Signalling and receptor crosstalk mediated by full and partial agonists of the muscarinic M\(_3\) receptor

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Muscarinic M₃ receptors couple predominantly to Gαq/11 while β₂-adrenoceptors primarily couple to Gαs. These receptor subtypes are expressed endogenously in several tissues including smooth muscle cells where they often mediate contrasting physiological effects. This study shows that stimulation of endogenously expressed Gαq/11-coupled muscarinic M₃ receptors in HEK 293 cells allows a subsequent robust Ca²⁺ response to stimulation of β₂-adrenoceptors. This study provides the first demonstration that despite the apparent inability of some partial agonists of the muscarinic receptor to mediate elevations of intracellular Ca²⁺, these agonists facilitate a Ca²⁺ response to the subsequent stimulation of β₂-adrenoceptors with noradrenaline that can be of greater magnitude than that facilitated by full agonists. This response was dependent on the concentration of both muscarinic receptor agonist and noradrenaline. Facilitation of noradrenaline-mediated Ca²⁺ responses occur only in the presence of activated muscarinic receptors, as treatment with atropine abolished responses to noradrenaline. Crosstalk was independent of the presence of extracellular Ca²⁺, but dependent on thapsigargin-sensitive intracellular stores. Investigation of potential mechanisms revealed a lack of enhanced PLC activity and ruled out a potential role for either PKA or PKC. An alternate mechanism for crosstalk was suggested based on the ability of adenylyl cyclase inhibitors to attenuate noradrenaline-mediated Ca²⁺ responses and the ability of forskolin or dbcAMP to mimic crosstalk. A role for Epac in contributing to the mechanism of crosstalk was implicated using the Epac specific activator, 8-pCPT-2'-O-Me-cAMP. It is possible that the ability of muscarinic receptor agonists to reveal Ca²⁺ signalling by noradrenaline is by Epac-cAMP signalling complex-mediated sensitisation of intracellular Ca²⁺ channels. Furthermore, the ability of noradrenaline to reveal Ca²⁺ signalling in the continued presence of muscarinic receptor agonists was demonstrated in rat tracheal smooth muscle cells and the human lung epithelial NCI H292 cell line. Given that noradrenaline influences Ca²⁺ oscillations in the presence of submaximal concentrations of muscarinic receptor agonists in HEK 293 cells, it is possible that such crosstalk could influence aspects of cell function by regulating changes in gene transcription.
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Abbreviations

HEK 293  human embryonic kidney cells 293
FCS    foetal calf serum
GFP    green fluorescence protein
FLIPR  fluorescence imaging plate reader
Fluo-3AM fluo-3-acetoxymethyl ester
BSS    basal salt solution
NA     noradrenaline
KHB    kreb's-hepes buffer
[^3]H-InsP3 total inositol phosphates
TCA    trichloroacetic acid
DTT    1, 4-dithiothreitol
mRNA   messenger RNA
DAG    diacylglycerol
PKA    protein kinase A
PKC    protein kinase C
PLC    phospholipase C
GRK    g-protein receptor kinase
EDTA   ethyl-n-diamine tetra acetic acid
CK1α   casein kinase 1α
ATP    adenosine tri-phosphate
PDE    phosphodiesterases
Ca2+   calcium
cAMP   cyclic adenosine monophosphate
CTX    cholera toxin
PTX    pertussis toxin
8-pCPT-2’-O-Me-cAMP  8-(4-chloro-phenylthio)-2’-O-methyladenosine-3’-5’-cyclic monophosphate
Chapter 1: General Introduction

1.1 G-Protein coupled receptors

The ability to sense and react to the environment is an important function of any organism and constitutes a major signalling event mediated mainly through plasma membrane receptors. The cellular responses can either be rapid, such as those involved in synaptic transmission that occur generally over the millisecond time-scale or occur over hours, such as those responses produced by insulin or growth factors. Of the different classes of membrane receptors, one of the largest families of receptors is the G-protein coupled receptor (GPCR) family. In the human genome ~80% of all known hormones and neurotransmitters activate cellular signal transduction mechanisms by activating GPCRs (Kristiansen, 2004). Of these, 400-500 GPCRs recognise ligands such as hormones, neurotransmitters and paracrine factors whilst the remaining code for receptors like taste or olfactory receptors (Wettschureck & Offermanns, 2005). The first mammalian non-visual receptor to be cloned and fully characterised was the β₂-adrenoceptor in 1986 (Dixon et al., 1986). Subfamilies of GPCRs cloned and characterised since then are detailed in Section 1.1.1.

The predicted tertiary structure of GPCRs is of seven hydrophobic α-helices (that form transmembrane domains) joined by hydrophilic loops with an extracellular amino-terminus (N-terminus) and a cytoplasmic carboxyl-terminus (C-terminus). Transmembrane domains represent areas of greatest homology of GPCRs (over 25% sequence homology; Pierce et al., 2002). These domains generally consist of 20-27 amino acids whilst the N and C-terminal domains vary between 7-595 and 2-359 amino acids respectively (Ji et al., 1998). To date, the only GPCR for which a crystal structure has been established is bovine rhodopsin (Palczewski et al., 2000). The crystallographic structure of this receptor confirmed the seven transmembrane helices connected by varying lengths of intracellular and extracellular loops. This structure also gave insight about a set of conserved cysteine residues on the cytoplasmic surface where G-protein activation occurs and which are likely to undergo conformational change upon photoactivation (Palczewski et al., 2000).
1.1.1 GPCR subfamilies

The International Union of Pharmacology Committee on Receptor Nomenclature and Drug Classification (NC-IUPHAR) of GPCRs classified GPCRs based on sequence similarity (Foord et al., 2005). The GPCR super family accordingly consists of 6 distinct groups (family A-F; Kolakowski, 1994). GPCRs present in metazoans consist of families: A, (rhodopsin type receptors); B, (secretin/glucagon type receptors); C, (metabotropic neurotransmitter type receptors) (Fredriksson et al., 2003; Gether, 2000: Kristiansen, 2004). Family D, E and F represent fungal pheromone receptors, cAMP receptors and archaeabacterial opsins respectively. Completion of the human genome sequence has revealed that more than 1% of the mammalian genome encodes for GPCRs (Foord et al., 2005). Many of the genes identified have sequences resembling known GPCRs, however their activating ligands and signalling mechanisms are relatively unclear. These ‘orphan’ receptors constitute a large proportion of the GPCR subfamily, hence the classification of GPCRs is not in any way complete. The focus of this thesis is receptors belonging to the Family A.

Family A, the rhodopsin type receptors, is by far the largest family of GPCRs. They are phylogenetically classified into six subgroups: the biogenic amine receptors, opsin receptors, bradykinin receptors, chemokine receptors, olfactory receptors and melatonin receptors. They are characterised by a set of highly conserved 20 amino acids, the majority of which are present in the transmembrane domains. Mutational analysis of these residues has shown that they are crucial for mediating conformational changes upon activation of the receptor (Wess et al., 1993). Arginine is the only conserved residue in the DRY motif (Asp-Arg-Tyr) at the boundary between the cytoplasmic side of the transmembrane domain 3 and intracellular loop 2 of family A receptors. This residue is crucial for signal transduction, since its mutation in receptors results in impaired signal transduction (Alewijnse et al., 2000; Jones et al., 1995). Using a fluorescent antagonist as a probe for the binding site of the prototype β2-adrenoceptor has revealed that the ligand-binding domain is located deep between transmembrane domains 3, 4, 5, 6 and 7 (Tota & Strader, 1990).

Family B consists of about 25 members including receptors for a variety of peptide hormones and neuropeptides (Pierce et al., 2002). These receptors have a relatively
large N-terminal extracellular domain containing six conserved cysteine molecules that play a key role in ligand binding (Pantaloni et al., 1996). The only conserved structural feature between family A and B is the disulphide bridge connecting the second and third extracellular loops (Wess, 1998). Site-directed mutagenesis studies have shown that the peptide-binding site is discontinuous and consists of contact points from the extended N-terminus and the extracellular loops. These receptors also share a highly conserved aspartic-acid residue located at the junction of transmembrane domain 2 (Kristiansen, 2004). Other differences between family A and B receptors include the absence of the DRY motif and conserved proline residues distinct from proline residues conserved in family A (Gether, 2000). Glucagon, secretin and calcitonin receptors are a few examples of this class of receptors.

Family C receptors are characterised by a long N-terminal tail (500-600 amino acids) that is crucial for ligand binding and activation. The N-terminal tail has a venus flytrap module (VFTM) that is involved in agonist binding and a cysteine-rich region connecting the VFTM and transmembrane domain (Pin et al., 2003). The arginine residue in the DRY motif is conserved only in a few subsets of this family, indicating that different molecular determinants control the activation of these receptors. Family A and family C share a few conserved residues suggesting that they may originate from a common ancestral gene (Pin et al., 2003). Like the other GPCR families, this class of receptor also has the conserved disulphide bridge connecting the second and third extracellular loops. Mammalian pheromone receptors, calcium receptors, GABA receptors and metabotropic glutamate receptors constitute this family (Gether, 2000).

More recently, the GRAFS classification based on a primary data set of 802 unique GPCRs from the human genome sequence was proposed (Fredricksson et al., 2003). Five main families were identified and named as glutamate, rhodopsin, adhesion, frizzled/taste2 and secretin. The glutamate family of receptors correlates with family C group of GPCRs. The rhodopsin family correlates with family A GPCRs, consists of the largest number of receptors and is further subdivided into four main groups (α, β, γ and δ) and 13 branches. The adhesion family of receptors consists of receptors with GPCR-like transmembrane domains fused together with one or several functional domains with adhesion-like motifs in the N-terminus, such as the EGF-like domain, mucin-like regions and conserved cysteine rich motifs. The N-terminus is variable ranging from
200-2800 amino acids, rich in glycosylation sites and proline residues. The family name adhesion refers to the long N-terminus, which contains motifs likely to participate in cell adhesion. The frizzled/taste2 receptors consist of two distinct receptor subtypes; frizzled and taste 2 receptors. They share several consensus motifs indicating close phylogenetic evolution. The secretin family of receptors bind rather large peptides and share a high amino acid identity and correlates with family B GPCRs.

