicted to display a lower binding affinity for constitutively active receptors than for their wild-type counterparts (Samama et al., 1993; Strange, 2002). However, in many cases little, if any, difference has been observed in the binding affinity of inverse agonists for constitutively active receptors (e.g. Wade et al., 2001). The binding affinities of a number of mACh receptor ‘antagonists’ (many of which have been demonstrated to act as inverse agonists at both M2 and M3 constitutively active receptor mutants; see
Figure 6.5
Effect of passage upon receptor expression level (a) and \({}^{3}H\)-NMS binding affinity constant (\(K_p\)) estimates (b) in membrane homogenates of the CHO-m3 MUT stable cell line. Data are shown as mean specific binding in fmol
receptor expression mg\(^{-1}\) membrane protein and \(K_p\) (nM). Results are expressed as mean ± s.e.m., n ≥ 2.
Chapter 5) were therefore determined in membrane homogenates prepared from wild-type and constitutively active mutant M\(_2\) and M\(_3\) receptor-expressing cells.

The mean competition binding curves, Hill slopes and binding affinity constants (pK\(_i\)) derived from a number of these experiments are given in Figure 6.6 and Table 6.2. For the M\(_2\) mACh receptor the corrected pK\(_i\) values were not significantly different between CHO-m2 WT- and CHO-m2 MUT-derived membranes for atropine, tolterodine, pirenzepine or methoctramine. However, darifenacin and oxybutynin both exhibited significantly lower affinity for the N410Y mutant receptor than for the wild-type M\(_2\) receptor in membrane homogenates (p < 0.05) (Table 6.2). In contrast, for the M\(_3\) mACh receptor, atropine, oxybutynin, tolterodine and methoctramine all displayed statistically significantly reduced affinity in CHO-m3 MUT membranes compared with membranes prepared from CHO-m3 WT cells (p < 0.05), while darifenacin and pirenzepine failed to significantly distinguish between wild-type and N514Y mutant receptors (Table 6.2).

In membranes prepared from all four cell lines, competition binding curves for methoctramine were characterised by Hill slopes of significantly greater than one (p < 0.05). However, for all other competition binding curves Hill slopes did not differ significantly from unity.

6.2.2.4 Guanine nucleotide sensitivity of agonist/[\(^{3}H\)]-NMS competition binding curves in membranes expressing wild-type and N410Y mutant M\(_2\) mACh receptors

[\(^{3}H\)]-NMS competition binding assays were performed with a range of muscarinic agonists in membranes prepared from CHO-m2 WT and CHO-m2 MUT cells. Mean competition binding curves from 3 or more experiments are shown in Figures 6.7 (for the full agonists methacholine and oxotremorine-M) and 6.8 (for the partial agonists oxotremorine and pilocarpine). The mean data from the curve-fitting to each individual data set are summarised in Table 6.3. In all cases, the data were fitted significantly better by a 2 site model than by a single site binding model (p < 0.05) and the corrected binding affinities of each of these two sites (pK\(_{H1}\) and pK\(_{L1}\)), as well as the proportion of the total number of sites that display high affinity binding, are given in columns 1-3 of Table 6.3. When fitted with a 1-site model, all curves
Figure 6.6

$[^3H]$-NMS competition binding curves at membrane homogenates of CHO-m2 WT, CHO-m2 MUT, 
CHO-m3 WT (vt-9) and CHO-m3 CAM cell lines for atropine (a), darifenacin (b), oxybutynin (c), 
tolterodine (d), pirenzepine (e) and methoctramine (f). Data are presented as mean percent of specific 
binding in the absence of competitor. Results are expressed as mean ± s.e.m., n ≥ 3.
Figure 6.6 (cont.)

$[^{3}H]$-NMS competition binding curves at membrane homogenates of CHO-m2 WT, CHO-m2 MUT, CHO-m3 WT (vt-9) and CHO-m3 CAM cell lines for atropine (a), darifenacin (b), oxybutynin (c), tolterodine (d), pirenzepine (e) and methoctramine (f). Data are presented as mean percent of specific binding in the absence of competitor. Results are expressed as mean ± s.e.m, n ≥ 3.
Table 6.2: Competition [H]NMS binding data for a range of mACh receptor antagonists in membrane homogenates prepared from CHO-m2 WT, CHO-m3 WT, CHO-m2 MUT and CHO-m3 MUT cells. Data are expressed as mean (s.e.m.) values from n ≥ 3 experiments. * indicates significant difference between wild-type and mutant (p < 0.05; Student's t test). ‡ indicates value significantly different from unity (p < 0.05; F-test).

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<tr>
<th></th>
<th>CHO-m2 WT</th>
<th></th>
<th>CHO-m2 MUT</th>
<th></th>
<th>CHO-m3 WT</th>
<th></th>
<th>CHO-m3 MUT</th>
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<td>pKᵢ</td>
<td>Hill slope</td>
<td>pKᵢ</td>
<td>Hill slope</td>
</tr>
<tr>
<td>atropine</td>
<td>8.56 (0.05)</td>
<td>1.07 (0.19)</td>
<td>8.51 (0.06)</td>
<td>0.99 (0.21)</td>
<td>8.91 (0.14)</td>
<td>0.79 (0.09)</td>
<td>8.43 (0.02)*</td>
<td>0.92 (0.09)</td>
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<tr>
<td>darifenacin</td>
<td>7.57 (0.16)</td>
<td>0.96 (0.03)</td>
<td>6.78 (0.07)*</td>
<td>1.01 (0.10)</td>
<td>8.75 (0.17)</td>
<td>1.22 (0.12)</td>
<td>8.57 (0.12)</td>
<td>1.16 (0.10)</td>
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<td>oxybutynin</td>
<td>7.66 (0.03)</td>
<td>1.05 (0.10)</td>
<td>7.32 (0.07)*</td>
<td>1.25 (0.26)</td>
<td>8.39 (0.09)</td>
<td>1.15 (0.09)</td>
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<td>tolterodine</td>
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<td>1.01 (0.02)</td>
<td>8.69 (0.06)</td>
<td>1.01 (0.09)</td>
<td>8.61 (0.02)</td>
<td>0.96 (0.03)</td>
<td>8.37 (0.11)*</td>
<td>1.02 (0.22)</td>
</tr>
<tr>
<td>pirenzepine</td>
<td>6.36 (0.05)</td>
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<td>6.41 (0.02)</td>
<td>0.91 (0.12)</td>
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<td>0.95 (0.11)</td>
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<tr>
<td>methoctramine</td>
<td>8.31 (0.02)</td>
<td>1.64 (0.10)‡</td>
<td>8.13 (0.16)</td>
<td>1.40 (0.06)‡</td>
<td>7.11 (0.03)</td>
<td>1.30 (0.22)‡</td>
<td>6.63 (0.05)*</td>
<td>1.47 (0.03)‡</td>
</tr>
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Figure 6.7

$[^{3}H]-NMS$ competition binding curves at membrane homogenates of CHO-m2 WT [(a) and (c)] and CHO-m2 MUT [(b) and (d)] cell lines for methacholine (MCh) [(a) and (b)] and oxotremorine-M (Oxo-M) [(c) and (d)] in the absence (closed symbols) and presence (open symbols) of GTP (100 μM). Data are presented as mean percent of specific binding in the absence of competitor. Results are expressed as mean ± s.e.m, n ≥ 3.
Figure 6.8

[^H]-NMS competition binding curves at membrane homogenates of CHO-m2 WT [(a) and (c)] and CHO-m2 MUT [(b) and (d)] cell lines for oxotremorine (Oxo) [(a) and (b)] and pilocarpine (Pilo) [(c) and (d)] in the absence (closed symbols) and presence (open symbols) of GTP (100 μM). Data are presented as mean percent of specific binding in the absence of competitor. Results are expressed as mean ± s.e.m, n ≥ 3.
Table 6.3 Competition $[^3H]$-NMS binding data in the absence and presence of GTP (100 μM) for a range of mACh receptor agonists in membrane homogenates prepared from CHO-m2 WT and CHO-m2 MUT cells. Data are expressed as mean (s.e.m.) values from n ≥ 3 experiments. * indicates significant difference between wild-type and mutant (p < 0.05; Student’s t test); † indicates value is significantly different in the presence of GTP (100 μM) (p < 0.05; Student’s t test).

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<th>CHO-m2 WT</th>
<th>CHO-m2 MUT</th>
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<tr>
<td></td>
<td>- GTP</td>
<td>+ GTP</td>
</tr>
<tr>
<td></td>
<td>pK$_H$</td>
<td>pK$_L$</td>
</tr>
<tr>
<td>MCh</td>
<td>6.94 (0.08)</td>
<td>4.94 (0.17)</td>
</tr>
<tr>
<td>Oxo-M</td>
<td>7.92 (0.17)</td>
<td>5.91 (0.25)</td>
</tr>
<tr>
<td>Oxo</td>
<td>8.27 (0.15)</td>
<td>6.02 (0.15)</td>
</tr>
<tr>
<td>Pilo</td>
<td>6.14 (0.22)</td>
<td>4.44 (0.29)</td>
</tr>
</tbody>
</table>

|        | - GTP                       | + GTP                       |
|        | pK$_H$  | pK$_L$  | % high affin | Hill slope | pK$_H$  | pK$_L$  | % high affin | pK$_i$ (1 site fit) | Hill slope |
| MCh    | 7.56 (0.23) | 5.58 (0.07)* | 42.0 (3.0) | 0.47 (0.06) | 6.71 (0.20) | 5.28 (0.13) | 39.7 (7.3) | 5.85 (0.02)* | 0.68 (0.03)‡ |
| Oxo-M  | 7.95 (0.09) | 6.09 (0.09) | 49.7 (5.4) | 0.47 (0.04) | 7.85 (0.15) | 6.13 (0.03) | 29.7 (4.8) | 6.50 (0.11)* | 0.62 (0.03)‡ |
| Oxo    | 8.36 (0.08) | 6.28 (0.07) | 40.3 (5.0) | 0.47 (0.03) | 6.73 (0.17)‡ | 5.34 (0.25)‡ | 64.5 (14.5) | 6.40 (0.06) | 0.76 (0.07)‡ |
| Pilo   | 6.17 (0.27) | 4.62 (0.39) | 54.3 (12.0) | 0.61 (0.01) | 5.20 (0.16) | 4.60 (0.49) | 50.3 (17.6) | 5.20 (0.02) | 0.84 (0.03)‡ |
displayed Hill slopes of significantly less than unity \((p < 0.05)\) and these are also included in Table 6.3 (column 4).

No significant differences in the relative proportions of 'high' and 'low' affinity receptor populations were detected between CHO-m2 WT and CHO-m2 MUT membranes. While MCh recognised the lower affinity binding site \((K_L)\) in CHO-m2 MUT membranes with a significantly higher affinity than in CHO-m2 WT membranes \((p < 0.05)\), the \(pK_H\) estimate for MCh was not significantly different between the two membrane preparations (Table 6.3). Indeed, for all other agonists tested, affinities for both high and low affinity receptor populations were approximately equivalent between CHO-m2 WT and CHO-m2 MUT membrane homogenates (Table 6.3).

As can be seen in Figures 6.7 and 6.8, inclusion of GTP (100 \(\mu\)M) in the assay caused a clear rightward shift and a steepening of the competition binding curve. This was supported by the finding that, when fitted to a 1-site model, the Hill slope estimates in the presence of GTP (100 \(\mu\)M) were significantly greater than those in the absence of GTP \((p < 0.05)\), in all cases except for pilocarpine in CHO-m2 WT membranes (Table 6.3). However, even in the presence of GTP, all of the Hill slopes remained significantly less than unity \((p < 0.05)\), so the corrected binding affinities from 2-site modelling of the curves \((pK_H\) and \(pK_L)\), as well as the proportion of the total number of sites that displayed high affinity binding, are given in columns 5-7 of Table 6.3. A significantly lower proportion of sites were recognised with high affinity by Oxo-M in CHO-m2 WT membranes in the presence of GTP relative to those in the absence of guanine nucleotide \((p < 0.05)\), but in all other cases, the relative proportions were not significantly altered in the presence of GTP (Table 6.3).

In most cases, the affinity constant estimates for both high and low affinity sites did not significantly differ in the presence of GTP either, although significant decreases in affinity were observed in some instances (see Table 6.3). In general (with the exception of Oxo-M), the high affinity sites appeared to be recognised with a slightly reduced affinity by agonists in the presence of guanine nucleotide, but in most cases statistical significance was not achieved (Table 6.3). In contrast, the low affinity population of receptors mostly displayed equivalent affinities for agonist in the absence and presence of GTP.
Columns 8 and 9 of Table 6.3 summarise mean pKᵢ and Hill slope estimates from curves fitted to a 1-site model, in the presence of GTP (100 μM). Both MCh and Oxo-M displayed a significantly higher affinity in CHO-m2 MUT membranes than in those prepared from CHO-m2 WT cells (p < 0.05). In contrast, the partial agonists Oxo and Pilo did not appear to discriminate between wild-type and N410Y mutant receptors expressed in CHO cell membranes (Table 6.3).