GPCRs are involved in a vast number of physiological roles and their dysfunction has been linked to many pathological conditions such as stationery night blindness, retinitis pigmentosa (rhodopsin receptor dysfunction) and familial precocious puberty (leutenizing hormone receptor dysfunction) (Flower, 1999). Due to their excellent potential for drug therapy, GPCR targets often represent ~30% of the portfolio of many pharmaceutical companies (Kristiansen, 2004). Classic examples of drug therapy targeted at GPCRs include some forms of severe hypertension (α1-adrenoceptor antagonists: prazosin, doxazocin), cardiomyopathy (β1-adrenoceptor antagonists: atenolol, metoprolol), asthma (β2-adrenoceptor agonists: salbutamol, salmeterol) and schizophrenia (dopamine D2 receptors antagonists: clozapine, spiperone) (Menzaghi et al., 2002). Given that known GPCRs are excellent drug targets, orphan receptors provide a rich group of targets for development of novel drugs.

1.2 G-proteins and receptor activation

G-proteins represent the primary mechanism by which G-protein coupled receptors transduce signals from a stimulus and consist of heterotrimeric and monomeric G-proteins. The monomeric G-proteins consist of over 100 members that can be classified into 5 subfamilies: Ras (regulate cell signalling events that leads to alteration in gene transcription), Rho (regulators of actin cytoskeleton and can also influence gene transcription), Arf and Rab (these GTPases (proteins that bind GTP) function in regulating formation, fusion and movement of vesicular transport between different membrane compartments of the cell) and Ran family GTPases (regulate microtubule organisation and nucleocytoplasmic protein transport; Bhattacharya et al., 2004).

The heterotrimeric G-proteins consist of α, β and γ subunits. To date at least 23 different Ga subunits have been described that are products of 16 different genes and splice
variants. The primary sequences of these subunits have about 20% invariant conserved sequence (Morris & Malbon, 1999). The Ga subunit consist of two domains, a GTPase domain involved in the binding and hydrolysis of GTP and a helical domain that buries the GTP within the core of the protein (Cabrera-Vera et al., 2003). The heterotrimeric G-proteins can be categorised into four groups: Gaα, Gaια, Gaq/11 and Gα12/13 based on the sequence identity of the α-subunit. This classification also roughly correlates with their function, with Gαα coupling to stimulation of adenylyl cyclases (Section 1.6.1.1), Gαια coupling to the inhibition of adenylyl cyclases and the activation of G-protein inwardly rectifying potassium channels (GIRKs: activated by Gβγ subunits; Yamada et al., 1998), Gaq/11 proteins coupling to the activation of phospholipase C-β (Section 1.4.1.3) and Gα12/13 proteins coupling to the activation of Rho guanine-nucleotide exchange factors (GEFs). In addition to these major families, other types of the Ga-subunits exists that bear considerable functional homology to the main family. These include Gα14, Gα15 and Gα16 that bear significant resemblance to Gαq/11, mediating their action through different isoforms of phospholipase C (PLC). The PLC family comprises a diverse group of enzymes that differ in structure and tissue distribution. Eleven distinct isoforms of phosphoinositide-specific phospholipase C (PLC) have been identified in mammals, which are grouped into four subfamilies (β, γ, δ, and ε; Rhee, 2001). These isozymes catalyze the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P2) to inositol 1,4,5-trisphosphate (Ins(1,4,5)P3) and diacylglycerol (DAG) in response to the activation of more than 100 different cell surface receptors. The α subunits of all four members of the Gq subfamily (αq, α11, α14, and α16) activate PLC-β isozymes but not PLC-γ, PLC-δ, or PLC-ε (Rhee, 2001).

Five genes encoding the β-subunit have also been identified (β1, β2, β3, β4, β5/5L). Each β-subunit consists of about 340 amino acids and has a molecular weight of about ~35,000Da. The amino acid sequence of the ubiquitously expressed β1-4 subunits is 80-90% identical. In contrast, the newly identified β5 and β5L expressed mainly in the brain and retina respectively, bear only 52% identity to the other β-subunits (Fletcher et al., 1998). The linear sequence of these proteins has seven to eight tandem repeats with a central conserved tryptophan-aspartic acid sequence called the WD-40 motif (McCudden et al., 2005; Vander Voorn & Ploegh, 1992). Of the three-heterotrimeric moieties, the γ-subunits are the most diverse in their amino acid sequence. Eleven
different γ-subunits are known (γ1, γ2, γ3, γ4, γ5, γ7, γ8, γcone, γ10, γ11 and γ12). The γ-subunits interacts with the β-subunits through an N-terminal coil and make extensive contacts along the base of the β-subunits (Cabrera-Vera et al., 2003).

Signal transduction was often seen as being primarily driven by the Ga subunit until functions of Gγ subunits were detailed (β and γ subunits are tightly associated and therefore often considered as a functional unit). The Gγ-subunit affects several signalling molecules including adenylyl cyclases, potassium channels and various isoforms of PLC (Hanoune & Defer, 2001; Rhee, 2001; Yamada et al., 1998). The mode of G-protein activation is identical in most GPCRs. In the 'resting state', the GDP bound G-protein α subunit has a high affinity for the βγ complex, forming the heterotrimeric moiety. Upon activation, changes in the tertiary structure of transmembrane domains are transmitted to the intracellular domains leading to a conformational change in the receptor. This conformational change in the secondary and tertiary structure of the receptor alters the ability of the receptor to couple to G-proteins and results in the subsequent dissociation of the heterotrimeric G-protein moiety. Several theories have been put forward to efficiently explain agonist binding and subsequent receptor signalling.

The ternary complex model of receptor activation explains that in the presence of GDP, agonist binding promotes the formation of a complex consisting of the agonist (A), receptor (R) and heterotrimeric G-protein (G), which exhibits high agonist binding affinity. This model states that there is a direct correlation between the efficacy of agonist at stabilising the ternary complex and at promoting multiple G-protein activation/deactivation cycles (Seifert et al., 2001). In the absence of G-proteins or when the presence of GTP allows for receptor catalysed G-protein activation, the A-R-G complex is dissociated and the receptor resides in a low affinity A-R state (reviewed by Maudsley et al., 2005). However studies with constitutive active receptor mutants suggested existence of two or more receptor conformations. Based on these observations, the extended ternary model proposes the existence of two receptor conformations. In the native state the receptor is maintained predominantly in the R-configuration by intramolecular interactions with transmembrane helixes. Agonist binding or selective mutagenesis which relieve these constraints allow the receptor to
‘relax’ into the R* configuration. This model suggests that the intrinsic efficacy of the ligand reflects its ability to alter the equilibrium between R and R* (Lefkowitz et al., 1993). According to this model, a full agonist stabilises the R* configuration shifting the equilibrium towards the active state of the receptor generating full receptor activation and maximal responses. However, a partial agonist with lower intrinsic efficacy cannot stabilise the R* equilibrium to a similar extent as a full agonist, thereby producing smaller responses. In contrast, inverse agonists preferentially bind to the R-configuration of the receptor shifting the equilibrium towards the inactive receptor configuration. Antagonists on the other hand, bind indiscriminately to both receptor configurations with no efficacy.

Although the extended ternary complex model can provide sufficient explanation for the properties of agonism, it is still limited to two receptor configurations. However, several lines of evidence suggest multiple active states of receptors. Agonists at the human serotonin receptor (2A and 2C) differentially activate two signal transduction pathways (Berg et al., 1998). The relative efficacy of the agonist varies depending on the signal transduction pathway measured, contradictory to existing theories that the relative degree of activation of each effector pathway by an agonist must be the same. Concentration-response curves to agonists were fitted using the three or multistate model. This model suggests that certain agonists can stabilise distinct conformations of the receptor by differentially exposing regions of the intracellular domain involved in coupling to different G-protein pools. This suggests that receptors can exist in multiple active configurations and an inactive configuration (Kenakin, 2002; Scaramellini & Leff, 1998). Fluorescence lifetime imaging of the β2-adrenoceptor has verified the existence multistate receptor configurations, confirming the multistate model (Ghanouni et al., 2001).

Following receptor activation, the signal must be switched off to retain fine control that GPCRs exerts over cellular events. The rate of GTP hydrolysis is often the rate-limiting factor in the reaction (Cabrera-Vera et al., 2003). The intrinsic GTP hydrolysis of the Ga subunits is regulated by a class of GTP-ase activating proteins (GAPs). GAP activity can sharpen the termination of a signal upon removal of the stimulus, attenuate a signal, promote regulatory association with other proteins or redirect signalling within a G-protein signalling network (Ross & Wilkie, 2000). Of these, regulators of G-protein
signalling (RGS) play a crucial role (Hollinger & Hepler, 2002). They are a family of highly diverse multifunctional signalling proteins, which share a conserved domain (RGS domain) that binds directly to the Ga subunit to modulate signalling. At least 37 different proteins are known, each characterised by the conserved domain, approximately 120 amino acid residues long (Ross & Wilkie, 2000). Two alternative nomenclatures have been proposed to classify RGS proteins into subfamilies. These include family A or RZ, family B or R4, family C or R7, family D or R12, family E or RA and family F or GEF (Siderovski & Willard, 2005). In addition to these regulatory proteins, G-protein effectors can also act as GAPs. The first identified G-protein effector GAP being PLC-β (Ross & Wilkie, 2000). The PLC-β family are activated by the Gaq/11-coupled receptors and all the PLC-β isoforms can effectively GAP Gaq/11 activity, but are ineffective for other G-proteins. Other effector GAPs include Rho-GEF and cyclic GMP phosphodiesterases (Ross & Wilkie, 2000). In addition, a plethora of modulatory proteins exists that are crucial in regulation of receptor signalling. These are described in more detail in the following sections.

1.3 Regulation of GPCR signalling

Signalling through GPCRs can be effectively regulated by either receptor desensitisation (receptors becomes ‘refractory’ within seconds of agonist stimulation due to phosphorylation of the receptors by serine-threonine kinases), sequestration (internalisation of receptors into clathrin coated or un-coated pits) and receptor down-regulation (on prolonged exposure of agonists, resulting in the degradation of the receptors in endosomes; Carman & Benovic, 1998). GPCRs, despite their diversity, appear to share common mechanisms for desensitisation. Typically, uncoupling of the G-proteins from the receptor is dependent on receptor phosphorylation mediated by intracellular second messenger kinases (e.g. protein kinase A; PKA and protein kinase C; PKC) or by a distinct family of GPCR kinases (GRKs) as discussed below.

1.3.1 GRK-mediated regulation of GPCR signalling

GRKs mediate a rapid, agonist-specific desensitisation of GPCRs (homologous desensitisation). Only the agonist-occupied receptor is in an appropriate receptor configuration to allow phosphorylation by GRKs, making this a mechanism that strictly desensitises agonist-occupied receptors (Chuang et al., 1996). Deletion or mutation of
putative phosphorylation sites of GPCRs in the third intracellular loop or the C-terminal tail impairs desensitisation (Bouvier et al., 1988). Coexpression of GRKs with GPCRs in heterologous cells enhances desensitisation of GPCRs (Freedman et al., 1995). Further more, use of a dominant negative receptor kinase inhibited phosphorylation of the β2-adrenoceptor by endogenous GRKs demonstrating the importance of GRKs in receptor desensitisation (Kong et al., 1994).