6.2.2.5 [³⁵S]-GTPγS binding in membranes expressing wild-type and N410Y mutant M₂ mACh receptors

Figure 6.9 (b) illustrates the mean basal [³⁵S]-GTPγS binding measured in CHO-m2 WT and CHO-m2 MUT cell membrane homogenates and the effect of pre-incubation with atropine (10 μM) on these basal levels. In both CHO-m2 WT and CHO-m2 MUT membranes, similar basal levels of [³⁵S]-GTPγS binding were observed and in each case, atropine failed to significantly reduce the agonist-independent binding (Figure 6.9 (b)).

[³⁵S]-GTPγS binding in response to MCh was measured in membranes prepared from CHO-m2 WT and CHO-m2 MUT cells. The mean concentration-response curves from 4 separate experiments are shown in Figure 6.9 (a). Maximal responses in equivalent quantities of membrane protein were significantly lower in CHO-m2 MUT membranes (9417 ± 607 c.p.m.) relative to those in CHO-m2 WT membranes (18070 ± 2043 c.p.m.), but this might be expected given the lower receptor expression levels in the mutant receptor-expressing cell line. The mean Hill slopes associated with the responses in CHO-m2 WT (0.75 ± 0.03) and CHO-m2 MUT (0.78 ± 0.05) did not significantly differ, but MCh was significantly more potent in stimulating [³⁵S]-GTPγS binding in N410Y mutant receptor-expressing membranes (pEC₅₀ = 6.98 ± 0.18) than those expressing wild-type M₂ mACh receptor (pEC₅₀ = 6.06 ± 0.04) (p < 0.05).
Figure 6.9
(a) Agonist (MCh) - stimulated total [35S]-GTPγS binding at membrane homogenates of CHO-m2 WT (closed symbols) and CHO-m2 MUT (open symbols) cell lines. Data are presented as mean percent stimulation over basal ± s.e.m, n = 3.
(b) Basal [35S]-GTPγS binding at membrane homogenates of CHO-m2 WT and CHO-m2 MUT cells in the absence and presence of atropine (10 μM) pre-incubation. Data are expressed as total [35S]-GTPγS binding (c.p.m.) at equivalent membrane protein concentrations. Results are expressed as mean ± s.e.m, n = 3.
6.2.2.6 Effects of inverse agonist treatment upon basal and forskolin-stimulated cyclic AMP accumulation in CHO cells stably expressing wild-type and N410Y mutant M2 mACh receptors

Basal cyclic AMP accumulation in CHO-m2 WT (1.41 ± 0.21 pmol mg⁻¹ protein; n = 6) and CHO-m2 MUT (1.31 ± 0.16 pmol mg⁻¹ protein; n = 6) cells was not significantly different. However, cyclic AMP accumulation in response to forskolin (10 μM) was significantly lower in CHO-m2 MUT (220 ± 16 pmol mg⁻¹ protein; n = 9) than in CHO-m2 WT (855 ± 126 pmol mg⁻¹ protein; n = 5) cells (p < 0.05), as illustrated in Figure 6.10 (a). This suggested that constitutive activation of G₁o proteins by the N410Y mutant M2 receptor might be tonically inhibiting adenylate cyclase activity, leading to an attenuated forskolin response. If this were the case, it would be anticipated that treatment with an inverse agonist would enhance the forskolin response in the CHO-m2 MUT cell line. Figure 6.10 summarises the effect of pre-treatment with (b) or the simultaneous addition of ((c) and (d)) a range of different mACh receptor antagonists/inverse agonists and a range of concentrations of the inverse agonist atropine (c) with forskolin (3 or 10 μM). However, it is clear that regardless of the choice or concentration of ligand, forskolin concentration or timing of addition, inverse agonist treatment failed to significantly enhance the forskolin-mediated accumulation of cyclic AMP. Figure 6.10 (Inset) illustrates that at high concentrations, methoctramine actually reduced the response to forskolin (3 μM) in CHO-m2 MUT cells.

Figure 6.11 (a) illustrates that at concentrations in excess of 10 μM, oxybutynin was capable of enhancing the forskolin-stimulated accumulation of cyclic AMP in CHO-m2 MUT cells. However, given that the binding affinity of oxybutynin at the N410Y mutant M2 receptor was approx. 50 nM (see Table 6.2), it is unlikely that this effect resulted from a specific action at the receptor. Figure 6.11 (c) indicates that a similar effect was observed in CHO-m2 WT cells, while in the same experiment, atropine (10 μM) failed to have any effect upon cyclic AMP accumulation. It is known that cyclic AMP is actively extruded from many cell types (Orlov & Maksimova, 1999; Bankir et al., 2002), so the possibility that oxybutynin might be interfering in this process was examined. The accumulation of cyclic AMP in the extracellular solution during the course of the experiment was measured and representative results are presented in
Figure 6.10
(a) Forskolin (10 μM)-mediated cAMP accumulation (10 min) in CHO-m2 WT and CHO-m2 MUT cell lines. Data are expressed as pmol cAMP accumulation mg⁻¹ protein.
(b) Effect of inverse agonist pre-incubation upon forskolin (10 μM)-mediated cAMP accumulation in CHO-m2 MUT cells.
(c) Effect of simultaneous addition of a range of concentrations of atropine with forskolin (3 μM) upon cAMP accumulation in CHO-m2 MUT cells.
(d) Effect of simultaneous addition of a range of inverse agonists with forskolin (3 μM) upon cAMP accumulation in CHO-m2 MUT cells.
Inset: Effect of simultaneous addition of a range of concentrations of methoctramine with forskolin (3 μM) upon forskolin-mediated cAMP accumulation in CHO-m2 MUT cells.
Data are presented as mean percent of maximal forskolin response (in absence of inverse agonist) ± s.e.m, n ≥ 3 unless indicated otherwise.
Figure 6.11 (b) and (d). At concentrations (50 and 100 μM) where an elevation in intracellular cyclic AMP accumulation was observed in both CHO-m2 WT and CHO-m2 MUT cells, a pronounced reduction in extracellular accumulation of cyclic AMP was observed in the solutions bathing CHO-m2 MUT (Figure 6.11 (b)) and CHO-m2 WT (Figure 6.11 (d)) cells. In contrast, a lower concentration of oxybutynin (10 μM), as well as a high concentration of atropine (10 μM), was without effect on the forskolin-mediated cyclic AMP accumulation (Figure 6.11 (b) and (d)). This suggests that concentrations of ≥50 μM oxybutynin significantly attenuated the extrusion of cyclic AMP from CHO cells. However, none of the inverse agonists tested significantly enhanced forskolin-stimulated cyclic AMP accumulation via a receptor-dependent mechanism. Additionally, atropine (10 μM) treatment had no effect upon basal cyclic AMP accumulation in either CHO-m2 WT or CHO-m2 MUT cells (data not shown).

6.2.2.7 Agonist-mediated inhibition of and augmentation of forskolin-stimulated cyclic AMP accumulation in CHO-m2 WT and CHO-m2 MUT cell lines

The pharmacology of a range of mACh receptor agonists at CHO-m2 WT and CHO-m2 MUT cells was examined by their ability to inhibit forskolin (10 μM)-stimulated cyclic AMP accumulation. Subsequent to pertussis toxin (PTx) pre-treatment (100 ng ml⁻¹; 20-24 h), the mean cyclic AMP accumulation response to forskolin (10 μM) was significantly attenuated in both CHO-m2 WT (129 ± 16 pmol mg⁻¹ protein; n = 5) and CHO-m2 MUT (56 ± 8 pmol mg⁻¹ protein; n = 8) cell lines (p < 0.05). Incubation with muscarinic agonist (in addition to forskolin) in PTx pre-treated cells caused a concentration-dependent increase in cyclic AMP accumulation, above the level resulting from forskolin treatment alone. It was therefore possible to investigate the ability of both wild-type and N410Y M₂ mACh receptors to couple through Gₛ or G₁₁ο to either augment or inhibit forskolin-stimulated cyclic AMP accumulation, using cells pre-treated with PTx or not treated, respectively. Mean maximal inhibitory (Iₘₐₓ) and stimulatory (Eₘₐₓ) responses (expressed as a percentage of the response to the reference agonist MCh), as well as an indication of the potency of both inhibitory (pIC₅₀) and stimulatory (pEC₅₀) responses for each agonist (in both CHO-m2 WT and CHO-m2 MUT cell lines) are summarised in Table 6.4. Mean concentration-response
Figure 6.11
(a) Concentration-dependent effect of oxybutynin upon forskolin (3 μM)-stimulated intracellular cAMP accumulation (10 min) in CHO-m2 MUT cells. Data are presented as mean percent of maximal forskolin response ± s.e.m. of 2 experiments, each performed in duplicate.
(b) and (d) Effects of atropine and oxybutynin upon forskolin (3 μM)-stimulated extracellular cAMP accumulation (10 min) in KHB bathing CHO-m2 MUT (b) and CHO-m2 WT (d) cells. Data are expressed as mean pmol cAMP mg⁻¹ protein ± s.e.m. and represent a single experiment performed in duplicate.
(c) Concentration-dependent effect of oxybutynin upon forskolin (3 μM)-stimulated intracellular cAMP accumulation (10 min) in CHO-m2 WT cells. Data are expressed as mean pmol cAMP mg⁻¹ protein ± s.e.m. and represent a single experiment performed in duplicate.
Table 6.4 Intact cell apparent binding affinity constant (pKₐ) values and inhibition of (- PTx) and enhancement of (+ PTx) forskolin-stimulated cAMP accumulation in CHO-m2 WT and CHO-m2 MUT cells. Data are expressed as mean (s.e.m.) values from n ≥ 3 experiments. * indicates significant difference between wild-type and mutant (p < 0.05; Student’s t test); † indicates significant difference between pEC₅₀ and pIC₅₀ values (p < 0.05; Student’s t test); # indicates that Eₘₐₓ is significantly less than 100% (p < 0.05; one-way ANOVA, Bonferroni’s post test).

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<td>pEC₅₀</td>
<td>Eₘₐₓ (%)</td>
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<td>68.3 (5.4) #</td>
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<td>Iₘₐₓ (%)</td>
<td>pEC₅₀</td>
<td>Eₘₐₓ (%)</td>
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<td>eᵣₑ (Gᵢ)</td>
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<td>37.2</td>
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<tr>
<td>Oxo-M</td>
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<td>7.79 (0.10)*</td>
<td>104.6 (2.5)</td>
<td>6.53 (0.04)* †</td>
<td>104.0 (3.8)</td>
<td>18.2</td>
<td>20.9</td>
<td>1.63</td>
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<tr>
<td>Oxo</td>
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<td>7.76 (0.06)*</td>
<td>103.5 (0.1)</td>
<td>5.82 (0.09)* †</td>
<td>66.8 (5.9) # *</td>
<td>87.1</td>
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<td>33.0 (6.0) # *</td>
<td>8.7</td>
<td>14.8</td>
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curves for MCh (Figure 6.12), Oxo-M (Figure 6.13), Oxo (Figure 6.14) and Pilo (Figure 6.15) are also presented in both CHO-m2 WT and CHO-m2 MUT cells, for both inhibitory and excitatory responses.

In all cases, with the exception of Pilo in CHO-m2 WT cells, the stimulatory cyclic AMP response exhibited a significantly lower potency for activation by muscarinic agonist than the corresponding inhibitory response ($p < 0.05$). This is summarised in Table 6.4 as EC$_{50}$/IC$_{50}$ ratios for each agonist and demonstrates that the ratio was generally lower for Pilo than for the other agonists investigated. However, since the stimulatory response to Pilo was particularly small, the accuracy of estimates of potency for this response is questionable, suggesting that such data should be interpreted with caution. Oxo displayed a particularly large disparity between potencies for the inhibition and stimulation of cyclic AMP accumulation respectively (EC$_{50}$/IC$_{50}$ ratio = 87.1) in CHO-m2 MUT cells. Also, whereas Pilo was the only agonist to produce a maximal inhibitory response that was significantly less than that observed for MCh (in both CHO-m2 WT and MUT cells), both Pilo and Oxo activated sub-maximal stimulatory responses ($p < 0.05$) (Table 6.4). However, in CHO-m2 MUT cells both Oxo and Pilo were significantly more efficacious in stimulating cyclic AMP accumulation than in CHO-m2 WT cells ($p < 0.05$).

Comparing the potencies of agonist-mediated inhibitory and stimulatory responses, some clear differences between CHO-m2 WT and MUT cell lines are apparent (Table 6.4). All four agonists tested exhibited significantly higher potency for the inhibition of cyclic AMP accumulation in CHO-m2 MUT cells than in CHO-m2 WT cells ($p < 0.05$). However, for the stimulatory response, only the full agonists MCh and Oxo-M displayed a significantly higher potency in N410Y mutant M2 receptor-expressing cells than in the wild-type-expressing cell line. While the rank order of potency (shown below) for the inhibitory (G$_i$) response was essentially similar between wild-type and N410Y mutant-expressing cell lines, a slight difference in the order of MCh and Oxo potencies was observed between CHO-m2 WT and MUT cell lines for the stimulatory (G$_s$) response. In the wild-type receptor-expressing cells, Oxo was significantly more potent than MCh for the stimulation of cyclic AMP accumulation ($p < 0.05$), but for the same response in CHO-m2 MUT cells, MCh was marginally (but statistically insignificantly) more potent than Oxo.
Figure 6.13
Concentration response curves for Oxotremorine-M (Oxo-M)-mediated inhibition or stimulation of forskolin-stimulated cyclic AMP accumulations in CHO-m2 WT (closed symbols) and CHO-m2 MUT (open symbols) cells with (stimulation) or without (inhibition) pre-treatment with PTx (100 ng ml⁻¹) for 20-24 h. Results are expressed as mean percent inhibition or stimulation, relative to that of MCh, of cyclic AMP accumulation. Data are expressed as mean ± s.e.m., n ≥ 3.
Figure 6.14
Concentration response curves for Oxotremorine (Oxo)-mediated inhibition or stimulation of forskolin-stimulated cyclic AMP accumulations in CHO-m2 WT (closed symbols) and CHO-m2 MUT (open symbols) cells with (stimulation) or without (inhibition) pre-treatment with PTx (100 ng ml\(^{-1}\)) for 20-24 h. Results are expressed as mean percent inhibition or stimulation, relative to that of MCh, of cyclic AMP accumulation. Data are expressed as mean ± s.e.m., n ≥ 3.
Figure 6.15
Concentration response curves for Pilocarpine (Pilo)-mediated inhibition or stimulation of forskolin-stimulated cyclic AMP accumulations in CHO-m2 WT (closed symbols) and CHO-m2 MUT (open symbols) cells with (stimulation) or without (inhibition) pre-treatment with PTx (100 ng ml⁻¹) for 20-24 h. Results are expressed as mean percent inhibition or stimulation, relative to that of MCh, of cyclic AMP accumulation. Data are expressed as mean ± s.e.m., n ≥ 3.
Rank order of potency

CHO-m2 WT (G\text{\alpha}o) : Oxo-M ≥ Oxo > MCh > Pilo
CHO-m2 WT (G\text{\alpha}) : Oxo-M ≥ Oxo > MCh > Pilo
CHO-m2 MUT (G\text{\alpha}o) : Oxo-M ≈ Oxo ≥ MCh > Pilo
CHO-m2 MUT (G\text{\alpha}) : Oxo-M > MCh ≥ Oxo > Pilo

Also included in Table 6.4 are 'relative efficacy' (e\text{rel}) estimates for each agonist, calculated according to the method of Ehlert (1985) (see Chapter 2: Materials and Methods). Since these estimates take into account receptor occupancy in addition to the potency displayed by an agonist in mediating a given response, it is possible to argue that this index provides a more valid comparison of the relative activity of an agonist in activating specific receptor-G-protein interactions. The generally lower potency and E\text{max} values observed for the stimulatory responses were reflected in the low relative efficacy values derived for the G\text{\alpha}-coupled response in both wild-type and N410Y mutant receptor-expressing cells (Table 6.4). The rank orders of relative efficacy (given below) indicate some differences, both between CHO-m2 WT and MUT cell lines and between G\text{\alpha}o- and G\text{\alpha}-mediated responses within the CHO-m2 MUT cell line.

Rank order of relative efficacy

CHO-m2 WT (G\text{\alpha}o) : Oxo-M > MCh >> Oxo > Pilo
CHO-m2 WT (G\text{\alpha}) : Oxo-M ≥ MCh > Oxo ≈ Pilo
CHO-m2 MUT (G\text{\alpha}o) : MCh > Oxo-M ≥ Oxo > Pilo
CHO-m2 MUT (G\text{\alpha}) : Oxo-M ≥ MCh > Pilo > Oxo

Comparing the inhibitory responses of wild-type and N410Y mutant receptor-expressing cells, there was a clear switch in the order of the relative efficacies of MCh and Oxo-M. Whereas Oxo-M (31.9) was more efficacious than MCh (25.0) in the wild-type cell line, the relative efficacy estimate for MCh (32.1) was greater than that for Oxo-M (20.9) in the CHO-m2 MUT cells. For the stimulatory responses, it was for the partial agonists Pilo and Oxo that a slight difference was observed between
CHO-m2 WT and MUT cell lines. In the N410Y mutant receptor-expressing cells, Pilo (0.82) was actually more efficacious than Oxo (0.47), whereas the relative efficacy estimates in CHO-m2 WT cells were virtually indistinguishable for Pilo (0.22) and Oxo (0.24) (Table 6.4). The combination of these changes in inhibitory and stimulatory response relative efficacy orders rendered the overall rank orders of efficacy for inhibitory and stimulatory responses in the CHO-m2 MUT cells quite different (see above).

6.2.2.8 \[^{3}H\]-NMS binding in intact CHO-m2 WT and CHO-m2 MUT cells

Apparent binding affinity constant (pK_a) estimates for each of the agonists investigated were determined in \[^{3}H\]-NMS competition binding assays at 4°C and the mean values from 3 or more experiments are given in Tables 6.4 and 6.5. MCh, Oxo-M and Oxo all bound with significantly higher affinity to CHO-m2 MUT cells than to CHO-m2 WT cells, but Pilo displayed similar affinity for both cell lines (Table 6.5). The mean Hill slopes (none of which differed significantly from unity), associated with the competition binding curves for each agonist, are given in Table 6.5. Figure 6.16 illustrates the mean competition binding curves for each agonist at CHO-m2 WT (a) and CHO-m2 MUT (b) cells.

In the calculation of the relative efficacy values given in Table 6.4, it was assumed that the binding affinity of the muscarinic agonists was not affected by pre-treatment with PTx, as the K_a values used in the derivation of relative efficacy estimates for both inhibitory and stimulatory responses were determined in cells not pre-treated with PTx. The validity of this assumption was confirmed by determining apparent affinity binding constant estimates (pK_a) for MCh and Oxo in cells pre-treated with PTx. Mean competition binding curves for these agonists in CHO-m2 MUT cells, with or without PTx pre-treatment are illustrated in Figure 6.17 and the corrected mean pK_a estimates are included in Table 6.5. For both MCh and Oxo, pK_a estimates did not differ significantly in CHO-m2 MUT cells pre-treated with PTx, compared with cells not treated with the toxin, and the associated mean Hill slopes were equivalent to unity in each case (Table 6.5).

It is clear from Figure 6.17, however, that in the absence of agonist, the specific \[^{3}H\]-NMS binding (expressed as a percentage of that in cells not treated with PTx) was
Table 6.5 Competition \(^{3}H\)-NMS binding data in intact CHO-m2 WT and CHO-m2 MUT cells with or without PTx pre-treatment (20-24 h). Data are expressed as mean (s.e.m.) values from \( n \geq 3 \) experiments. * indicates significant difference between wild-type and mutant \((p < 0.05; \) Student's \( t \) test).

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<td>5.92 (0.07)</td>
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<td>*</td>
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</table>
Figure 6.16
\[^{3}H\]-NMS competition binding curves at intact CHO-m2 WT and CHO-m2 MUT cells, for the mACh receptor agonists MCh, Oxo-M, Oxo and Pilo. Data are expressed as mean percent control specific binding ± s.e.m., n ≥ 3.
Figure 6.17
Effect of PTx pre-treatment (100 ng ml⁻¹; 20-24 h) upon [³H]-NMS competition binding curves at intact CHO-m2 MUT cells for the mACh receptor agonists MCh (a) and Oxo (b). Data are expressed as mean percent of specific binding in cell not pre-treated with PTx ± s.e.m., n ≥ 3.
significantly lower in PTx-treated cells. This was confirmed in [³H]-NMS saturation binding assays in CHO-m2 MUT cells with and without PTx pre-treatment ($p < 0.05$) (Figure 6.18 (b)). In contrast, PTx pre-treated CHO-m2 WT cells expressed similar levels of $M_2$ mACh receptor to those cells not treated with toxin (Figure 6.18 (a)). Receptor expression ($B_{max}$) and [³H]-NMS binding affinity ($K_D$) estimates in CHO-m2 WT and MUT cell lines with and without PTx pre-treatment are summarised in Table 6.6. PTx pre-treatment had no effect on the affinity of [³H]-NMS for either wild-type or N410Y mutant $M_2$ mACh receptors, although the radioligand displayed a slightly (but statistically significantly) higher affinity in CHO-m2 MUT cells relative to CHO-m2 WT cells ($p < 0.05$) (Table 6.6).

Pertussis toxin therefore appeared to selectively reduce the cell surface expression of N410Y mutant $M_2$ mACh receptors expressed in CHO cells, without influencing the expression of wild-type $M_2$ mACh receptors expressed in the same cell background. If this were a reflection of the enhanced constitutive activity (and perhaps decreased stability) of the N410Y mutant receptor, it might be anticipated that the effect would be reversed by the interaction of an inverse agonist with the mutant receptor. Both wild-type and N410Y mutant $M_2$ mACh receptor expression was therefore monitored in cells exposed to PTx in the absence and presence of atropine (300 nM), as well as in response to atropine (300 nM) alone. The mean data from 3 or more similar experiments are summarised in Table 6.6 and Figure 6.18 (c). Atropine slightly, but insignificantly, enhanced the expression level in CHO-m2 MUT cells when present alone, while PTx alone significantly reduced the cell surface expression of the N410Y mutant $M_2$ mACh receptor ($p < 0.05$). The inclusion of atropine (300 nM) in addition to PTx failed to significantly attenuate the effect of PTx. No significant effects upon receptor expression in CHO-m2 WT cells were observed with any of the treatments.
Figure 6.18
Effect of PTx pre-treatment (100 ng ml^{-1}; 20-24 h) on mACH receptor expression levels in CHO-m2 WT (a) and CHO-m2 MUT (b) cells, assessed by \(^{3}H\)-NMS saturation binding analysis in intact cells. Data points were performed in duplicate and curves shown are representative of 3 or more experiments.

(c) Effect of incubating atropine (300 nM) alone or in combination with PTx (100 ng ml^{-1}) for 24 h upon mACH receptor expression levels in CHO-m2 WT and CHO-m2 MUT cells. Results are expressed as mean specific binding in fmol receptor expression mg^{-1} protein ± s.e.m., n ≥ 3.
Table 6.6 \([^3]H\)-NMS binding affinity constant (K_0) and receptor expression (B_{max}) estimates at intact CHO-m2 WT and CHO-m2 MUT cells with or without PTx (100 ng ml\(^{-1}\); 20-24 h) and atropine (300 nM; 24 h) pre-treatment. Data are expressed as mean (s.e.m.) values from n ≥ 3 experiments. * indicates significant difference between wild-type and mutant (p < 0.05; Student’s t test); † indicates significant difference between un-treated and PTx pre-treated cells (p < 0.05; Student’s t test).

<table>
<thead>
<tr>
<th></th>
<th>K_0 (nM)</th>
<th>B_{max} (fmol mg(^{-1}) protein)</th>
<th>- atropine</th>
<th>+ atropine</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHO-m2 WT</td>
<td>0.22 (0.02)</td>
<td>1234.8 (62.1)</td>
<td>1197.0 (28.0)</td>
<td></td>
</tr>
<tr>
<td>CHO-m2 WT + PTx</td>
<td>0.21 (0.02)</td>
<td>1359.3 (69.7)</td>
<td>1262.5 (41.0)</td>
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</tr>
<tr>
<td>CHO-m2 MUT</td>
<td>0.16 (0.004) *</td>
<td>637.1 (52.5)</td>
<td>725.0 (89.9)</td>
<td></td>
</tr>
<tr>
<td>CHO-m2 MUT + PTx</td>
<td>0.16 (0.01)</td>
<td>426.9 (46.8) †</td>
<td>475.9 (94.5)</td>
<td></td>
</tr>
</tbody>
</table>
6.3 Discussion

This study has detailed the pharmacological characterisation of CHO cell lines stably expressing wild-type (CHO-m2 WT) and N410Y constitutively active mutant (CAM) (CHO-m2 MUT) \( M_2 \) mACh receptors. Although the N410Y mutant \( M_2 \) receptor has previously been demonstrated to possess a greater tendency to spontaneously activate G-proteins in the absence of agonist stimulation (see Chapter 5), constitutive activity was not consistently observed in CHO-m2 MUT cells, assayed by either cyclic AMP accumulation or \(^{35}\)S-GTP\(_y\)S binding. However, several features consistent with a constitutively activating mutation were confirmed in this cell model, including enhanced agonist binding affinity and potency in functional assays. Increased efficacy of muscarinic partial agonists was also observed in some cases at the N410Y mutant receptor-expressing cells.