Seven mammalian GRK genes have so far been identified (GRK1-7), which includes the extensively studied GRK2 (Ferguson, 2001). The GRK family members share significant sequence homology and are composed of three domains: a highly conserved catalytic domain flanked by a conserved amino-terminal domain that contains an RGS like domain (implicated in receptor recognition) and a variable C terminal domain (that mediates membrane association of GRKs). Based on sequence and function homology, the family of GRKs can be further subdivided into three groups: GRK 1 and 7; GRK 2 and 3; and GRK 4, 5 and 6 (Ferguson, 2001).

Several factors influence the activity of these enzymes towards receptors. This includes the conformation of the receptor and association of kinases with the membrane (Pierce et al., 2002). GRKs 2 and 3 are primarily cytosolic, which translocate to the plasma membrane upon agonist stimulation. In contrast, GRKs 4, 5 and 6 are associated with the plasma membrane (Pitcher et al., 1998). Despite the differences between the GRK families, membrane association of these kinases appear to be mediated at least in part by determinants in their carboxyl-terminal domains (Stoffel et al., 1997). The various mechanisms responsible for membrane targeting of enzymes include isoprenylation (Koch et al., 1993), protein acylation (Stoffel et al., 1994) and interaction with lipid ligands (PtdIns(4,5)P2 and phosphatidyserine; PS). Each GRK utilises distinct mechanisms for membrane association, making it unique within the family.

1.3.2 Second messenger kinase-mediated regulation of GPCR signalling

PKA and PKC mediate a heterologous or "non-agonist-specific" desensitisation (i.e. do not require an agonist occupied receptor) by uncoupling the receptors from the G-proteins (Lefkowitz, 1998). The ability of these second messenger kinases to phosphorylate both agonist occupied and non-occupied receptors is responsible for the
concentration-response curves for homologous phosphorylation to be similar to the activation of second messenger kinases but not the agonist occupancy curve (Tobin, 1997).

In addition to GPCRs, second messenger kinases are known to phosphorylate effectors such as adenylyl cyclases and PLC-β (Section 1.4). PKC activation leads to the phosphorylation and desensitisation of many Gαq/11 and Gαi-coupled receptors (Liang et al., 1998; Tang et al., 1998). Recent studies have shown that besides regulating receptor signalling, PKA can mediate switching in G-protein coupling specificity. For example, the β2-adrenoceptor is a Gαs-coupled GPCR. However, PKA mediated phosphorylation of the receptor allows an increase in Gαi coupled signalling and a decrease in Gαs coupled signalling. This facilitates activation of pathways such as stimulation of ERK/MAPK pathway (Section 1.6.1.1; Zamah et al., 2002).

1.3.3 GRK and second messenger independent kinase-mediated regulation of GPCR signalling

A variety of GPCRs including the β2-adrenoceptor, muscarinic M3 receptor and bradykinin B2 receptor can be phosphorylated by kinases distinct from GRKs in vitro (Blaukat et al., 2001; Budd et al., 2001; Fan et al., 2001). For example, casein kinase 1α (CK1α) phosphorylates the third intracellular loop of the muscarinic M3 receptor in an agonist-dependent manner. The ability of this kinase to drive receptor phosphorylation is not limited to the muscarinic M3 receptor, as it has also been shown to phosphorylate rhodopsin and muscarinic M1 receptors (Tobin et al., 1997; Waugh et al., 1999). There are three consensus sequences for CK1α phosphorylation, designated as Type I, Type II and Type III sites (Tobin, 2002). In a number of GPCRs, the C-terminal tail has the consensus sequences for phosphorylation by CK1α including angiotensin II AT1 receptors, bradykinin B2 receptors and α1D-adrenoceptors. This indicates the possible broad receptor substrate specificity of CK1α.

CK1α belong to a family of kinases broadly classified as CK1 and CK2. Despite similarity in their names these kinases do not share any structural relationship. CK2, traditionally classified as a messenger-independent protein kinase, is found ubiquitously distributed in eukaryotic organisms. Like CK1, CK2 regulates a myriad of cellular
functions including maintenance of cell viability, cell cycle regulation and cell division (Litchfield, 2003). The CK1 family consist of seven members (CK1 α, β, γ1-3, δ and ε) that are expressed ubiquitously. CK1α is the smallest member of the family consisting of only 325 amino acids (Tobin, 2002). In addition to its well-known function in phosphorylating cellular substrates, recent studies have highlighted the importance of the CK1 family in the regulation of proteins involved in control of cellular differentiation, proliferation, chromosome segregation and circadian rhythms. Mutations in the coding sequences of CK1 isoforms have been implicated in neurodegenerative diseases and cancer (Knippschild et al., 2005). CK1 is also involved in the regulation of synaptic transmission in the brain (Chergui et al., 2005). Using CK1 inhibitors, evidence was provided for the involvement of CK1 in decreasing NMDA receptor activity in the striatum via activation of protein phosphatases 1 and 2A resulting in increased dephosphorylation of NMDA receptors (Chergui et al., 2005).

1.3.4 Role of arrestins in receptor regulation

Activation of a GPCR by ligands often results in the loss of responsiveness or desensitisation of the receptor. Phosphorylation of the receptor promotes the binding of arrestins to the receptor, which sterically interferes with the receptor-G-protein interaction (Lefkowitz, 1998). Four different families of arrestins have been cloned and studied including visual arrestin, β-arrestin 1 and 2 and cone arrestin (Attramadal et al., 1992; Craft et al., 1994; Lohse et al., 1990; Wilden et al., 1986). Visual arrestin and cone arrestin are expressed in the retina while β-arrestin 1 and 2 are distributed throughout the body, with higher expression in the spleen and brain (Attramadal et al., 1992). In addition to their roles in receptor desensitisation, arrestins have also been implicated in receptor internalisation (Ferguson et al., 1996). Once the receptors are internalised they either undergo dephosphorylation and are recycled to the plasma membrane or are targeted for degradation (down-regulation).

The involvement of arrestins in receptor internalisation varies significantly depending on the receptor, agonist and cell type (Luttrell & Lefkowitz, 2002). Of the four family members, only β-arrestins are known to be involved in GPCR endocytosis. Chimeric constructs of arrestins consisting of the carboxyl-tail of β-arrestins replaced by the equivalent regions of visual arrestins fail to promote receptor internalisation (Krupnick
et al., 1997). A β2-adrenoceptor mutant defective in its ability to sequester was utilised to show that over-expression of β-arrestins rescued the sequestration phenotype of the mutant, where over-expression of GRKs alone failed to rescue sequestration. However, an enhancement of sequestration of the mutant was observed on simultaneous expression of GRK 2 and arrestins, highlighting a coordinating role for arrestins and GRKs in receptor internalisation (Ferguson et al., 1996).

Phosphorylated receptors are internalised by several pathways. They include clathrin-coated pit-mediated endocytosis (mediated by β-arrestins), caveolae-mediated endocytosis and endocytosis mediated through uncoated pits (Claing et al., 2002). β-Arrestins 1 and 2 show a significant difference in their ability to mediate the internalisation of GPCRs. β-arrestins are composed of two domains, N and C, which allow them to function as adapter proteins linking GPCRs onto the clathrin-dependent endocytotic machinery. Further, β-arrestins were demonstrated to interact with clathrin cages in vitro (Goodman et al., 1997). Studies using cells from arrestin knockout mice, transfected with β2-adrenoceptors, showed a loss of receptor internalisation following agonist stimulation. Although both arrestins are equivalent in their ability to mediate desensitisation of β2-adrenoceptors, β-arrestin 2 is slightly more efficient in promoting internalisation (Kohout et al., 2001). This may reflect the higher affinity of β-arrestin 2 for clathrin and therefore more efficient recruitment to the phosphorylated receptor (Oakley et al., 2000). β-arrestins interact with the clathrin heavy chain, β2-adaptin, of the clathrin adaptor protein AP-2. When arrestin is recruited to the plasma membrane it becomes rapidly dephosphorylated. This is crucial for clathrin binding and the subsequent targeting of receptors to clathrin coated pits. These pits are then pinched off from the cell surface by the action of the GTPase, dynamin, allowing the receptors to be targeted for degradation or recycling.

Based on their ability to internalise, GPCRs can be divided into two distinct categories; class A receptors and class B receptors. For class A receptors, β-arrestin 2 translocates to the activated receptor more rapidly than β-arrestin 1. The receptor and arrestin co-localise in clathrin-coated pits and rapidly dissociate before the GPCR is targeted to endosomes. For class B receptors, β-arrestin 1 and 2 bind with equal affinity. However, the receptor-arrestin complex is more stable and is found to co-localise in endosomes.
(up to 4 hours before the receptor is finally recycled). Furthermore, class A receptors are also found to recycle more rapidly in comparison to class B receptors (Oakley et al., 2000).

Endocytosis of the receptors by clathrin dependent and independent mechanisms delivers the receptors to early endosomes. These are a heterogeneous population of membrane compartments with tubular-vesicular morphology located along the cell periphery (Hopkins et al., 1985). Receptors are either recycled to the plasma membrane from peripheral and perinuclear endosomes (a process referred to as resensitisation) or progress to the lysosomes for degradation (a process referred to as down-regulation); (Sorkin & von Zastrow, 2002). Down-regulation refers to a decrease in the total number of receptors present in cells or tissues, which is typically induced over a period of hours to days after prolonged or repeated exposure to an agonist. Although endocytosis is required for both rapid receptor internalisation and the slower receptor down-regulation it is still unclear whether these processes are mediated via a shared or separate membrane pathway (Tsao et al., 2001). However, emerging evidence suggest the presence of cytoplasmic sequences in certain GPCRs that promote sorting, of internalised GPCRs to lysosomes as well as sequences that promote or prevent the rapid recycling of receptors from the endocytotic vesicles to the plasma membrane (von Zastrow, 2003).

1.4 Heterologous interactions

As our understanding of the cellular pathways of GPCRs has advanced, it has become increasingly apparent that besides regulating linear transduction pathways, receptor activation also results in complex signalling patterns within cells. Such complexity may in part be a result of interactions between different signalling pathways, interactions between receptors or interactions at the level of G-proteins (Cordeaux & Hill, 2002). There is considerable evidence for the ability of GPCRs which couple preferentially to one signalling pathway to be regulated by inputs from another. This mode of regulation influences receptor specificity and efficacy, having huge implications for the pathophysiological functioning. Heterologous interactions or crosstalk between GPCRs can result in either enhancement or loss of receptor function (desensitisation). Enhanced receptor functioning may result from, for example, receptor-receptor interactions
Aspects of heterologous interactions including desensitisation and dimerisation will be discussed briefly below. Heterologous desensitisation can be mediated by PKA or PKC. This is independent of agonist stimulation and has the potential to induce phosphorylation and desensitisation of GPCRs (Vazquez-Prado et al., 2003). For example, phosphorylation by PKA of the Ins(l,4,5)P_3 receptors have been implicated in the potentiation of acetylcholine-mediated secretion from parotid acinar cells by cAMP elevating agents, like forskolin (Bruce et al., 2002).

Similarly PKC can mediate a key role in heterologous desensitisation. In Rat-1 cells expressing endogenous endothelin receptors and recombinantly the α_1B-adrenoceptor, the latter are phosphorylated rapidly in response to endothelin-1 (Vazquez-Prado et al., 1997). The effect of endothelin-1 was of a greater magnitude than noradrenaline suggesting rapid heterologous regulation in these cells. Similarly, in CHO cells expressing the muscarinic M_3 receptors and β_2-adrenoceptors, stimulation with carbachol resulted in a dose-dependent phosphorylation of β_2-adrenoceptors. This heterologous receptor phosphorylation coincided with receptor desensitisation, measured by loss of agonist binding and reduction of cAMP responses (Budd et al., 1999). A unidirectional regulation of GPCRs was also demonstrated in CHO cells co-expressing the bradykinin B_2 receptor and the muscarinic M_3 receptor. Stimulation of these cells with bradykinin induced muscarinic receptor phosphorylation. However, no alteration in the functional response to the stimulation of the muscarinic receptors was observed (Willars et al., 1999). Another instance of heterologous desensitisation, activation of the muscarinic M_3 receptors induced α_1B-adrenoceptor desensitisation and phosphorylation via PKC in cells expressing the muscarinic M_3 receptor and the α_1B-adrenoceptor. The activation of α-adrenoceptors desensitised the muscarinic receptors without influencing receptor phosphorylation (Bundey & Nahorski, 2001).

Enhanced receptor function may also result from dimerisation of GPCRs. Biochemical and fluorescence studies have demonstrated that GPCRs can form and function as dimers or oligomers (Angers et al., 2002; Rios et al., 2001; Terrillon & Bouvier, 2004). Dimerisation may be between the same receptor subtypes (homodimers) or between receptors of different subtypes (heterodimers). A classic example of dimerisation required for receptor functioning is the GABA receptors. When genes encoding the
GABA_{b}R1 and GABA_{b}R2 were cloned and expressed individually, low affinity receptors were obtained in comparison with the endogenous receptors expressed in brain membranes. However, when expressed together, fully functional receptors with affinity equivalent to the endogenous receptors were observed (Pin et al., 2005; White et al., 1998). This example demonstrates that GABA receptors require dimerisation to traffic and function. However, the requirement for dimerisation to allow receptor functioning may not be obligatory for all GPCRs.

Heterodimerisation between the angiotensin II AT_{1} receptor and bradykinin B_{2} receptor is an example of dimerisation resulting in enhanced receptor signalling. Dimerisation between the two receptor subtypes results in enhanced G-protein coupling, inositol phosphate responses and subsequent Ca^{2+} signalling to vasoactive hormones. Although heterodimerisation influenced G-protein coupling, it was agonist-independent. Potentiated responses required co-expression, but not co-stimulation (AbdAlla et al., 2000). Similarly, heterodimerisation between the \beta_{2}-adrenoceptor and the \alpha_{1D}-adrenoceptor also resulted in an enhancement of cell surface expression, Ca^{2+} mobilisation and internalisation of the \alpha_{1D}-adrenoceptor that is normally poorly trafficked to the cell surface and largely non-functional when expressed by itself in a variety of cell types (Uberti et al., 2005). These findings give a clear example of the functional relevance of dimerisation in receptor trafficking, expression and signalling.

Heterologous interactions resulting in the gain of receptor functions, (referred to as crosstalk), were traditionally known to be mediated by levels of effector activation and second messenger production. Activation of GPCRs results in a range of concurrent effects on signalling proteins including PLC, adenylyl cyclase and ion channels. The nature of this depends on intracellular signalling proteins, the threshold for activation and levels at which signalling pathway is measured. In the following sections crosstalk between differentially coupled GPCRs and potential mechanisms by which they occur will be discussed.

1.4.1 *Crosstalk at effector levels*

Crosstalk between different pathways allows cells to integrate information from several sources, providing a system for regulation of cellular functions. Crosstalk between GPCRs has been demonstrated by the co-ordinated measurement of a number of
different functional outputs including cAMP, phosphoinositide signalling and Ca\(^{2+}\) mobilisation. Research in immortalised model cell lines such as HEK 293 cells or CHO-K1 cells recombinantly expressing GPCRs have demonstrated crosstalk between differentially coupled receptors. However, evidence is also emerging for this phenomena in primary cells, reflecting that crosstalk is physiologically relevant and not simply due to over expression of receptors in immortalised cells.

Cytosolic Ca\(^{2+}\) signal exerts control over a broad range of cellular processes. The specificity of these signals is encoded by frequency, amplitude and subcellular localisation of responses (Bruce *et al.*, 2003). Intracellular Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_{i}\)) stores influence Ca\(^{2+}\) signals evoked by Ca\(^{2+}\) entry by sequestering incoming Ca\(^{2+}\), by regulating store operated capacitative Ca\(^{2+}\) entry and through their ability to increase [Ca\(^{2+}\)]\(_{i}\), thereby exerting stimulatory or inhibitory effects on Ca\(^{2+}\) entry. Ca\(^{2+}\) entry in turn influences [Ca\(^{2+}\)]\(_{i}\), stores as the intracellular Ca\(^{2+}\) channels (Ins(1,4,5)P\(_3\) or ryanodine receptors) are regulated by cytosolic Ca\(^{2+}\) and Ca\(^{2+}\) induced Ca\(^{2+}\) release (CICR), allowing amplification of responses (Taylor & Broad, 1998). Crosstalk between GPCRs resulting in enhanced mobilisation of Ca\(^{2+}\) may be due to the regulation of the processes described above or by concomitant regulation of additional signal transduction pathways, like cAMP. The effects of Ca\(^{2+}\) on cAMP are of particular significance as they allow co-ordination between two of the most ubiquitous intracellular signalling pathways.

Ca\(^{2+}\) can regulate components of the cAMP signalling machinery by either activating or inhibiting different adenylyl cyclases and phosphodiesterases (Section 1.6.1.1). Recent studies have highlighted the ability of these distinct pathways to co-ordinate signalling at multiple levels (Gorbunova & Spitzer, 2002; Rich *et al.*, 2001). A good example of crosstalk that results in synergism of cAMP on Ca\(^{2+}\) signalling is the study in parotid acinar cells (Bruce *et al.*, 2002). cAMP elevating agents such as forskolin exert synergistic control over fluid secretion and exocytosis in acinar cells, stimulated by muscarinic activation of [Ca\(^{2+}\)]\(_{i}\) signalling. Crosstalk that results in inhibition of adenylyl cyclases by Ca\(^{2+}\) release has also been demonstrated. In aortic smooth muscle cells, inhibition of adenylyl cyclases were found to be mediated by stimulation of the vasopressin receptor (V\(_{1a}\)) by arginine\(^8\)-vasopressin by the Ins(1,4,5)P\(_3\)-mediated transient release of Ca\(^{2+}\) (Dyer *et al.*, 2005). These examples highlight the roles of
[Ca\textsuperscript{2+}]\textsubscript{i} in mediating crosstalk in physiologically relevant cells. A review detailing aspects of crosstalk resulting in enhanced release of [Ca\textsuperscript{2+}]\textsubscript{i}, including potential mechanisms by which they may occur has been recently published (Werry et al., 2003). There are considerable diversity in the mechanisms by which crosstalk occurs between Ga\textsubscript{s}-Ga\textsubscript{q11}, Ga\textsubscript{q11}-Ga\textsubscript{i} and Ga\textsubscript{i}-Ga\textsubscript{s} receptors. The following sections highlight some potential mechanisms by which crosstalk occur between differentially coupled receptors.

1.4.1.1 Crosstalk between Ga\textsubscript{s} and Ga\textsubscript{q11}-coupled receptors

Signalling mediated by the activation of Ga\textsubscript{s} and Ga\textsubscript{q11}-coupled receptors activate two distinct pathways however, these can interact at various levels. For example, elevation of intracellular Ca\textsuperscript{2+} following PLC activation by Ga\textsubscript{q11} could lead to an increase or decrease in cAMP production by influencing Ca\textsuperscript{2+} sensitive adenylyl cyclases (Section 1.6.1.1). Other possible levels of interaction between the two signalling pathways are depicted in Figure 1.4.1.1.

Depending on the type of adenylyl cyclase present, G\textsubscript{pr} subunits regulate its activity either positively through adenylyl cyclase 2, 4 and 7 or negatively through adenylyl cyclase 1, 5 and 6 (Hanoune & Defer, 2001). In type-1 cultured rat cerebellar astrocytes, the potentiation (by adenosine mediated through Ga\textsubscript{s}-coupled adenosine A\textsubscript{2B} receptor) of Ga\textsubscript{q11}-coupled purinergic receptor mediated Ca\textsuperscript{2+} response is due to the action of G\textsubscript{pr} subunits released from Ga\textsubscript{s}, as pre-treatment of cells with forskolin or dibutyryl-cAMP did not potentiate the Ca\textsuperscript{2+} responses [Figure 1.4.1.1; Blue arrow 1; (Jimenez et al., 1999)]. Similarly the G\textsubscript{pr} subunits released by Ga\textsubscript{q/11}-coupled receptors can activate adenylyl cyclases as shown by the ability of muscarinic M\textsubscript{1} receptors to potentiate the stimulation of adenylyl cyclase by corticotrophin-releasing hormone in rat frontal cortex [Figure 1.4.1.1; Blue arrow 2; (Onali & Olianas, 2001)].
Fig. 1.4.1.1. Potential interactions between signalling pathways of Ga and Ga$_{q/11}$-coupled receptors. Stimulation of Ga and Ga$_{q/11}$-coupled receptors results in the activation of adenylyl cyclase and PLC respectively resulting in second messenger production (black arrows). Numbered blue arrows represent potential interactions between the two signalling pathways. 1. $\beta$ subunits from Ga-coupled receptors may activate PLC potentiating a Ga$_{q/11}$-mediated response 2. Likewise $\beta$ subunits from Ga$_{q/11}$ may stimulate particular adenylyl cyclase isoforms 3. PKA may phosphorylate and deactivate PLC 4. PKA may phosphorylate and modulate the activity of Ins(1,4,5)P$_3$ receptors 5. PKC may phosphorylate and activate adenylyl cyclase isoforms 6. Ca$^{2+}$ may activate or inhibit adenylyl cyclase isoforms (Cordeaux & Hill, 2002). Further details in text.