6.3.1 Investigation of the constitutive activity in CHO-m2 WT and CHO-m2 MUT stable cell lines

Initial functional characterisation of the selected CHO-m2 WT and CHO-m2 MUT clones demonstrated that the N410Y mutant receptor-expressing cell line exhibited a significantly lower level of cyclic AMP accumulation in response to treatment with the adenylate cyclase activator forskolin. Theoretically, this might be expected in a cell line expressing constitutively active \( G_{ib} \)-protein-coupled receptors, when compared with an identical cell background in which the wild-type \( M_2 \) mACh receptor is expressed. Indeed, agonist-independent inhibition of adenylate cyclase activity in cells expressing either wild-type (e.g. Shryock et al., 1998) or CAM (Ren et al., 1993; Wade et al., 2001) \( G_{ib} \)-coupled receptors has been reported. Notably, Wade et al. (2001) observed that CHO cells stably expressing a constitutively active mutant \( \alpha_2A \)-adrenoceptor exhibited a cyclic AMP production in response to forskolin of approx. 20-25 % of that measured in wild-type or mock-transfected cells, providing strong evidence that the CAM receptor was tonically inhibiting adenylate cyclase and thereby reducing it responsiveness to forskolin.

However, to demonstrate that the reduced forskolin response in CHO-m2 MUT cells was due to the constitutive activity of the mutant \( M_2 \) receptor population, cells were
treated with the inverse agonist atropine in an attempt to reverse the constitutive inhibitory influence upon adenylate cyclase. The failure to detect any significant effects upon forskolin-stimulated cyclic AMP accumulation with atropine or a range of other putative inverse agonists suggests that the difference in forskolin response is not related to differences in M2 receptor constitutive activity. Even when using a range of different timings of inverse agonist addition (i.e. addition before, after and simultaneously with forskolin) and lower forskolin concentrations, none of the muscarinic inverse agonists tested significantly increased cyclic AMP accumulation across concentration ranges at which they would be expected to bind M2 receptors. It is possible that the difference in forskolin responses between cell lines merely reflects clonal variation in either the quantity or forskolin-sensitivity of adenylate cyclase present. However, a number of N410Y mutant receptor-expressing cell lines displayed comparable forskolin responses to those observed in mutant clone # 87 (CHO-m2 MUT), when originally screened for functional activity (data not shown). Additionally, it is unlikely that the forskolin response of the CHO-m2 WT cell line selected for further study was anomalously high, since the CHO-m2 cell line, routinely used by our lab and others, exhibits a similar level of cyclic AMP accumulation in response to forskolin.

Methoctramine did appear to influence forskolin-mediated cyclic AMP accumulation in CHO-m2 MUT cells, but by inhibiting the forskolin response and only at concentrations of ≥10 μM. Previous reports have suggested that at concentrations of > 5 μM, methoctramine is capable of activating phosphoinositide hydrolysis independently of muscarinic receptors, in a manner that is sensitive to PTx treatment (Lee et al., 1989; Chahdi et al., 1998). Daeflner et al. (1999b) subsequently examined the effects of micromolar concentrations of methoctramine upon GTPase activity in pig sarclemma and found methoctramine to inhibit GTPase activity at these concentrations. Based upon these data and the polyamine structure of methoctramine, it has been proposed that at micromolar concentrations, methoctramine interacts with activated G_iw-proteins and, by inhibiting the intrinsic GTPase activity of the G-protein α-subunit, prevents its re-association with the βγ-subunit (Daeflner et al., 1999b). In CHO-m2 MUT cells, therefore, high micromolar concentrations of methoctramine might actually be enhancing a modest activation of G_iw-proteins, leading to the observed decrease in cyclic AMP accumulation.
Oxybutynin was also observed to influence the forskolin response at high micromolar concentrations, enhancing the cyclic AMP accumulation in response to forskolin treatment in both CHO-m2 WT and CHO-m2 MUT cells. Similar concentrations of oxybutynin were found to reduce the extracellular accumulation of cyclic AMP, so it seems likely that the effect of oxybutynin is to inhibit the extrusion of cyclic AMP from CHO cells, rather than to influence adenylate cyclase activity. The active transfer of cyclic AMP out of a variety of cell types has been reported and is believed to occur via an organic acid co-transporter (Orlov & Maksimova, 1999; Bankir et al., 2002). Several other agents have been reported to inhibit this cyclic AMP extrusion pathway, including prostaglandins (Rindler et al., 1978; Brunton & Mayer, 1979) and probenacid (Davoren & Sutherland, 1963) so it is possible that oxybutynin might be acting similarly. However, oxybutynin only significantly increased intracellular cyclic AMP at concentrations of > 10 μM, while therapeutic levels of oxybutynin are in the nanomolar range, so effects upon the active transport of cyclic AMP and other ions are unlikely to have significant clinical implications.

The failure to observe enhanced basal levels of [³⁵S]-GTPγS binding in membranes prepared from CHO-m2 MUT cells than in the equivalent membranes prepared from CHO-m2 WT cells indicates that the N410Y mutant receptor might not be causing a significant level of G-protein activation in these cells. Although similar basal levels of [³⁵S]-GTPγS binding in equivalent quantities of membrane homogenate prepared from CHO-m2 MUT cells expressing approx. 50% of the muscarinic receptor expression level in CHO-m2 WT cells might be interpreted as representing a significantly higher level of constitutive G-protein coupling, the inability of atropine to concentration-dependently reduce the basal [³⁵S]-GTPγS binding in CHO-m2 MUT membranes would suggest otherwise. Evidence from the [³⁵S]-GTPγS binding assay is therefore consistent with that presented from the cyclic AMP accumulation assay, particularly in the failure to demonstrate inverse agonism at the CAM M₂ receptor.

6.3.2 The role of receptor expression levels in the constitutive activity of the N410Y mutant M₂ mACh receptor

It is possible that the level of constitutive activity in the CHO-m2 MUT cell line was lower than that observed when the receptor was transiently expressed as a result of the
moderate expression level of N410Y receptor in CHO-m2 MUT cells. It is well established that constitutive activity is proportional to receptor density (de Ligt et al., 2000) and this has been verified for other CAM mACh receptors (Ford et al., 2002). Receptor expression level in intact CHO-m2 MUT cells was estimated to be approx. 600 fmol mg⁻¹ protein, compared with levels of between 1 and 2 pmol mg⁻¹ protein in experiments performed with transiently expressed mutant receptor (see Chapter 5). However, the latter estimate is likely to be a substantial under-estimation of the receptor expression in those cells successfully transfected with receptor (since less than 50% of the total cell population is likely to be expressing receptor protein). Additionally, G-protein (Goq2) was over-expressed in the transient expression system and this is known to enhance the constitutive activity of muscarinic receptors (Burstein et al., 1997). It is therefore likely that this would have augmented the level of constitutive activity in CHO cells transiently expressing the N410Y mutant M₂ receptor, an influence that would have been lacking in the CHO-m2 MUT cell line.

It is interesting to note that an expression level of approx. 19 pmol mg⁻¹ protein was measured for the CAM α₁₃-adrenoceptor expressed stably in CHO cells (Wade et al., 2001). In contrast, Ford et al. (2002) reported significant constitutive activity in all five muscarinic receptor subtypes upon transient expression of < 1 pmol mg⁻¹ protein (although expression levels were not determined for the M₂ receptor mutant). While such comparisons are likely to be compromised by differences in cell background and receptor subtype, it does seem likely that higher expression levels might be required to observe detectable constitutive activity in stable expression systems. It might, therefore, have been preferable to use a stable cell line expressing higher levels of N410Y mutant M₂ receptors, to facilitate the detection of constitutive activity.

However, although a greater proportion of mutant receptor-transfected clones (relative to wild-type receptor-transfected cells) expressed significant levels of muscarinic receptor in the primary screen, upon detailed analysis it was found that only one of these clones expressed in excess of 1 pmol mg⁻¹ protein (clone # 96). Preliminary functional characterisation of mutant clone # 96 (approx. 3-4 pmol mg⁻¹ protein) revealed a lack of inhibitory cyclic AMP response to methacholine (data not shown). In fact, at higher levels of stimulation, a modest enhancement of forskolin-stimulated cyclic AMP accumulation was observed, but this was not large enough to permit further analysis.
6.3.3 The influence of passage and cell preparation upon receptor expression levels in CHO-m2 WT and CHO-m2 MUT cells

The paucity of highly expressing N410Y mutant clones might suggest that higher levels of CAM receptor expression were not well tolerated by the cell, causing adaptive changes in the extent of mutant receptor expression. Numerous previous studies have observed lower levels of CAM receptor expression than wild-type upon both transient (Rasmussen et al., 1999; Alewijnse et al., 2000; Pauwels & Tardif, 2002; Zeng et al., 2003) and stable (Pei et al., 1994; Stevens et al., 2000; Huang et al., 2001; Li et al., 2001) expression. Evidence from the transient expression of the N410Y mutant M2 mACh receptor (see Chapter 5) indicates that this constitutively active mutant is expressed at significantly lower levels than the wild-type M2 receptor, so it might be expected that persistent expression of the receptor might not yield high levels of cell surface receptor expression. Expression levels in membranes prepared from CHO-m2 WT and MUT cell lines over a range of different passages were assayed. In CHO-m2 WT cells, the M2 receptor expression appeared to increase in proportion to the passage (at least up to P33), perhaps as a result of the continued selection pressure (occurring in the presence of G418) in favour of those cells expressing the pcDNA3- M2 plasmid. In contrast, CHO-m2 MUT cells expressed slightly (but statistically insignificantly) lower levels of N410Y mutant M2 receptor with increasing passage, in spite of facing the same selection pressure as CHO-m2 WT cells. A rapid, significant decrease in CAM M3 receptor expression was observed in the CHO-m3 MUT stable cell line, which is consistent with the apparently greater de-stabilising effect of the N514Y mutation in the M3 receptor subtype than of the homologous mutation in the M2 receptor (i.e. the greater level of constitutive activity observed in the CAM M3). Nonetheless, these data suggest that the stably expressed N410Y M2 mACh receptor might be under the influence of two opposing effects: the selection-driven increase in receptor expression, as observed for the wild-type M2 receptor, and a down-regulation of receptor expression resulting from the constitutive activation and/or the structural instability of the CAM M2 receptor. However, overnight (24 h) treatment with atropine failed to significantly increase cell surface receptor expression in intact CHO-m2 MUT (or CHO-m2 WT) cells, in contrast to the robust up-regulation observed in CHO-K1 cells transiently expressing CAM M2.
6.3.4 The effect of PTx pre-treatment upon receptor expression levels in CHO-m2 WT and CHO-m2 MUT cells

Another interesting observation relating to receptor expression levels was the effect of pertussis toxin (PTx) pre-incubation upon the apparent cell surface muscarinic receptor expression in CHO-m2 MUT cells. 20-24 h incubation with PTx significantly reduced the level of N410Y mutant M2 mACh receptor expression in these cells, while similar treatment was without effect upon the wild-type M2 receptor. This suggested that the effect of PTx was somehow related to the influence of the mutation upon the M2 mACh receptor. Perhaps by uncoupling the receptor from G_{i/o}-proteins, PTx treatment further de-stabilised the CAM M2 receptor structure, leading to an enhanced susceptibility to degradation. If this were the case, it might be anticipated that co-incubation with inverse agonist might counteract the de-stabilisation and 'rescue' CAM M2 receptor expression. However, similar levels of receptor expression were observed upon PTx treatment in the presence or absence of atropine, suggesting that either atropine is not capable of sufficiently stabilising the receptor structure, or that the effect of PTx treatment upon receptor expression is not related to either constitutive activity or structural instability of the N410Y mutant M2 mACh receptor.

There is little precedent for such an effect of PTx treatment upon the expression of wild-type or CAM GPCR. Indeed, Roseberry et al. (2001) reported that pre-treatment of HEK-293 cells stably expressing M2 mACh receptors with PTx caused a significant delay in the onset of agonist-induced internalisation of the M2 receptor, without affecting the overall extent of internalisation. PTx pre-treatment caused a modest increase in receptor expression and also facilitated an agonist-mediated recruitment of intracellular receptors to the cell surface upon agonist treatment (Roseberry et al., 2001). Importantly, no effect of PTx was observed upon the level or rate of constitutive internalisation; i.e. in the absence of agonist treatment (Roseberry et al., 2001). Although these findings suggest the opposite effect of PTx upon the cell surface expression levels of agonist-activated M2 mACh receptor to those findings in the present study with a constitutively active M2 receptor, both studies found no effect
upon quiescent receptor trafficking, suggesting similarities in the mechanisms occurring in each case. Perhaps differences in cell background, the availability of intracellular pools of receptor or the adaptive effects of long-term activation of the CAM receptor might underlie the apparently opposing results of PTx pre-treatment in these two studies.

If the constitutively active receptor were being down-regulated as a result of its persistent activation of G\textsubscript{io}-proteins, it would be anticipated that the uncoupling of the receptor from G\textsubscript{io} (as will occur upon PTx pre-treatment) might, if anything, cause an increase in the cell surface receptor expression. If the failure of atropine to reverse the down-regulation of receptor expression in response to PTx indicates that receptor instability is not responsible for the loss of receptor expression, a more complex mechanism is implied. There is some evidence in the literature that G\textsubscript{io}-proteins might have alternative roles in the endocytosis/intracellular trafficking of proteins (Helms, 1995; Nürnberg & Ahnert-Hilger, 1996). PTx sensitive G-proteins have been localised to the endoplasmic reticulum, Golgi complex, endosomes and the membranes of secretory granules (see Helms, 1995) and experiments with PTx-treated CD8 cells indicated that G\textsubscript{io}-proteins stimulate or facilitate the re-distribution of the water channel aquaporin 2 (AQP2) from an intracellular compartment to the apical membrane (Valenti et al., 1998). It is therefore conceivable that the effect of PTx might be to inactivate intracellular G\textsubscript{io}-proteins involved in the constitutive shuttling of CAM M\textsubscript{2} receptors to the plasma membrane. The inhibition of a pathway replenishing the cell surface expression of CAM M\textsubscript{2} receptors could explain the lower apparent expression of the N410Y mutant M\textsubscript{2} mACh receptor at the plasma membrane in cells pre-treated with PTx. However, in the absence of direct experimental evidence for such a role for G\textsubscript{io}-proteins in CHO cells, this remains purely speculative.

6.3.5 Effect of the N410Y mutation upon agonist binding affinity at M\textsubscript{2} mACh receptors

Despite the inability to demonstrate significant constitutive activity in the stable cell model, this system did permit the extensive characterisation of agonist binding affinities and functional potencies. The extended ternary complex model (ETC)
predicts both of these properties to be enhanced at a constitutively active receptor (relative to wild-type) and this has been experimentally confirmed for a wide variety of CAM GPCRs (e.g. Samama et al., 1993; Hwa et al., 1997; Ford et al., 2002). In the present study, apparent binding affinity estimates for the muscarinic agonists methacholine, oxotremorine-M and oxotremorine were significantly higher at intact CHO-m2 MUT than at CHO-m2 WT cells. As would be predicted by the ETC model (Samama et al., 1993), the agonists investigated in this study displayed increases in apparent binding affinity for the CAM M₂ receptor that approximately correlated with the intrinsic activity of the agonists, with no increase evident for the weak partial agonist pilocarpine, a small increase apparent for the partial agonist oxotremorine and the largest increases observed for the full agonists methacholine and oxotremorine-M.

However, data obtained from experiments performed in membrane homogenates of the same cell lines suggest a more complex situation. Competition radioligand binding curves for each of the four agonists at both CHO-m2 WT and MUT cell membranes were fitted significantly better by a two-site model, with high and low affinity sites differing in binding affinity by 50-200 fold. However, comparing these binding affinity estimates between CHO-m2 WT and CHO-m2 MUT cell membranes, it is clear that, with the exception of methacholine, the muscarinic agonists investigated here displayed almost identical affinities in CHO-m2 WT and MUT membranes, in terms of both high and low affinity sites. Methacholine was the only agonist that displayed significantly higher affinity in CHO-m2 MUT membrane homogenates than in CHO-m2 WT membranes. This would suggest that the nature of the high and low affinity binding sites, which might be expected to represent G-protein-coupled and un-coupled receptors, is not substantially altered by the N410Y mutation, with only methacholine discerning a conformational difference in the low affinity site to a statistically significant level. This is consistent with a model in which the N410Y mutation increases the extent to which receptors functionally couple to G-proteins, but does not influence the conformation with which they do so. However, in such a model it might be expected that the proportion of high affinity sites might be greater in the mutant receptor population, but this is not the case in the present study, where if anything, a smaller (though not statistically significantly different) proportion of the total population of N410Y mutant receptors than wild-type receptors were bound with high affinity by agonist.
These observations therefore pose the question of what the high and low affinity populations actually represent in each cell line. This was addressed by examining the competition radioligand binding curves in the presence of guanosine triphosphate (GTP), which would be anticipated to uncouple receptors from their cognate G-proteins, as has previously been reported for the M2 mACh receptor (Berrie et al., 1979). In agreement with previous studies comparing the effects of guanine nucleotides upon competition binding curves (e.g. Berrie et al., 1979; Vanderheyden et al., 1990), those generated in the presence of GTP at both CHO-m2 WT and MUT cell membranes were shifted to the right and were mostly significantly steeper than the corresponding curves in the absence of GTP. However, in most cases, Hill slopes associated with the competition binding curves remained significantly less than unity even in the presence of guanine nucleotides, suggesting that either GTP at the concentration used in these studies (100 µM) was not sufficient to fully uncouple receptor-G-protein complexes, or that some of the complexity in the binding curves is independent of G-protein coupling. In support of the former explanation, Huang et al. (1998) reported that while in the presence of 100 µM GppNHp (a non-hydrolysable GTP analog) the competition binding curves for acetylcholine and arecoline at the (Ser388Tyr, Thr389Pro) mutant M1 mACh receptor were fitted best with a one-site model, that of choline retained a two-site binding profile. Increasing the concentration of GppNHp to 200µM, however, produced a competition binding curve for choline that exhibited a one-site binding profile (Huang et al., 1998b). However, the use of the non-hydrolysable GTP analog, GTPγS (100 µM), failed to elicit more significant shifts in the agonist binding curves of the M2 mACh receptors studied here (data not shown), indicating that GTP hydrolysis was not reducing the effectiveness of GTP to uncouple receptor-G-protein complexes.

In a later study by Huang and colleagues, it was found that the binding of acetylcholine to the high affinity binding site at the Ser388Tyr mutant M1 mACh receptor (the homologous mutant to the N410Y M2 receptor) was insensitive to even 400 µM GTP pre-treatment (Huang et al., 1999). Numerous other examples of guanine-nucleotide insensitive (or partially sensitive) multiple site competition binding curves have been reported at constitutively active mutant GPCRs (Hwa et al., 1997; Alewijnse et al., 2000; Huang et al., 2001) and receptors exhibiting enhanced agonist binding affinity and potency (Malmberg & Strange, 2000). In light of these...
reports in receptor mutants displaying similar characteristics to the N410Y mutant M2 receptor, it is interesting to note that the effect of GTP upon the steepness and complexity of the agonist competition binding curves presented herein, was less pronounced in the CHO-m2 MUT cell membranes than in those prepared from CHO-m2 WT cells. This is particularly evident when comparing the apparent shifts in the presence of GTP between CHO-m2 WT and CHO-m2 MUT curves for any given agonist (particularly the full agonists methacholine and oxotremorine-M). It is therefore conceivable that the N410Y mutation facilitated the inter-conversion of the M2 receptor between multiple affinity states, even in the absence of G-protein coupling.

6.3.6 Effect of the N410Y mutation upon inverse agonist binding affinity at M2 mACh receptors

As discussed in Chapter 5, the ETC model predicts that inverse agonists would be anticipated to display lower affinities at CAM receptors than at the corresponding wild-type receptors (Samama et al., 1993; Strange, 2002). However, in many experimental systems such affinity shifts have not been observed (Kjelsberg et al., 1992; Ren et al., 1993; Ford et al., 2002) and more detailed theoretical analysis suggests that it is only when a significant proportion of receptors are present in the active state that detectable shifts in inverse agonist potency will be observed (Wade et al., 2001; Strange, 2002). In the present study, it was found that, of the six inverse agonists tested, only darifenacin and oxybutynin displayed significantly different affinities for membranes derived from CHO-m2 WT and CHO-m2 MUT cells. Although not achieving statistical significance, a small difference in binding affinity was observed in some other cases and it is possible that such small (< 2-fold) shifts might represent quite substantial populations of active receptor in the CHO-m2 MUT membranes, but clearly demonstrates the lack of sensitivity of such radioligand binding assays in detecting inverse agonism.

In contrast, four out of the six ligands exhibited significantly lower affinity for CHO-m3 MUT membranes than for those prepared from CHO-m3 WT cells. This would suggest that a relatively small proportion of receptors exist in the active state even in CHO-m2 MUT cells and provides further evidence that the N410Y mutation in the
M2 receptor has a less severe effect upon the equilibrium between inactive and active receptor species than the homologous mutation in the M3 mACh receptor. The data obtained at the CAM M2 and M3 receptors with darifenacin appear contradictory, in that darifenacin displays the greatest shift in binding affinity between wild-type and CAM M2 receptors, but only a modest, statistically insignificant shift between wild-type and CAM M3 mACh receptors. This could be interpreted as indicating that darifenacin displayed greater selectivity between active and inactive receptor species (i.e. was a more potent inverse agonist) at the M2 than at the M3 receptors. However, there was little evidence in support of this in the [3H]-inositol phosphate accumulation assays, where darifenacin exhibited similar capabilities as a ‘full’ inverse agonist at both M2 and M3 constitutively active mutant receptors (see Chapter 5). It would have been interesting to compare the potencies of these responses, but as this was not possible at the CAM M2 receptor, the implications and relevance of the radioligand binding data may only be speculated upon.

6.3.7 Functional characterisation of CHO-m2 WT and CHO-m2 MUT cell lines

Agonist potency was initially examined by measuring the methacholine-stimulated total [35S]-GTPyS binding in membranes prepared from CHO-m2 WT and MUT cell lines, as this functional output was also investigated in membranes prepared from cells transiently expressing the wild-type and N410Y mutant M2 mACh receptors (see Chapter 5). As would be predicted for a constitutively active mutant receptor, methacholine was significantly more potent in stimulating [35S]-GTPyS binding in CHO-m2 MUT membranes than in those prepared from CHO-m2 WT cells. The absolute potency values for methacholine were lower at both wild-type and mutant cell lines than when the corresponding receptors were expressed transiently, but this is likely to result from the significantly higher expression levels achieved upon transient expression. In support of this, both the absolute potency values and the ‘fold-change’ in potency between wild-type and mutant are comparable in the stable CHO cell lines and in HEK-293 cells transiently expressing the M2 receptors, where more comparable expression levels were observed. Maximal responses to methacholine were also significantly (approx. 2-fold) higher in CHO-m2 WT than in CHO-m2 MUT membranes, approximately correlating with the ratio of receptor expression.
between these cell lines, suggesting similar efficacies at wild-type and mutant M₂ receptors in this system.

A more extensive investigation of agonist potency and efficacy was performed in CHO-m₂ WT and MUT cell lines by measuring agonist-induced inhibition of forskolin-stimulated cyclic AMP. All four agonists tested exhibited significantly higher potency in CHO-m₂ MUT than in CHO-m₂ WT cells. Comparing the data for methacholine between cyclic AMP and [³⁵S]-GTPγS binding assays suggests consistency in the ratios of wild-type and mutant potencies (~ 10-fold difference in each case) and a 3-4 fold higher potency at the level of cyclic AMP inhibition, which is likely to reflect signal amplification between G-protein coupling and effector (adenylate cyclase) inhibition. Smaller shifts in potency between wild-type and mutant were observed for oxotremorine and pilocarpine (approx. 5-6 fold in each case). This might be expected for partial agonists as, according to the ETC model (Samama et al., 1993), the magnitude of the shift in agonist potency is proposed to be proportional to agonist efficacy, as has been demonstrated previously for the muscarinic receptors (Burstein et al., 1997). It is therefore surprising that the full agonist oxotremorine-M exhibited such a modest shift in potency (approx. 2.5-fold) between CHO-m₂ WT and MUT cells. However, despite also behaving as a full agonist at both (Ser₃₈₈Tyr) and (Ser₃₈₈Tyr, Thr₃₈₉Pro) mutant M₁ receptors, oxotremorine-M displayed significantly smaller increases in potency than acetylcholine and carbachol (Huang et al., 1999), so it is perhaps not surprising that a smaller shift than methacholine is observed in the present study. It should be noted, however, that the shift in oxotremorine-M potency at each of the M₁ receptor mutants discussed above was significantly greater than that observed for oxotremorine, in contrast to the findings at the N₄₁₀Y M₂ receptor mutant (Huang et al., 1999). Given the substantial increase in apparent binding affinity measured for oxotremorine-M at the CHO-m₂ MUT cells (compared with the minor changes in affinity observed for oxotremorine and pilocarpine), the modest potency exhibited for the inhibition of cyclic AMP accumulation does seem anomalous.

Another feature commonly reported at constitutively active mutant receptors is an increase in efficacy, of partial agonists in particular (Samama et al., 1993). In the present study, oxotremorine elicited a similar maximal response to methacholine and oxotremorine-M in CHO-m₂ WT cells, suggesting that the system is sensitive enough
to permit full activation even to a moderate partial agonist such as oxotremorine. Previous studies reported similar findings for oxotremorine at CHO cells expressing M₂ (McKinney et al., 1991) and M₄ (Richards & van Giersbergen, 1995) mACh receptors, while sub-maximal responses were observed for oxotremorine in cell lines expressing M₁, M₃ and M₅ mACh receptor subtypes (Richards & van Giersbergen, 1995; Huang et al., 1999). In contrast, pilocarpine displayed a partial agonist activity in CHO-m2 WT cells, in agreement with previous studies detailing the weak partial agonist behaviour of pilocarpine at all muscarinic receptor subtypes (McKinney et al., 1991; Richards & van Giersbergen, 1995).