Using mutational analysis of G$\beta$ residues that interact with Ga-GDP, the G$\beta_7$ subunits in particular, have been shown to stimulate and modulate both PLC and adenylyl cyclase activity (Ford et al., 1998). Analysis of the mutant’s ability to regulate the activity of PLC-β and adenylyl cyclase revealed that G$\beta$ residues are required for the activation of each effector and provide evidence for partially overlapping domains for regulation of these effectors. This organisation provides an explanation as to why subunit dissociation is crucial for signal transmission through the G$\beta_7$ subunits (Ford et al., 1998).
The ability of PKA to regulate PLC-β was proposed by Liu and Simon (1996). They reconstituted the G_{βγ} stimulated PLC pathway in COS-7 cells in the presence or absence of the catalytic subunit of PKA. Co-transfection of G_{βγ} subunits and PLC-β2 lead to an approximate 5-fold increase in PLC activity that was completely inhibited by the catalytic subunit of PKA (Figure 1.4.1.1; Blue arrow 3). However, PKA failed to regulate Ga_{q11} stimulated PLC isoforms and PKC failed to mimic the effect of PKA. A recent emerging regulatory role of PKA is by mediating phosphorylation of Ins(1,4,5)P_{3} receptors (Figure 1.4.1.1; Blue arrow 4). In mouse parotid acinar cells, PKA-mediated phosphorylation of Ins(1,4,5)P_{3} receptors results in potentiation of Ca^{2+} signalling to cAMP elevating agents such as forskolin (Bruce et al., 2002). Recently, an extension of this study in human parotid acinar cells similarly demonstrated a role for PKA-mediated phosphorylation of Ins(1,4,5)P_{3} in mediating crosstalk (Brown et al., 2004).

PKC stimulated by the production of DAG can activate certain isoforms of adenylyl cyclases [Figure 1.4.1.1; Blue arrow 5; (Hanoune & Defer, 2001)]. These isoforms are the Ca^{2+} insensitive adenylyl cyclase 2, 4 and 7 [Section 1.6.1.1; (Cooper, 2003)]. Interactions between the two pathways were studied using recombinantly expressed Ga_{s}-coupled vasopressin V_{2} receptors and Ga_{q11}-coupled vasopressin V_{1α} receptors. The simultaneous activation of both receptors by vasopressin resulted in the accumulation of cAMP that was twice the level induced by vasopressin V_{2} receptor activation alone. This potentiation of cAMP responses was mimicked by the activation of PKC by PMA and abolished in the presence of PKC inhibitors. It was also unaffected by the presence of phosphodiesterases, suggesting that an alteration of cAMP hydrolysis was not responsible for the enhanced cAMP responses (Klingler et al., 1998).

Similarly, in CHO cells recombinantly expressing the Ga_{q11}-coupled-angiotensin (AT_{1α}) receptor and vasopressin V_{2} receptors, co-stimulation with angiotensin and vasopressin potentiated the production of cAMP in comparison to cells stimulated with vasopressin alone. This potentiation was shown also to be mimicked by phorbol esters and inhibited by PKC inhibitors (Klingler et al., 1998). More recently, evidence for the ability of PKC to regulate adenylyl cyclase was demonstrated in rat pancreatic islets (Tian & Laychock, 2001). The ability of cAMP to stimulate secretion of insulin in the presence of glucose in rat pancreatic islet cells is well documented (Tian & Laychock, 2001). Carbachol stimulation of these cells, in the presence of glucose, resulted in a
concentration-dependent potentiation of cAMP accumulation, which was abolished by PKC inhibitors (GF109203XC and RO-32-0432) or by PKC down-regulation using 20h phorbol ester (PMA) treatment.

1.4.1.2 **Crosstalk between Gαi and Gαi-coupled receptors**

Activation of Gαi-coupled receptors inhibits cAMP accumulation and activity of PKA in contrast to Gαs-coupled receptors. The Gβγ subunits also have a significant role in effector modulation (Figure 1.4.1.2; Blue arrow 1). It has been demonstrated that activation of Gαi-coupled receptors sensitises adenylyl cyclases to subsequent Gαs stimulation (reviewed by Watts & Neve, 2005). This agonist-induced sensitisation is dependent on the isoform of adenylyl cyclase expressed and the ability of Gβγ subunits to enhance its activity (Thomas & Hoffman, 1996). While all isoforms of adenylyl cyclase are activated by Gαs, isoforms 2, 4 and 7 are activated by Gβγ in the presence of Gαs and isoforms 1, 5 and 6 are inhibited by Gβγ subunits (Cooper, 2003; Cordeaux & Hill, 2002). In rat brain frontal cortex, prestimulation of GABAB receptors with either baclofen or GABA induced a concentration-dependent potentiation of cAMP responses to corticotrophin-releasing hormone (Onali & Olinas, 2001). This potentiation was pertussis toxin-sensitive implicating a role for Gai0 G-proteins, while the use of the βγ scavenger Ga4 abolished the potentiation implicating Gβγ in this process. The ability of Gai-coupled receptors to potentiate Gαs-mediated adenylyl cyclase activity has also been demonstrated for a number of other receptors including muscarinic M4 receptors, dopamine D4 receptors and α2 adrenoceptors (Mhaouty-Kodja et al., 1997; Onali & Olinas, 1995; Watts & Neve, 1997).

In addition to isoform specific regulation by G-protein subunits, the second messenger kinases PKA and PKC can also regulate adenylyl cyclase activity (Figure 1.4.1.2; Blue arrow 2). In HEK 293 cells transfected with adenylyl cyclase 2, stimulation of PKC by phorbol esters facilitated the activation of this isoform by Gαi-coupled receptors (Tsu & Wong, 1996). Similarly in HEK 293 cells expressing the Gαi-coupled dopamine D2L receptors and adenylyl cyclase 6, activation of PKC robustly enhanced dopamine-receptor mediated adenylyl cyclase sensitisation resulting in potentiation of the cAMP responses (Beazely & Watts, 2005). Possible levels of interaction between the two signalling pathways are depicted in Figure 1.4.1.2.
Fig. 1.4.1.2. **Potential interactions between signalling pathways of Ga, and Ga,-coupled receptors.** Stimulation of Ga, and Ga,-coupled receptors results in the activation or inhibition of adenylyl cyclase activity respectively (black arrows). Numbered blue arrows represent potential interactions between the two signalling pathways. 1. βγ subunits from Ga,-linked receptors may activate adenylyl cyclase isoforms potentiating Ga, mediated responses 2. PKC may phosphorylate and regulate adenylyl cyclase activity. Further details in text.

1.4.1.3 **Crosstalk between Gaq11 and Ga,-coupled receptors**

Activation of Gaq11 and Ga,-coupled receptors results in the stimulation of PLC that mediates the regulation of various cellular functions. There are 11 isoforms of PLC, which catalyse the hydrolysis of PtdIns(4,5)P2 to Ins(1,4,5)P3 and DAG. The PLC isoforms identified to date are all single polypeptides and consist of five subtypes: β(1-4), γ(1-2), δ(1-4), ε and ζ (Rhee, 2001; Saunders et al., 2002). The extensively studied PLC-β isoforms are activated by either the Ga and βγ subunits of Gaq11-coupled receptors or the βγ-subunits most usually from Ga,-coupled receptors (Rhee & Bae, 1997). Although all PLC-β(1-4) isoforms are activated by Ga (rank order for activation: PLC-β1 ≥ PLC-β3 ≥ PLC-β4 ≥ PLC-β2) and βγ subunits, the relative sensitivity with which they are activated varies, with PLC-β1 being least sensitive to Gβγ subunits.
(Rhee, 2001). There is a wealth of literature investigating crosstalk between the \( \text{G} \alpha_{q/11} \) and \( \text{G} \alpha_{i} \)-coupled receptors, resulting in enhanced phosphoinositide responses (Werry et al., 2003). The extent to which \( \text{G} \alpha_{i} \)-coupled receptors can augment \( \text{G} \alpha_{q/11} \)-mediated responses often depends on receptor expression levels. For example, \( \text{G} \alpha_{i/0} \)-coupled adenosine receptors recombinantly expressed in CHO cells were able to potentiate \( \text{G} \alpha_{q/11} \)-coupled purinergic receptor-mediated phosphoinositide responses. This potentiation could not be mimicked by the activation of endogenously expressed \( \text{G} \alpha_{i/0} \)-coupled 5-HT\(_{1B} \) receptors with receptor levels undetectable by saturation antagonist binding (Dickenson & Hill, 1996). However, in cells expressing ‘moderate’ receptor numbers (as determined by saturation antagonist binding) 5-HT\(_{1B} \) receptor agonist sumatriptan enhanced purinergic receptor mediated phosphoinositide responses. This indicates that levels of receptor expression influence crosstalk (Dickenson & Hill, 1998). The various levels of interactions between the two signalling pathways are depicted in Figure 1.4.1.3.

Regulation of PLC activity is perhaps one of the most straightforward means by which crosstalk is mediated via \( \text{G} \alpha_{q/11} \) and \( \text{G} \alpha_{i} \)-coupled receptors subtypes. The extensively studied PLC-\( \beta(1-3) \) isoforms have distinct binding sites for \( \text{G} \alpha \) and \( \text{G} \beta_{y} \) subunits (Lee & Fraser, 1993; Smrcka & Sternweis, 1993). Several studies have attempted to address the importance of \( \text{G} \beta_{y} \) subunits in coupling of \( \text{G} \alpha_{i} \)-coupled receptors to PLC activity. Transfection of \( \text{G} \beta_{y} \) subunits in COS-M6 cells significantly increased basal inositol phosphate accumulation, demonstrating the ability of this subunit to activate PLC-\( \beta \) (Zhu & Birnbaumar, 1996). Activation of muscarinic M\(_{2} \) receptors in cells over expressing only \( \text{G} \beta_{y} \) subunits elicited agonist-mediated PLC responses that were pertussis toxin insensitive, in comparison to cells expressing the \( \text{G} \alpha_{i/0} \beta_{y} \) subunits. These results indicate that \( \text{G} \beta_{y} \) subunits can stimulate PLC. Using constitutive active mutants of different \( \text{G} \alpha_{q/11} \) members, it was demonstrated that preactivation of PLC-\( \beta \) is required for \( \text{G} \alpha_{i} \)-mediated potentiation of inositol phosphate responses mediated via the \( \text{G} \beta_{y} \) subunits (Chan et al., 2000). In CHO-K1 cell expressing the neuropeptide Y\(_{2} \) receptors, transfection of the \( \text{G} \beta_{y} \) scavengers \( \alpha \)-transducin or GRK2 residues 495-689, abolished augmentation of P\(_{2} \)Y\(_{2} \) purinergic receptor stimulated responses by neuropeptide Y\(_{2} \) receptor. This provides evidence that \( \text{G} \beta_{y} \) subunits of the \( \text{G} \alpha_{i} \)-coupled neuropeptide receptor are involved in the amplification of ATP stimulated PLC responses (Selbie et
In addition to enhanced PLC activity, crosstalk may also result in the ability to augment \( \text{Ga}_{\text{q11}} \)-mediated arachidonic acid release (Selbie et al., 1995; Selbie et al., 1997). Crosstalk between the \( \text{Ga}_{\text{i}} \)-coupled neuropeptide \( \text{Y}_1 \) receptor and the \( \text{Ga}_{\text{q11}} \)-coupled \( \alpha_{1B} \)-adrenoceptor resulted in potentiation of arachidonic acid release mediated via the \( \alpha_{1B} \)-adrenoceptor in the presence of peptide YY. Depletion of PKC following chronic treatment with PMA abolished any potentiation of arachidonic acid. This demonstrates that the ability of \( \alpha \)-adrenoceptor agonist to generate arachidonic acid is mediated by both the activation of PKC and direct coupling of the receptor to phospholipase A2 (PLA2; Selbie et al., 1995). Using the alpha transducin subunit, the augmentation of arachidonic acid release by \( \alpha_{1B} \)-adrenoceptor was demonstrated to be dependent on \( G_{\beta Y} \) subunits of neuropeptide \( \text{Y}_1 \) receptors (Selbie et al., 1997).

**Fig. 1.4.1.3. Potential interactions between signalling pathways of \( \text{Ga}_{\text{i}} \) and \( \text{Ga}_{\text{q}} \)-coupled receptors.** Stimulation of \( \text{Ga}_{\text{i}} \) and \( \text{Ga}_{\text{q11}} \)-coupled receptors results in the inhibition of adenylyl cyclase activity and activation of PLC respectively (black arrows). Numbered blue arrows represent potential interactions between the two signalling pathways. 1. \( \beta Y \) subunits released by \( \text{Ga}_{\text{i}} \) activation may stimulate PLC potentiating responses mediated by \( \text{Ga}_{\text{q11}} \). Alternatively, the phosphorylation of PLC by PKC may be regulated by the \( \beta Y \)-subunits. 2. \( \beta Y \) subunits may also be exchanged between the two-receptor subtypes (Cordeaux & Hill, 2000). Further details in text.
1.4.2 Physiological and experimental relevance of crosstalk

Heterologous interactions between differentially coupled receptors can result in the enhancement or reduction of physiological responses. In prefrontal cortex pyramidal neurons, application of dopamine D1 receptor agonists causes a significant increase of steady state N-methyl-D-aspartate receptor (NMDAR) evoked current (Chen et al., 2004). This interaction is of importance as this region of the brain is associated with control of cognition and emotions. Moreover, increase of NMDAR activity influences schizophrenia-like behaviour, and regulation of NMDAR function via the dopamine D1 receptors has great therapeutic implication. Physiologically relevant crosstalk also occur between purinergic Goq11-coupled P2Y1 and Gao-coupled P2Y12 receptors in human platelets (Hardy et al., 2004). P2Y12 receptors contribute to platelet signalling by potentiation of P2Y1-induced Ca2+ responses. This crosstalk provides a delicate interaction, crucial for platelet activation and inhibition during normal hemostasis. Recently, in type-1 cerebellar astrocytes, the activation of Gao-coupled adenosine A2B receptors was demonstrated to potentiate Ca2+ mobilisation in response to ATP, mediated by the Goq11-coupled purinergic receptors (Jimenez et al., 1999). Astrocytes maintain close proximity to neurons and other cell types in the nervous system and their functions are affected by release of ATP and other dinucleotides, suggesting that these may be important mediators of communication (Jimenez et al., 1999). In yet another example of a physiologically relevant crosstalk, activation of Gao-coupled parathyroid hormone in a rat osteoblast cell-line potentiates the Ca2+ release by Goq11-coupled purinergic receptor (Buckley et al., 2001). This potentiation was also demonstrated for the phosphorylation of the transcription factor CREB and induction of c-fos. Based on their observations, the authors suggest that release of nucleotides may act to sensitise osteoblasts to systemic factors such as parathyroid hormone resulting in the localised remodelling of bone (Buckley et al., 2001). Crosstalk can similarly augment a physiological response like smooth muscle contraction (refer to Section 1.6.1). Smooth muscle cells co-express both Gai and Goq11-coupled muscarinic receptors. The roles of Gao-coupled receptors in smooth muscle relaxation are detailed in section 1.6.1 Contraction of smooth muscle is regulated by changes in cytosolic Ca2+. The Gai-linked muscarinic receptor can amplify the Goq11-stimulated PLC activity resulting in enhanced Ca2+ release and subsequent potent contraction (Figure 1.4.2; Rang and Dale, Pharmacology, Fifth Edition).
The above examples illustrate the relevance of examining crosstalk in a recombinant or endogenous system. GPCRs are ubiquitously expressed and often multiple subtypes are co-expressed. Therefore the potential for crosstalk in every cell is enormous and of great interest. In this thesis, crosstalk between Ga_q/11-coupled muscarinic M_3 receptors and Ga_o-coupled β_2-adrenoeceptors were investigated in an attempt to characterise the molecular interactions. These receptor subtypes are co-expressed extensively in several tissues including smooth muscle cells. The following sections describe these receptor families and particular receptor subtypes.

**Fig.1.4.2. Interactions between Ga_q/11 and Ga_o-coupled receptors resulting in enhanced smooth muscle contraction.**

### 1.5 The Muscarinic receptor family

Acetylcholine is a major neurotransmitter in the central and peripheral nervous systems. This neurotransmitter mediates its actions through two distinct classes of plasma membrane receptors, the ionotropic nicotinic receptors and the metabotropic muscarinic receptors. Nicotinic receptors function as ligand-gated ion channels. Muscarinic receptors are GPCRs belonging to the biogenic amine family (family A). These receptors are able to mediate the actions of acetylcholine, regulating many physiological responses including smooth muscle contraction, glandular secretion, motor control,
temperature control, the cardiovascular system and memory (Caulfield & Birdsall, 1998; Wess et al., 1993).

Historically, existence of several muscarinic receptor subtypes was revealed by the cardioselective properties of gallamine (reviewed by Caulfield & Birdsall, 1998). Five distinct muscarinic receptors have since been cloned and identified. The muscarinic M₁ and M₂ receptor subtypes were cloned by screening cDNA libraries from porcine cerebral and cardiac tissues respectively (Kubo et al., 1986). While, muscarinic M₃, M₄ and M₅ receptors were subsequently cloned by screening genomic and cDNA libraries from brain tissue (Bonner et al., 1987; Bonner et al., 1988; Peralta et al., 1987). The chromosomal localisations of the muscarinic m₁-m₅ genes are 11q12-13, 7q35-36, 1q43-44, 11p12-11.2 and 15q26 respectively (Bonner et al., 1987).

The distribution of muscarinic receptors in central and peripheral tissues has been investigated mainly by pharmacological studies with subtype selective agents or by molecular, immunohistochemical or hybridisation studies (Caulfield, 1993; Matsui et al., 2004; Wess, 2004). Messenger RNA (mRNA) for muscarinic M₁ receptors is abundantly expressed in the brain including the cerebral cortex, hippocampus and corpus striatum. This subtype contributes 40-50% of the muscarinic receptor population in the brain (Matsui et al., 2004). Predominant expression of muscarinic M₄ receptors in the rat brain striatum was verified by immunoprecipitation studies with selective polyclonal sera (Yasuda et al., 1993). These studies identified that muscarinic M₂ receptors constitutes the majority of muscarinic receptors in rat brain stem (Levey et al., 1991). Muscarinic M₃ receptor mRNA have been detected in the endocrine glands, heart and smooth muscles while muscarinic M₅ mRNA is least abundant in the brain. Tissue expression of the receptor subtypes is summarised in Table 1.5.

In most tissues, different muscarinic receptors are often co-expressed. However, the most abundant subtype does not necessarily exert the predominant biological effects. For example, in most smooth muscle cells, muscarinic M₃ receptors and muscarinic M₂ receptors are expressed in a ratio of 1:4 (Eglen et al., 1994). However, the muscarinic M₃ receptors have been largely implicated in smooth muscle contraction and cholinergic transmission despite the predominant expression of muscarinic M₂ receptors (Ehlert et al., 1999).
Table 1.5. Tissue distribution of muscarinic receptors based on mRNA distribution and immunoprecipitation studies of receptor proteins using antibodies (Hulme et al., 1990; Wess, 2004).

<table>
<thead>
<tr>
<th>Receptor Subtype</th>
<th>Tissue Distribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>$M_1$</td>
<td>Cortex, hippocampus, glands and sympathetic ganglia</td>
</tr>
<tr>
<td>$M_2$</td>
<td>Heart, hindbrain, ileum, uterus and smooth muscle</td>
</tr>
<tr>
<td>$M_3$</td>
<td>Smooth muscles, secretory glands, heart and forebrain</td>
</tr>
<tr>
<td>$M_4$</td>
<td>Cortex, striatum and hippocampus</td>
</tr>
<tr>
<td>$M_5$</td>
<td>Eye, brain stem nuclei and hippocampus</td>
</tr>
</tbody>
</table>

Consistent with characteristics of the family A GPCRs, these receptors share conserved features including the disulphide bridge linking the first and second extracellular loops (Kurtenbach et al., 1990) and the DRY motif at the cytoplasmic interface between the third transmembrane domain and second intracellular loop (Zhu et al., 1994). Evidence from biochemical and site directed mutagenesis studies have shown that the disulphide bridge links the two cysteine molecules that are conserved in most GPCRs (Boyd et al., 1996; Cook et al., 1996). This disulphide bond is crucial for ligand binding, while the cysteine molecules are crucial for cell-surface receptor expression (Zeng et al., 1999).