In CHO-m2 MUT cells, although pilocarpine displayed a slightly enhanced maximal response, it was still significantly less than that induced by methacholine, suggesting that the effect of the N410Y mutation on partial agonist efficacy was relatively mild. However, to facilitate comparison of the effects of the constitutively activating mutation on the efficacy of all four tested agonists, the method of Ehlert (1985) was used to determine ‘relative efficacy’ values. This calculation takes into account both the ratio of maximal response to that of a reference agonist (in this case, methacholine) and the occupancy-response relationship, based upon $K_B$ and $EC_{50}$ estimates derived under similar conditions (Ehlert, 1985). The relative efficacy estimates defined by this method provide an indication of the relative abilities of agonists to evoke a response in the chosen test system and by accounting for occupancy, it might be argued that these values are a more valid index of agonist intrinsic activity. Comparison of the relative efficacy estimates for both pilocarpine and oxotremorine indicates an enhanced efficacy for each partial agonist in CHO-m2 MUT cells, relative to CHO-m2 WT cells. This is likely to reflect the increase in potency exhibited by both pilocarpine and oxotremorine, in the absence of large increases in apparent binding affinity, at the N410Y mutant receptor.

The other interesting point to arise from the relative efficacy estimates for the inhibition of cyclic AMP accumulation is that, in contrast to the situation observed in CHO-m2 WT cells, methacholine is more efficacious than oxotremorine-M in CHO-m2 MUT cells (this is also summarised in the rank order of relative efficacies displayed in section 6.2.2.7). This reflects the relatively modest increase in oxotremorine-M potency observed at the mutant receptor (see earlier discussion), leading to a relative efficacy estimate for oxotremorine-M in CHO-m2 MUT cells that
is similar to that determined for oxotremorine. This might be interpreted as an indication that oxotremorine-M is relatively poor at stimulating the N410Y mutant M<sub>2</sub> receptor to couple to G<sub>q/11</sub>-proteins. Earlier studies investigating CAM mACh receptors have not compared relative efficacy values for muscarinic agonists at wild-type and mutant receptors, so it is not clear whether this is common to similar mutants of other mACh receptor subtypes, but as discussed earlier, oxotremorine-M was reported to display relatively modest shifts in potency at M<sub>1</sub> mACh receptors bearing the homologous mutation to that investigated in the present study (Huang et al., 1999).

Although predominantly coupled to G<sub>q/11</sub>-proteins, the M<sub>2</sub> mACh receptor has been reported to couple to G<sub>q</sub>-proteins under certain experimental conditions (Michal et al., 2001). Following pertussis toxin (PTx) pre-treatment to inactivate the G<sub>q/11</sub>-protein population, many G<sub>q/11</sub>-coupled receptors, including the muscarinic M<sub>2</sub> and M<sub>4</sub> receptors (Michal et al., 2001; Mistry et al., in press), robustly augment forskolin-stimulated cyclic AMP accumulation, via G<sub>q</sub>-protein coupling. This strategy was therefore utilised in the present study to investigate agonist pharmacology at wild-type and N410Y mutant M<sub>2</sub> mACh receptors coupled to the G<sub>q</sub> pathway. In CHO-m2 WT cells, the full agonists methacholine and oxotremorine-M produced robust, concentration-dependent increases in cyclic AMP accumulation in the presence of forskolin. The partial agonist nature of oxotremorine was apparent in this system as oxotremorine elicited less than 40 % of the maximal response observed upon methacholine treatment, while pilocarpine produced a very weak (~ 5 % of methacholine) response. However, in CHO-m2 MUT cells, significantly enhanced maximal responses were observed to both partial agonists, relative to those observed in the wild-type receptor-expressing cells, suggesting an increase in efficacy as predicted for a constitutively active receptor. Indeed, comparison of the relative efficacy estimates for all agonists tested, indicated that small increases in efficacy were observed at the N410Y mutant M<sub>2</sub> receptor. In line with the predictions of the Ehlert relative efficacy equation, methacholine and oxotremorine-M, displaying relative efficacies of close to unity, exhibited approximately equivalent K<sub>B</sub> and EC<sub>50</sub> values, indicating a lack of receptor reserve for these responses (Ehlert, 1985).

With the exception of pilocarpine in CHO-m2 WT cells, all agonists displayed lower potencies (by between 10- and 100-fold) for the stimulation of cyclic AMP accumulation, compared with their inhibitory potencies, in agreement with previous
studies (Michal et al., 2001; Mistry et al., in press). Interestingly, both methacholine and oxotremorine-M displayed significantly greater potencies in CHO-m2 MUT cells than in CHO-m2 WT cells, indicating that even in the absence of G\textsubscript{i/o}-coupling, the N410Y mutant receptor displays greater potency and affinity for agonists than the wild-type receptor. This suggests that the observed effects result from mutation-induced changes in receptor conformation and do not depend upon coupling to G\textsubscript{i/o}-proteins, in agreement with previous studies on the effects of constitutively activating mutations in other GPCRs (e.g. Ren et al., 1993).

Comparison of the ratio of EC\textsubscript{50}/IC\textsubscript{50} values can provide further insight into the relative potencies of the agonists for inhibitory and stimulatory responses at wild-type and mutant receptors. In CHO-m2 WT cells, the ratios are fairly consistent, with the exception of pilocarpine, which displayed similar potencies for both responses. However, the difficulty in accurately determining the EC\textsubscript{50} for such a small response as was observed for pilocarpine necessitates caution in the interpretation of these data. EC\textsubscript{50}/IC\textsubscript{50} ratios in CHO-m2 MUT cells are considerably more varied, with oxotremorine-M displaying an apparently modest difference in potency for G\textsubscript{i/o} and G\textsubscript{s}-coupling, once again reflecting the modest increase in potency of this agonist for the activation of G\textsubscript{i/o}-proteins by the N410Y mutant M\textsubscript{2} receptor. In contrast, oxotremorine displays an anomalously large EC\textsubscript{50}/IC\textsubscript{50} ratio, largely resulting from the relatively weak activation of G\textsubscript{s} by this agonist at the N410Y mutant M\textsubscript{2} receptor; oxotremorine displayed only a small, statistically insignificant increase in potency for the stimulation of cyclic AMP accumulation in CHO-m2 MUT cells relative to CHO-m2 WT cells. Taken together, these data might be taken as evidence for agonist-directed trafficking of signalling (Kenakin, 1995; 1997) at the N410Y mutant M\textsubscript{2} receptor, with oxotremorine-M apparently displaying some degree of selectivity for the stimulation of G\textsubscript{s} over G\textsubscript{i/o}-coupling to the mutant M\textsubscript{2} receptor, while oxotremorine exhibited the opposite profile. The EC\textsubscript{50}/IC\textsubscript{50} ratio has previously been used to investigate agonist-directed trafficking of signalling at a variety of other GPCRs (Berg et al., 1998; Bonhaus et al., 1998; Brink et al., 2000). Agonist-specific trafficking has also been previously demonstrated at the wild-type M\textsubscript{2} mACh, where pilocarpine was found to be more effective in activating G\textsubscript{r2} compared to G\textsubscript{r1/2} in CHO cells using a [\textsuperscript{35}S]-GTP\textsubscript{S} binding/immunoprecipitation strategy (Akam et al.,
2001). It is clear that further investigation is required to fully explore this concept at the muscarinic receptors.

6.3.8 Concluding remarks

The investigation of the agonist pharmacology of the wild-type and N410Y mutant M₂ mACH receptors expressed stably in a CHO cell background has confirmed several key findings about the nature of the mutation. Enhanced agonist affinity, potency and in many cases (particularly for the partial agonists) efficacy has been observed, all of which are hallmark characteristics of a constitutively active mutant receptor (Samama et al., 1993; Lefkowitz et al., 1993). Coupled with the evidence of significantly higher constitutive activity of the N410Y mutant M₂ receptor, even when expressed at equivalent or lower levels than the wild-type receptor, presented in the previous chapter, data presented here confirms that the mutation of asparagine 410 to tyrosine induces an increase in the tendency of the M₂ receptor to spontaneously couple to its cognate G-proteins, as has been reported for similar mutations in other muscarinic receptor subtypes (Spalding et al., 1995, 1997; Huang et al., 1998b, 1999; Ford et al., 2002; Dowling, PhD thesis, 2004).

However, comparison of the present data with previous studies investigating similar mutations at the other muscarinic receptors indicates that the N410Y mutation in the M₂ receptor appears to have a less substantial effect than might be predicted from these earlier studies. For instance, no significant constitutive activity was recorded in the stable CHO cell lines expressing approx. 600 fmol mutant receptor mg⁻¹ protein, while previous studies have reported considerable levels of constitutive activity of other CAM mACH receptors expressed at similar or even lower levels (e.g. Ford et al., 2002). Reasons for this might include differences in cell background, which has been shown previously to significantly influence muscarinic receptor signalling (Richards & van Giersbergen, 1995) and levels of constitutive activity (see Milligan, 2003) but significant constitutive activity has been previously reported for even wild-type M₂ receptors expressed in CHO cells (e.g. Jakubik et al., 1995), so this would seem an unlikely explanation.

The choice of mutation is obviously a critical factor in designing a constitutively active mutant receptor. Although Ford et al. (2002) used the double mutation (N410Y,
T411P) to generate a CAM M₂ receptor, the use of the single N410Y mutant receptor in the present study was considered sufficient given the substantial levels of constitutive activity observed in the homologously mutated CAM M₃ receptor (Dowling, PhD thesis, 2004). Mutation of the homologous site in the M₃ mACh receptor was also found to generate CAM receptors in 11 out of the 13 mutations investigated (Spalding et al., 1997), while the S388Y mutation in the M₁ receptor subtype was considered to be that which was predominantly responsible for generating enhanced constitutive activity in M₁ receptor mutants (Huang et al., 1998b, 1999). Also, in the latter study, it was observed that effects on agonist affinity, efficacy and potency were similar or very slightly less at S388Y M₁ receptor mutants than at the double mutated (S388Y, T389P) receptor (Huang et al., 1999). In addition, the equivalent double mutation at the M₂ receptor caused only modest (4-5 fold) shifts in agonist affinity (compared with up to 10-fold shifts in the present study), while no significant agonist-mediated responses were detectable (Ford et al., 2002). The present study therefore provides the first rigorous characterisation of a constitutively active mutant M₂ mACh receptor. By comparison with the homologous mutation in the M₁ receptor (Dowling, PhD thesis, 2004) and previously published examples in other muscarinic receptor subtypes, it is proposed that the M₂ mACh receptor might be less susceptible to constitutive activation by mutations at the junction of TM6 and the third extracellular loop than other members of the muscarinic receptor family. However, the N410Y mutation M₂ mACh receptor investigated in the present study also appeared to influence receptor-G-protein coupling in an agonist-dependent manner, suggesting that it might provide a suitable model for the investigation of agonist-directed trafficking of signalling (Kenakin, 1995, 1997) in addition to constitutive activity and inverse agonism.
Chapter 7. Concluding discussion

7.1 Functional selectivity of antagonists at the mACh receptors

Based upon evidence presented in this thesis, and in previous studies, it is clear that a sub-set of muscarinic antagonists are capable of displaying ‘functional selectivity’ for the inhibition of M₃ muscarinic receptor-mediated responses in urinary bladder, relative to those mediated by the same receptor subtype in salivary glands. Previous reports have indicated that darifenacin (Newgreen & Naylor, 1996; Gupta et al., 2002), tolterodine (Nilvebrant et al., 1997a; Gillberg et al., 1998) and oxybutynin (Gupta et al., 2002) display such selectivity under conditions where the reference compound atropine exhibits identical affinities for both bladder and salivary gland responses. Experimental data presented in this thesis detail the characterisation of the functional affinities of darifenacin, oxybutynin, tolterodine and atropine for the inhibition of muscarinic agonist-stimulated phosphoinositide turnover in guinea-pig urinary bladder and submandibular salivary gland. A key finding of this study was that darifenacin, oxybutynin and tolterodine all displayed significantly higher affinities for the inhibition of functional responses in bladder than in submandibular glands, while atropine exhibited similar affinities in each tissue. This provides persuasive evidence that, at the level of second messenger turnover, certain muscarinic agonists can display functional selectivity in a tissue-dependent manner.

As with earlier studies reporting such selectivity, the equivalent activity of atropine in each tissue clearly indicates that the observed selectivity does not simply represent a ‘frame-shifting’ of antagonist affinities between preparations. The present study therefore provides the first thorough comparison of the affinities of these three clinically important ligands in second messenger assays.