Other conserved residues of importance are aspartate 105 in transmembrane domain 3, threonine 231 and 234 in transmembrane domain 5, and tyrosine 506 and asparagine 507 in transmembrane domain 6 (numberings refer to the rat $M_3$ muscarinic receptor). These residues are crucial for either agonist binding and/or receptor activation (Huang et al., 1998).

Sequence alignment of the human muscarinic receptor subtypes have shown a 67% identity, the differences being amassed mainly by variations in the extracellular amino-terminus, cytoplasmic carboxyl-terminus and the third intracellular loop (3i loop). The greatest divergence between the sequences of $M_1$, $M_3$ and $M_5$ muscarinic receptors and $M_2$ and $M_4$ muscarinic receptors is found within the 3i loops; which determine the specific $G_{q/11}/G_{i/o}$ coupling preferences of the two sub-groups. Muscarinic $M_1$, $M_3$ and $M_5$ receptors demonstrate higher homology with each other in comparison to muscarinic $M_2$ and $M_4$ receptors. The muscarinic $M_1$ and $M_3$ receptor sequences show greater homology than the muscarinic $M_5$ receptors and this is more apparent in the 3i loop. The
muscarinic M_5 receptor sequences are marginally more similar to the muscarinic M_3 receptors than the muscarinic M_1 receptors (Hulme et al., 1990). Furthermore, the muscarinic M_1, M_3 and M_5 receptors couple preferentially to \( \text{G}_{i/11} \) family of G-proteins, leading to the activation of PLC, while muscarinic M_2 and M_4 receptors couple preferentially to \( \text{G}_{i/0} \) proteins leading to the inhibition of adenylyl cyclases (Caulfield, 1993).

The N-terminal region of the 3i loop is crucial for determining G-protein coupling specificity (Cotecchia et al., 1990). This was demonstrated using chimeric receptor constructs of muscarinic M_2/M_1 and M_2/M_3 receptors. When the 3i loop of the receptors was exchanged, the chimeric receptors exhibited coupling specificity similar to the receptor from which the 3i loop was derived (Kubo et al., 1988; Wess et al., 1989). The importance of the second intracellular loop and the carboxyl-terminal portion of the 3i loop in G-protein recognition have also been demonstrated (Blin et al., 1995). Studies with the rat M_1 muscarinic receptor have suggested that the ligand binding domain is a cavity enclosed by the transmembrane helices and ligand binding is initiated by ion-ion interaction between the positively charged amine moiety of the ligand and negatively charged aspartate residues present in transmembrane domains 2 or 3. Mutagenesis of aspartate-99 to asparagine results in an inability of the M_1 muscarinic receptor to bind the antagonist quinuclidinyl benzilate (QNB; Fraser et al., 1989). Further studies using site-directed mutagenesis of conserved threonine (Thr^{234}) and tyrosine (Tyr^{506}) residues of the rat M_3 muscarinic receptor have shown these residues to be critical for ligand binding affinity (Wess et al., 1992). Muscarinic receptors have considerable potential as therapeutic targets. Muscarinic receptor antagonists are currently used in the treatment of bronchial asthma, peptic ulcer and overactive bladder (Felder et al., 2001; Matsui et al., 2004). Despite the wide range of therapeutic applications, the clinical use of anti-muscarinic drugs is often limited by undesirable side effects.

1.5.1 The Muscarinic M_3 receptor

The focal point of this thesis is the muscarinic M_3 receptor. They are distributed widely in the peripheral autonomic organs with largest expression in the exocrine glands and smooth muscle tissues. Recently, the expression of the M_3 muscarinic receptors in the heart, atria and intrinsic cardiac neurons was also reported (Hassall et al., 1993; Wang et al., 2004; Hellgren et al., 2000; Wess, 2004). This receptor has been largely
implicated in the smooth muscle contractile functions in the airway, urinary bladder, stomach and ileum (Eglen et al., 1994; Wess, 2004). Muscarinic M₃ receptor knockouts have enabled the study of receptor subtype specific effects in the organs where muscarinic receptors are co-expressed. The muscarinic M₃ receptor was mapped to Chrm3 of the mouse genome. Using a specific targeting vector for Chrm3 disruption, muscarinic M₃ receptor genes were disrupted in mouse embryonic stem cells (Matsui et al., 2000). Gene disruption of the muscarinic M₃ receptor did not result in a significant upregulation of other muscarinic receptor subtypes (Yamada et al., 2001). However, these knockouts exhibited slower growth and weight gain in comparison to their wildtype littermates. There is conflicting evidence for the role of muscarinic M₃ receptors in salivary secretion. This is thought to be one of the contributing factors for differences in weight and growth rate of knockouts. One study reported that a single dose of muscarinic receptor agonist, pilocarpine (1mg/kg), induced salivation in wild type but not in homozygous knockouts, implicating an exclusive role for muscarinic M₃ receptors in salivation (Matsui et al., 2000). However, other studies have implicated a joint role for muscarinic M₁ and M₃ receptors in salivation (Gautam et al., 2004; Yamada et al., 2001).

Studies have also addressed the roles of muscarinic M₃ receptors in smooth muscle functioning in various tissues including the bladder, aorta and trachea. In homozygous mutants, all male animals suffered from severely distended urinary bladder in comparison to females that exhibited mild bladder distension (Matsui et al., 2002). Despite this, the urinary output and analysis were completely normal. Examination of detrusor smooth muscle function revealed a reduction of carbachol-induced contraction by 95% in homozygous animals in comparison to wild types (Matsui et al., 2000; Stengel et al., 2002). In mouse aorta, a role for muscarinic M₃ receptors was implicated in acetylcholine-induced nitric oxide release leading to vasodilation (Khurana et al., 2004). However, an independent study by Fisher et al., (2004) reported that bradycardia caused by vagal stimulation or administration of acetylcholine was abolished in muscarinic M₂ receptor knockouts. In contrast, heart rate was unaffected in muscarinic M₃ receptor knockouts (Fisher et al., 2004).

Tracheal and bronchial constrictions are also mediated by muscarinic M₃ receptors. Muscarinic agonist-induced bronchoconstriction consist of three phases. A robust
bronchoconstriction develop within minutes of agonist administration followed by a transient relaxation phase and finally, a sustained bronchoconstriction phase (Struckmann et al., 2003; Stengel et al., 2002). In muscarinic M₃ receptor knockout animals, bronchoconstriction induced by lower concentrations of agonists were comparable to wild types. However, with increasing concentration of agonists, bronchoconstriction was significantly reduced. The transient relaxation phase was unaffected in both genotypes. These results indicate that the muscarinic M₃ receptor affects peripheral airway tone, although involvement of other muscarinic receptor subtypes could not be ruled out (Struckmann et al., 2003). Other aspects of the muscarinic M₃ receptor pharmacology including ligand binding, G-protein coupling, signalling and regulation will be discussed in the following sections.

1.5.1.1 Structural basis of ligand binding and G-protein coupling

Accumulating evidence has suggested that in family A GPCRs, the endogenous ligands bind to the target receptor in a narrow cleft enclosed by the transmembrane domains, 10-15Å away from the membrane surface (Wess et al., 1995). Ligand binding causes a change in the arrangement of the transmembrane domains enabling the receptor to couple to and activate specific classes of G-proteins (Gether, 2000). Mutational studies and more recently, disulphide cross-linking studies have been used extensively to characterise the ligand binding domains of GPCRs. Site directed disulphide cross-linking studies involve two cysteine molecules situated in close proximity to form a disulphide link spontaneously or under mild oxidising conditions (Zeng et al., 1999). A rat muscarinic M₃ receptor was used as a model system to identify receptor structure and activation. The muscarinic M₃ receptor has 13 cysteine residues (Cys₁₁₁, ₁₄₀, ₂₂₀, ₅₁₆, ₅₁₉, ₅₃₂, ₅₄₂, ₅₄₆ and ₅₆₀), 9 of which are conserved across the muscarinic M₁-₅ receptor subtypes. Replacement of the three non-conserved cysteine residues (Cys²⁸⁹, ³₁₀ and ⁴₁⁹) in the 3i loop of the receptor did not affect ligand binding or functional properties of the receptor. However, the individual replacement of Cys¹⁴₀, ₂₂₀ or ₅₃₂ with alanine or serine residues completely obliterated ligand binding. This indicates that the 3i loop offers a high degree of conformational flexibility to enable receptor/ G-protein coupling on ligand binding.

More recent in situ cross-linking studies using these mutant receptors have shown that the structural changes associated with ligand binding bring the Cys²⁵⁴ in transmembrane
domain 5 closer to Cys\textsuperscript{489-492} of transmembrane domain 6, facilitating the formation of disulphide bonds (Ward \textit{et al.}, 2002). Studies in cysteine mutants were used to identify any agonist induced conformational changes in the ligand-binding pocket of the muscarinic M\textsubscript{3} receptor (Han \textit{et al.}, 2005). In the presence of an agonist, a serine to cysteine mutation (S151C) within transmembrane domain 3 lead to the formation of disulphide bond with Cys\textsuperscript{532} in transmembrane domain 7. It was proposed that acetylcholine binding to the receptor causes a conformational change in the extracellular segment of transmembrane domain 3 and 7, bringing them together (Han \textit{et al.}, 2005).

Mutational analysis of specific amino acids at the junction between transmembrane domain 6 and the third extracellular loop of the receptor have attempted to provide further insights to the process of ligand binding. Mutation of the serine-threonine residues conserved between muscarinic receptors M\textsubscript{1} and M\textsubscript{5} or asparagine-threonine conserved between muscarinic M\textsubscript{2}, M\textsubscript{3} and M\textsubscript{4} receptors to a tyrosine-proline residues, resulted in constitutive activity of all 5 receptor subtypes (Ford \textit{et al.}, 2002). The responses to carbachol and acetylcholine were increased by at least 5 fold in each mutated receptor subtype. The constitutive activity was inhibited by atropine; however, increasing concentrations of agonists overcame the inhibition. It was concluded that interactions between the transmembrane domain 3 and 6 stabilises the conformation of the receptor and transmembrane domain 6 acts as a ligand-dependent switch (Ford \textit{et al.}, 2002).