An obvious question to arise from these observations is: could the expression of multiple muscarinic receptor subtypes account for the functional selectivity of certain antagonists? The non-subtype-selective nature of atropine might suggest that the functional selectivity of the other ligands reflects their subtype selectivity profiles. However, although darifenacin and oxybutynin display some degree of muscarinic receptor subtype selectivity (Noronha-Blob & Kachur, 1991; Gillberg et al., 1998; Wallis & Napier, 1999), tolterodine exhibits no selectivity between muscarinic receptor subtypes (Nilvebrant et al., 1997a). It would therefore seem unlikely that
subtype selectivity could be solely responsible for the observed functional selectivity profiles of these antagonists.

Nonetheless, there is considerable evidence for the expression of multiple \(G_{q11}\)-coupled mACh receptor subtypes in the salivary glands of various species (Wess, 2004). An investigation of the mACh receptor subtype expression profiles of the major salivary glands of the guinea-pig was therefore undertaken. A significant finding of the present study was that none of the three major salivary glands of the guinea-pig express detectable levels of \(M_1\) receptor subtype and it is likely that they in fact express a near-homogeneous population of \(M_3\) mACh receptors. This contrasts with numerous studies in mice lacking individual genes for specific muscarinic receptor subtypes, which have demonstrated significant expression of several additional mACh receptor subtypes, including \(M_1\), \(M_4\) and \(M_5\) receptors (Yeomans et al., 2001; Bymaster et al., 2003). An assessment of the literature relating to earlier biochemical studies reveals a distinct lack of consensus with regard to the muscarinic receptor subtypes expressed in the parotid, sublingual and submandibular salivary glands (Dai et al., 1991; Dorje et al., 1991; Watson & Culp, 1994; Watson et al., 1996; Culp et al., 1996; Moriya et al., 1999; Barras et al., 1999). However, it is evident from consideration of the literature, in the context of data presented in this thesis, that there is significant inter-species variation in mACh receptor subtype expression. The absence of a significant \(M_1\) mACh receptor population in the salivary glands of the guinea-pig is particularly compelling, given the report of a substantial \(M_1\) receptor complement in the same glands of the dog, determined using almost identical techniques to those implemented in the present investigation (Clarke et al., 2003a). Such inter-species differences highlight the importance of the choice of animal model in pharmacological studies and perhaps underline the limitations of such models in predicting pharmacological effects in man.

The findings of this study, therefore, suggest that the measurements of phosphoinositide turnover in both urinary bladder and submandibular salivary gland represent indices of \(M_3\) mACh receptor activation in these tissues. The different functional affinities determined for certain muscarinic antagonists in these two tissues therefore indicate that either the \(M_3\) receptors expressed in the tissues differ in some way, or that an as yet undefined cellular factor(s) modulates their pharmacology in a tissue-dependent manner. Since the muscarinic receptors are encoded by intronless
genes, the potential for genetic heterogeneity is limited. Potential mechanisms include post-transcriptional (e.g. RNA editing) and post-translational (e.g. palmitoylation) modifications of the M3 receptor in a tissue-dependent manner, but our understanding of the dynamic regulation of such processes in relation to the muscarinic receptors and other GPCRs is still limited (Seeburg & Hartner, 2003; Qanbar & Bouvier, 2003).

Given the growing evidence that GPCRs do not function in isolation, but instead may form component parts of multi-protein complexes, facilitating multiple protein-protein interactions (Milligan & White, 2001; Bockaert et al., 2003), it is quite conceivable that the prevailing protein complement of the cellular milieu will modulate the interactions available to a given GPCR. Thus, differences in cell background might come to have a bearing upon the functional and pharmacological behaviour of that receptor. The challenge for GPCR researchers in the future is to dissect the multitude of potential interacting partners and demonstrate those that have not only the potential to impart a measurable influence upon receptor behaviour, but also those responsible for many of the apparently anomalous behaviours observed in vivo.

It has been demonstrated that the association of distinct GPCRs with each other (as hetero-oligomers) can generate a novel unit, with distinct functional and pharmacological properties (Jordan & Devi, 1999; Yoshioka et al., 2001). The potential that M2 and M3 mACh receptors, co-expressed in the urinary bladder, might interact in such a way to generate the anomalous pharmacology observed in the present study and others, has been discussed in this thesis. However, the failure to observe significant effects upon the pharmacology of the M3 receptor when co-expressed with an M2 receptor population in CHO cells, coupled with the paucity of evidence for muscarinic receptor hetero-oligomerisation (Zeng & Wess, 2000) would suggest otherwise. A recent study also directly explored the potential for distinct regions (N- and C-termini, intra- and extra-cellular loops) of a number of muscarinic receptor subtypes to physically associate, but found no evidence for such interactions (Kang et al., 2003). However, Kang et al. (2003) did not investigate the hydrophobic transmembrane domains and evidence has recently been provided (Bakker et al., 2004a) for the physical association of histamine H1 receptors by the process of 'domain-swapping', which involves the exchange of membrane-spanning domains between the two protomers (Gouldson et al., 2000). Interestingly, the authors
proposed that a H₄ receptor-selective ligand also displayed some selectivity for the domain-swapped dimer over the monomeric H₄ receptor (Bakker et al., 2004a). The potential for muscarinic receptors to undergo similar interactions remains to be fully investigated and could provide further information regarding the nature of the atypical pharmacologies observed in the present study. Given that previous studies on oligomerised muscarinic receptors have highlighted some anomalous pharmacological profiles (Chiacchio et al., 2000), this would certainly appear worthy of further investigation.

The roles of specific plasma membrane microdomains in the regulation of GPCR signalling cascades are becoming increasingly well understood (Steinberg & Brunton, 2001). Previous studies have demonstrated that muscarinic receptors are capable of binding to caveolin isoforms in cardiac myocytes (Feron et al., 1997) and smooth muscle (Murthy & Makhlof, 2000), and given that other components of the PLC signalling pathway are also selectively enriched in caveolae, it is possible that these specialised lipid rafts might form signalling microdomains in certain cell types. Given the relative abundance of caveolae in smooth muscle (Okamoto et al., 1998), it is tempting to speculate that such localisation may occur preferentially in these tissues. Clearly, further research is required to elucidate the roles of caveolae in GPCR signalling, particularly that of the muscarinic receptors, and to investigate whether the assembly of such signalling scaffolds impacts upon the observed tissue-dependent pharmacology of certain ligands.

The identification of the functional selectivity of certain muscarinic receptor antagonists at the level of second messenger signalling in the present study, provides an in vitro correlate of in vivo findings previously reported in guinea-pig and other species (Nilvebrant et al., 1997a; Gillberg et al., 1998; Gupta et al., 2002). However, the question remains as to how relevant these findings in animal models are to the use of these compounds in the clinic. Clinically, both tolterodine (Abrams et al., 1998; Malone-Lee et al., 2001) and darifenacin (Haab et al., 2004) have demonstrated similar efficacy but superior side-effect profiles relative to oxybutynin. It is therefore perhaps a little surprising that in the present study, all three antagonists display comparable bladder selectivity. Given the likely species-dependent differences in salivary gland muscarinic receptor populations it is conceivable that the situation in humans might be quite different to that observed in any of the laboratory animal
models investigated thus far. Comparator studies in human tissues would therefore significantly advance our understanding of what is occurring clinically. Other factors such as the direct smooth muscle relaxant properties of oxybutynin (Kachur et al., 1988) and its highly active metabolite N-desethyl oxybutynin (Waldeck et al., 1997), must also be considered in relation to the clinical actions of these drugs. Thus, although studies such as those presented herein significantly enhance our appreciation of the pharmacological actions of these compounds, it is anticipated that the many questions raised by this work, and that of others, will stimulate further research in this area.

7.2 Constitutive activity and inverse agonism at mACh receptors

The primary aim of this study was to generate a constitutively-active, mutant (CAM) M$_2$ mACh receptor, to facilitate an investigation of the inverse agonist properties of a number of muscarinic antagonists, including those of clinical relevance in the management of overactive bladder. The introduction of a single amino acid change (N410Y) in the M$_2$ mACh receptor (at the junction between TM 6 and the third extracellular loop) generated a mutant receptor displaying many of the hallmark features of a CAM receptor (Samama et al., 1993; Lefkowitz et al., 1993): significantly enhanced agonist-independent activity; increased binding affinity and potency of agonists; augmented partial agonist efficacy. The N410Y mutant receptor was also expressed at significantly lower levels than the wild-type M$_2$ receptor in CHO-K1 cell transiently transfected with equivalent amounts of cDNA for each receptor. The N410Y mutant M$_2$ receptor therefore behaved largely as would be predicted for a CAM receptor.

All of the ligands investigated, which were previously classified as muscarinic antagonists, exhibited similar levels of inverse agonism at the CAM M$_2$ receptor. Similar experiments performed in this and previous studies, indicated that these ligands also behaved as full inverse agonists at the CAM M$_3$ receptor (harbouring the homologous mutation to that in the CAM M$_2$ receptor; Dowling, PhD thesis, 2004). In light of recent estimates of the prevalence of inverse agonism at GPCRs in general (Kenakin, 2004), it would be anticipated that the vast majority of muscarinic ‘antagonists’ would in fact be inverse agonists. Different levels of negative efficacy
have been defined for inverse agonists at the β2-adrenoceptor (Chidiac et al., 1994), but neither the present study, nor previously published work, have presented convincing evidence that muscarinic inverse agonists exhibit a significant range in negative efficacies. However, this may represent the lack of sensitivity of presently used stimulus-biased assays/mutations rather than a uniform efficacy profile of muscarinic inverse agonists. Conceptually, it is no easier to accept that all muscarinic inverse agonists possess similar ratios of affinity for inactive and active receptor species (as would be predicted by the ETC model for a number of ligands of equal negative efficacy) than it is to believe that a large number of ligands will fail to discriminate between active and inactive receptor conformations at all (i.e. will be true neutral antagonists). It is important to note that negative efficacy is a property inherent to the ligand, while inverse agonism is a phenotypic behaviour (Kenakin, 2004) and therefore failure to observe differences in inverse agonism does not prove that all of the ligands investigated possess similar levels of negative efficacy. A better understanding of the range of negative efficacies achievable might permit the design of therapeutic agents to exhibit different degrees of inverse agonism in different systems e.g. it may be possible to selectively reduce the abnormally high levels of constitutive activity in one system, without affecting the more modest basal activity of another system by the administration of a drug with only intermediate negative efficacy.

Surprisingly, not all of the ligands found to act as inverse agonists in second messenger assays caused a significant up-regulation of CAM M2 receptor expression levels. Whereas 24 h incubation with atropine, darifenacin and, to a lesser extent pirenzepine, significantly enhanced N410Y mutant M2 receptor expression, similar exposure to a number of inverse agonists, including tolterodine and oxybutynin, failed to effect receptor expression. This contrasts with other studies using CAM receptors, including that with the CAM M3 receptor (Dowling, PhD thesis, 2004), where all inverse agonists (and even in some cases neutral antagonists and agonists) significantly stabilize the receptor at the cell surface, preventing the down-regulation/degradation of the structurally unstable mutant receptor (Li et al., 2001; Pauwels & Tardif, 2002; Zeng et al., 2003). The comparison of inverse agonist-mediated up-regulation of M2 and M3 receptors therefore indicated that different mechanisms might underlie the observed increases in expression of each subtype. It is
therefore proposed that the N514Y mutation in the CAM M_3 receptor has a greater destabilising effect upon the receptor than the homologous (N410Y) mutation does upon the M_2 receptor. Thus, while ligands (including the agonist carbachol) tend to stabilize the CAM M_3 receptor and thereby enhance its cell surface expression, only those ligands that reduce the constitutive activity of the mutant M_2 receptor increase its plasma membrane localisation, by preventing a constitutive internalisation of the receptor that is presumably initiated by the constitutive activity of the mutant M_2 receptor. In such a model, the inability of certain ligands to cause a significant up-regulation of the CAM M_2 receptor would be interpreted as an indication that the ligands are not sufficiently negatively efficacious to exert an effect. It is therefore possible that such an assay might provide more detailed information about the negative efficacy of the ligands investigated than the inhibition of the accumulation of [^H]-inositol phosphates i.e. a classical constitutive activity/inverse agonist assay. The lower ‘potency’ observed for the concentration-dependent up-regulation of CAM M_2 receptor by atropine (compared with binding affinity and inverse agonist ‘potency’ estimates) tends to support the notion that, by analogy to systems used in the definition of positive agonist efficacies, the ‘stimulus-response’ coupling is weaker, allowing lower levels of efficacy to be discerned. However, further evidence that the observed up-regulatory responses correlate with negative efficacy is required, especially since other possible mechanisms (including the ‘chaperoning’ of intracellular receptor pools to the plasma membrane) discussed within this thesis have yet to be eliminated.

In addition to the hallmark features of a constitutively-active, mutant receptor exhibited by the N410Y mutant M_2 receptor, some interesting differences in agonist pharmacology were observed, particularly the findings that oxotremorine and oxotremorine-M appeared to display contrasting preferences for G_{i/o} and G_s-mediated responses in CHO cells stably expressing the N410Y mutant M_2 receptor. Notably, these agonists failed to display such anomalous behaviour at the wild-type M_2 mACh receptor, suggesting that the N410Y mutation might alter aspects of receptor-G-protein coupling in addition to stabilizing the active receptor conformation(s). In this respect, the work of Beinborn et al. (2004) is of interest, as they propose that constitutively-activating mutations may be sub-divided into two categories: type I CAMs strictly adhere to the classical criteria outlined earlier, including enhanced
constitutive activity, agonist affinity and potency and increased partial agonist efficacy (Samama et al., 1993; Lefkowitz et al., 1993); type II CAMs, however, are those which exhibit compound-dependent changes in ligand affinity/activity. Beinborn et al. (2004) attribute the pharmacological characteristics of these two classes of CAM to the location of the amino acid substitutions, with type I mutations resulting from substitution of residues within the third intracellular loop. In contrast, type II mutations were found to result from substitutions in the transmembrane domains and in particular at residues in close proximity to the extracellular surface of the receptor (Beinborn et al., 2004). The N410Y mutation at the junction of TM 6 and the third extracellular loop would therefore be classified as a type II CAM, as would the majority of CAM mACH receptors studied to date (Spalding et al., 1995, 1997, 1998; Ford et al., 2002; Dowling, PhD thesis, 2004). In accordance with the proposals of Beinborn et al. (2004), agonist-dependent alterations in potency and efficacy were observed in the present study and although many of the hallmark criteria for a CAM were met by the N410Y mutant, the constitutive activity was moderate (constitutive activity was not detectable in stable cell lines expressing < 1 pmol mg⁻¹ protein) and consistent increases in binding affinity were not observed in all cases. In addition, inverse agonists did not uniformly display decreased affinity for the N410Y mutant, as would have been predicted for a CAM receptor.

The data presented here therefore support the theory that mutations in the transmembrane domains of GPCRs might not produce classical CAMs. Such a theory has significant implications for the study of constitutive activity and inverse agonism, as it suggests that type I mutations might provide better models for predicting the activity of inverse agonists, without the added complications of alterations in affinity/activity observed in many type II mutations (Beinborn et al., 2004). It would therefore be interesting to compare the effects of mutation in the third intracellular loop of the M₂ mACh receptor with those in transmembrane domains, such as the N410Y mutation, particularly in relation to the determinations of inverse agonist efficacy. One of the few studies to investigate the effect of mutations in the C-terminal tail of the third intracellular loop in the muscarinic receptors, found that insertion of one or more alanine residues into this region caused robust constitutive activation of the M₂ mACh receptor (Liu et al., 1996), suggesting that substitutions in this region are likely to generate CAMs. Whether these mutants would fit the
hallmark criteria more accurately than substitutions in the transmembrane domains remains to be determined. However, it is also interesting to note that mutation of residues within the highly conserved DRY motif (in the cytoplasmic end of the third transmembrane domain) of the M₁ mACh receptor failed to generate constitutively-active, mutant receptors (Lu et al., 1997), providing further evidence that mutations within the transmembrane domains of the muscarinic receptors might not generate classical constitutively-active mutants.

The vast majority of studies investigating the phenomena of constitutive activity and inverse agonism have concentrated on manipulating receptor-G-protein coupling in recombinant systems (Milligan, 2003). This is not without good reason, as such systems permit genetic manipulation of GPCRs and their downstream signalling intermediates with relative ease and allow constitutive activity to be monitored in the absence of endogenous agonist (Milligan, 2003). However, as a result our understanding of the prevalence and extent of constitutive activity in vivo is limited. Many early reports of constitutive activity and inverse agonism came from studies on endogenous muscarinic receptors (Giles & Noble, 1976; Berrie et al., 1979; Soejima & Noma, 1984) and later studies even presented evidence for constitutive activity of muscarinic receptors in the absence of endogenous agonist (Hilf & Jakobs, 1992; Hanf et al., 1993; Jakubik et al., 1995). More recently, the in vivo roles of constitutively active cannabinoid CB₁ (Richardson et al., 1997) and histamine H₃ (Morisset et al., 2000) receptors have been demonstrated.

However, it is clear that the challenge in the future is to take forward the findings in heterologous expression systems and apply them to the physiological roles of GPCRs in vivo. The situation in vivo is of course complicated by the myriad interactions of GPCRs (Brady & Limbird, 2002), but the influence of such interactions must be considered if we are to fully appreciate the behaviour of GPCRs in vivo. Indeed, a recent study by Bakker et al. (2004b) reported that co-expression of a variety of Gₒ- coupled receptors with the naturally constitutively-active histamine H₁ receptor revealed a constitutive functional coupling of the Gₒ-linked receptors, which was not apparent when expressed alone. It is therefore likely that Gₒ-coupled receptors endogenously co-expressed in tissues with naturally constitutively-active receptors might exhibit levels of basal activity that would not be predicted from their examination in isolation in a recombinant system.
Conversely, it is quite conceivable that other interactions, including direct protein-protein interactions, might reduce the level of constitutive activity of a receptor system, as recently proposed by Milligan (2003). For instance, interactions between ligand-gated ion channels and GPCRs of the dopamine receptor family have been reported to occur via the C-terminal tail of the receptors (Liu et al., 2000; Lee et al., 2002). It might be anticipated that such interactions would compromise G-protein coupling and could therefore limit the extent of constitutive G-protein activation (Milligan, 2003). Further investigation of the influences of the cellular environment upon constitutive activity might be anticipated to reveal both constraining and facilitating interactions in vivo, providing us with a better understanding of the cellular regulation of basal receptor activity.

De Deurwaerdere et al. (2004) have provided the first evidence for a role for constitutive activity of 5-HT2C receptors in vivo, in the tonic inhibition of mesencephalic dopamine neurones. If constitutive GPCR activity is a generalised mechanism of neuronal regulation in the CNS, a potential role of muscarinic receptor constitutive activity might be in the well-recognised interaction of the cholinergic and dopaminergic systems in striatal function (Zhou et al., 2003). In addition, it is tempting to speculate upon a pathophysiological role for constitutive muscarinic receptor activity in the overactive bladder, where enhanced detrusor excitability has been implicated and where muscarinic 'antagonists', determined in the present study to be inverse agonists, are often effective therapies (Wyndaele, 2001). It is anticipated that future research in the field of constitutive activity and inverse agonism might be directed towards the investigation of such pathophysiological circumstances in which the specific targeting of inverse agonists might be of therapeutic benefit.


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Burford NT, Tobin AB and Nahorski SR (1995) Coupling of muscarinic M_1, M_2 and M_3 acetylcholine receptors, expressed in Chinese hamster ovary cells, to pertussis toxin-sensitive/insensitive guanine nucleotide-binding proteins. Eur J Pharmacol 289:343-351.

Burford NT, Tobin AB and Nahorski SR (1995) Differential coupling of M_1, M_2 and M_3 muscarinic receptor subtypes to inositol 1,4,5-trisphosphate and adenosine 3',5'-
cyclic monophosphate accumulation in Chinese hamster ovary cells. *J Pharmacol Exp Ther* 274:134-142.


Cheng Y and Prusoff WH (1973) Relationship between the inhibition constant (Kᵢ) and the concentration of inhibitor which causes 50 per cent inhibition (I₅₀) of an enzymatic reaction. *Biochem Pharmacol* **22**:3099-3108.


Huang XP, Williams FE, Peseckis SM and Messer WS, Jr. (1998b) Pharmacological characterization of human m1 muscarinic acetylcholine receptors with double mutations at the junction of TM VI and the third extracellular domain. J Pharmacol Exp Ther 286:1129-1139.

Huang XP, Williams FE, Peseckis SM and Messer WS, Jr. (1999) Differential modulation of agonist potency and receptor coupling by mutations of Ser388Tyr and Thr389Pro at the junction of transmembrane domain VI and the third extracellular loop of human M₁ muscarinic acetylcholine receptors. Mol Pharmacol 56:775-783.


$\alpha_{1D}$-adrenoceptor mediating the contractile response of rat aorta to noradrenaline. Br J 

potency of the hydroxylated metabolite of darifenacin in its ability to decrease 
salivary flow using pooled population pharmacokinetic-pharmacodynamic data. Br J 

receptors mediating contractions of circular and longitudinal muscle of human 

Kilts JD, Akazawa T, Richardson MD and Kwatra MM (2002) Age increases cardiac 
$G_{q2}$ expression, resulting in enhanced coupling to G protein-coupled receptors. J Biol 
Chem 277:31257-31262.

Suppression of the carbachol-activated nonselective cationic current by antibody 
against alpha subunit of Go protein in guinea-pig gastric myocytes. Pflugers Arch 
436:494-496.

Kjelsberg MA, Cotecchia S, Ostrowski J, Caron MG and Leflcowitz RJ (1992) 
Constitutive activation of the $\alpha_{1B}$-adrenergic receptor by all amino acid substitutions 
at a single site. Evidence for a region which constrains receptor activation. J Biol 
Chem 267:1430-1433.

of solifenacin succinate (YM905) and current antimuscarinic drugs in bladder and 

Koenig JA and Edwardson JM (1994) Kinetic analysis of the trafficking of muscarinic 
acetylcholine receptors between the plasma membrane and intracellular 

Koenig JA and Edwardson JM (1996) Intracellular trafficking of the muscarinic 
acetylcholine receptor: importance of subtype and cell type. Mol Pharmacol 49:351- 
359.

Koenig JA and Edwardson JM (1997) Endocytosis and recycling of G protein- 


of $G_{a}$ is critical for constraining the selectivity of receptor coupling. J Biol Chem 
272:19107-19110.

Kostenis E, Gomez J, Lerche C and Wess J (1997) Genetic analysis of receptor- 


Lee NH and el-Fakahany EE (1991) Allosteric interactions at the m1, m2 and m3 muscarinic receptor subtypes. J Pharmacol Exp Ther 256:468-479.


Pike LJ and Casey L (1996) Localization and turnover of phosphatidylinositol 4,5-
bisphosphate in caveolin-enriched membrane domains. *J Biol Chem* 271:26453-
26456.

Pike LJ and Miller JM (1998) Cholesterol depletion delocalizes phosphatidylinositol
bisphosphate and inhibits hormone-stimulated phosphatidylinositol turnover. *J Biol


MM, Caron MG and Leffkowitz RJ (1992) Role of βγ-subunits of G proteins in
targeting the β-adrenergic receptor kinase to membrane-bound receptors. *Science*
257:1264-1267.

domain-mediated membrane association and activation of the β-adrenergic receptor
kinase requires coordinate interaction with Gβγ-subunits and lipid. *J Biol Chem*
270:11707-11710.

Plakidou-Dymock S, Dymock D and Hooley R (1998) A higher plant seven-

protein G13 activates a phospholipase D isozyme by a pathway requiring Rho family

Pommier B, Da Nascimento S, Dumont B, Bellier B, Million E, Garbay C, Roques BP
and Noble F (1999) The cholecystokininB receptor is coupled to two effector
pathways through pertussis toxin-sensitive and -insensitive G proteins. *J Neurochem*
73:281-288.

Porter AC, Bymaster FP, DeLapp NW, Yamada M, Wess J, Hamilton SE, Nathanson
NM and Felder CC (2002) M₃ muscarinic receptor signaling in mouse hippocampus
and cortex. *Brain Res* 944:82-89.

relationships of calcitonin analogues as agonists, antagonists, or inverse agonists in a constitutively activated receptor cell system. *Mol Pharmacol*
51:658-665.

Prakash YS, Kannan MS, Walseth TF and Sieck GC (1998) Role of cyclic ADP-
ribose in the regulation of [Ca²⁺]ᵢ in porcine tracheal smooth muscle. *Am J Physiol*
274:C1653-1660.


Shehnaz D, Ansari KZ and Ehlert FJ (2001) Acetylcholine-induced desensitization of the contractile response to histamine in Guinea pig ileum is prevented by either pertussis toxin treatment or by selective inactivation of muscarinic M_{3} receptors. J Pharmacol Exp Ther 297:1152-1159.


White TA, Kannan MS and Walsh AF (2003) Intracellular calcium signaling through the cADPR pathway is agonist specific in porcine airway smooth muscle. *FASEB J* 17:482-484.


Appendix 1. Interpreted reflection seismic lines

The interpreted reflection seismic lines are enclosed on the CD-ROM. Labelled

Appendix 1. Archive of seismic lines.

The CD-ROM is accessible using a PC with 128 MB of ram
and a CD-ROM reader. The software required to view the
reflection seismic lines is Corel Draw 9 or higher.
Appendix 3 Sedimentary logs recorded in the Midland Valley of Scotland

The Sedimentary logs recorded in the MVS were logged in various opencast coal mines and from natural sections. The logs are enclosed on the CD-ROM. Labelled

The CD-ROM is accessible using a PC with 128 MB of ram and a CD-ROM reader. The software required to view the reflection seismic lines is Corel Draw 9 or higher.

The CD-ROM contains three folders:

Key to all logs

All sedimentary logs in a spatial and temporal framework

Individual logs