1.5.1.2 Signalling and regulation of the muscarinic M\textsubscript{3} receptor

Muscarinic M\textsubscript{3} receptors couple predominantly to the pertussis toxin insensitive G\textsubscript{aq/11} (Caulfield, 1993). Activation of this class of G-proteins leads to the stimulation of membrane-bound PLC-\(\beta\), which accelerates the hydrolysis of PtdIns(4,5)P\textsubscript{2} into DAG and Ins(1,4,5)P\textsubscript{3}. DAG activates membranes-bound protein kinase PKC, which in turn catalyses the phosphorylation of a variety of intracellular proteins. Ins(1,4,5)P\textsubscript{3} released acts on an Ins(1,4,5)P\textsubscript{3} receptors that are ligand gated Ca\textsuperscript{2+} channels present on the membrane of the endoplasmic reticulum, resulting in release of intracellular Ca\textsuperscript{2+}.

Recombinant muscarinic M\textsubscript{3} receptors have also been shown to couple to and activate pertussis toxin sensitive G\textsubscript{ao} G-proteins. This was demonstrated using an agonist
stimulated GTPyS binding to membrane preparations from cells and subtype specific immunoprecipitation of Ga subunits, photolabelled with [α-32P]GTP azidoanilide (Nahorski et al., 1997). Recently, this receptor has been implicated in the activation of PLC-ε (Evellin et al., 2002). Stimulation of Gaα-coupled receptors was demonstrated to activate a Ca2+ signalling pathway mediated through Epac (exchange protein directly activated by cAMP) that serves as a cAMP effector, inducing GTP-loading and subsequent activation of Rap2B, which in turn results in the specific activation of PLC-ε (Schmidt et al., 2001). Similarly, in cells expressing the muscarinic M3 receptor, using cAMP inhibitors and catalytically inactive GTPases, its signalling was also demonstrated to be dependent on cAMP and GTPase Rap2B. Transfection of PLC-ε potentiated total inositol phosphates accumulation and the subsequent Ca2+ flux. This potentiating effect was nullified in the presence of a catalytically inactive mutant of PLC-ε (Evellin et al., 2002).

Stimulation of the muscarinic M3 receptor with carbachol can also result in the activation of phospholipase D (PLD), measured as an accumulation of its transphosphatidyllation product, phosphatidyl ethanol (PtdEtOH; Schmidt et al., 1995). PLD hydrolysates the phospholipid, phosphatidylcholine, resulting in the formation of choline and phosphatidic acid. Phosphatidic acid in turn acts as a messenger activating cellular kinases, vesicular trafficking and certain proto-oncogenes (English, 1996; Schmidt et al., 1999). Studies in HEK 293 cells recombinantly expressing muscarinic M3 receptors, have also implicated the activation of Ga12 proteins in PLD activation (Rumenapp et al., 2001). PLD activation is independent of PLC activation and muscarinic receptor-mediated stimulation of PLD is rapidly desensitised in comparison to the PLC, indicating that distinct pathways activate these phospholipases.

Following agonist stimulation, the muscarinic M3 receptor is rapidly phosphorylated in an agonist dependent manner by several kinases including PKC, GRKs or CK1α (May et al., 1999; Tobin & Nahorski, 1993; Tobin et al., 1997). Agonist-mediated receptor phosphorylation by members of the GRK family is an established model for GPCR phosphorylation resulting in receptor desensitisation (Section 1.3.1). A range of GRKs has been implicated in phosphorylation of the muscarinic receptor. Initially, only GRK 2 and 3 were demonstrated to phosphorylate the receptor in an agonist-dependent
manner (Debburman et al., 1994; Wu et al., 2000). More recently, studies of endogenous muscarinic receptor populations in the human neuroblastoma cell line SH-SY5Y have shown that GRKs 3 and 6 also play a role in receptor phosphorylation (Willetts et al., 2001; Willets et al., 2002).

An alternative route to phosphorylation of the muscarinic M₃ receptor is involving CK1α. Expression of a catalytically inactive mutant of CK1α that acts in a dominant negative manner, inhibits agonist-mediated receptor phosphorylation by ~40% in COS-7 and HEK 293 cells. The functional role of phosphorylation of the muscarinic M₃ receptor was investigated using a mutant of the receptor that showed a ~80% reduction in agonist-mediated phosphorylation. This mutant underwent agonist-mediated desensitisation suggesting that, unlike many GPCRs, desensitization of the muscarinic M₃ receptor is not mediated by receptor phosphorylation. Contrary to the expectations that receptor phosphorylation is responsible for desensitisation of peak Ins(1,4,5)P₃ responses, as the mutant induced Ins(1,4,5)P₃ responses with identical temporal characteristic. Moreover, the peak Ins(1,4,5)P₃ response appears to be dramatically potentiated in the phosphorylation-deficient mutant. Pre-stimulation of the mutant also resulted in desensitisation of peak Ins(1,4,5)P₃ response indicating that phosphorylation may instead control the magnitude of the initial inositol phosphate responses (Budd et al., 2000). These results indicate that for muscarinic M₃ receptors, phosphorylation of the receptor may not be crucial for desensitisation.

Although arrestins binds to muscarinic M₃ receptors, the receptor can undergo an arrestin-independent, dynamin-dependent internalisation (Lee et al., 1998). Using a dominant negative mutant of clathrin, Hub, that causes the depletion of the light chain of clathrin causing the clathrin coated pits to be frozen at the membrane, the internalisation of the receptors was shown to occur via clathrin-coated pits. Recently, the importance of tubulins in GPCR signalling and regulation have been highlighted. Tubulins are 100kDa structural proteins associated with the cytoskeleton, that have been shown to influence amongst others, the functioning of adenylyl cyclases and PLC-β1 (Popova & Rasenick, 2000). Upon agonist stimulation, a simultaneous recruitment of tubulin and clathrin from the membrane occurs which is mirrored by a decrease in Gβγ subunits. Immunoprecipitation studies demonstrated that the clathrin, tubulin and the
Gβγ subunits internalised together. This interaction between tubulin and the Gβγ subunits is necessary for muscarinic M₃ receptor internalisation via clathrin-coated pits (Popova & Rasenick, 2004). This occurs through the 3i loop of the muscarinic M₃ receptor that has a Gβγ-docking site (Wu et al., 2000).

1.6 The β-adrenoceptor family

The existence of two adrenoceptors was proposed as early as 1948 by Ahlquist based on the different potencies amongst a series of phenethylamines in a variety of in vitro and in vivo preparations from various species (Rang and Dale, Pharmacology, Fifth Edition). Ahlquist postulated the existence of two distinct adrenoceptor subtypes, α and β. With the discovery of adrenoceptor antagonists the existence of two α-adrenoceptors (α₁ and α₂) and two β-adrenoceptors (β₁ and β₂) was confirmed. Other adrenoceptor subtypes were cloned and characterised subsequently. The adrenoceptors belong to the prototypical class of Family A GPCRs. The two α-adrenoceptors are coupled to two distinct G-proteins leading to the stimulation of either PLC-β (α₁-adrenoceptor) or inhibition of adenylyl cyclases and calcium channels (α₂-adrenoceptors).

β-Adrenoceptors are coupled to the activation of adenylyl cyclases and production of cAMP. They mediate the actions of adrenaline or noradrenaline resulting in positive ionotropic and chronotropic effects of the catecholamines. Adrenoceptors are involved in development, behaviour, heart function, smooth muscle tone and energy metabolism. The hamster β₂-adrenoceptor was the first nonvisual mammalian GPCR to be cloned (Dixon et al., 1986). This receptor consisted of 418 amino acids. The complete nucleotide sequence for the human β₂-adrenoceptor was cloned a year later (Kobilka et al., 1987). The human receptor subtype showed an overall sequence homology of 87% with the hamster β₂-adrenoceptor. Sequence analysis also revealed that the carboxyl tail was the most divergent region of the molecule (Kobilka et al., 1987). Later, screening of a human placenta lambda gt11 library, led to the isolation of the human β₁-adrenoceptor, consisting of 477 amino acid residues (Frielle et al., 1987). However, the existence of another β-adrenoceptor was speculated based on the findings that in adipose tissue, ileum and skeletal muscles, selective β₁ or β₂-adrenoceptor agonists showed relatively low potency. Screening of a genomic library with an avian β₁-adrenoceptor and human β₂-adrenoceptor probes identified a protein consisting of 402
amino acids (Emorine & Marullo, 1989). This protein showed a sequence homology of 40-50% to the human \( \beta_1 \) and \( \beta_2 \)-adrenoceptors. Functional characterisation of the cloned product using \( \beta \)-agonists and antagonists confirmed this protein to be the third subtype of \( \beta \)-adrenoceptor and was termed the \( \beta_3 \)-adrenoceptor (Emorine & Marullo, 1989). Although the \( \beta \)-adrenoceptors are structurally similar and exhibit the ability to activate the same G-proteins, they are products of distinct genes. The human \( \beta_2 \)-adrenoceptor gene is situated at \( q31-q32 \) position on the long arm of chromosome 5 (Kobilka \textit{et al.}, 1987), whilst the \( \beta_1 \)-adrenoceptor is at \( 10q24-q26 \) and the \( \beta_3 \)-adrenoceptor is at \( 8p12-p11.2 \) (Yang-Feng \textit{et al.}, 1990). The \( \alpha_1 \) and \( \alpha_2 \)-adrenoceptors genes are in close proximity to the \( \beta_2 \)-adrenoceptor and \( \beta_1 \)-adrenoceptors, suggesting a common lineage for both adrenoceptor subtypes.

\( \beta \)-Adrenoceptors are distributed ubiquitously in the human body. A range of biochemical and quantitative studies has attempted to address the tissue localisation of \( \beta \)-adrenoceptors. These studies have identified the expression of \( \beta_1 \)-adrenoceptors and \( \beta_2 \)-adrenoceptors in heart and lung with no detectable mRNA levels for \( \beta_3 \)-adrenoceptors (Mak \textit{et al.}, 1996). \textit{In situ} hybridisation studies in rat brain confirmed \( \beta_1 \)-adrenoceptor localisation in cerebral cortex, anterior olfactory nucleus, intermediate grey matter of the spinal cord, pineal gland and medullary reticular formations (Nicholas \textit{et al.}, 1993). The localisation of \( \beta_2 \)-adrenoceptors is mainly in the cerebellar cortex, olfactory bulb, hippocampal formation and thalamic intralaminar nuclei. In addition, both receptor subtypes are expressed in smooth muscle cells, skeletal muscle, liver, nerve terminals, salivary glands and platelets. The expression of \( \beta_3 \)-adrenoceptors were detected in adipose tissues, gall bladder and to a much lower extend in colon (Krief \textit{et al.}, 1993). Low levels of \( \beta_3 \)-adrenoceptors were also detected in human adult brain including the brain stem, cerebellum, temporal cortex, parietal cortex and occipital cortex, while 100-fold more \( \beta_3 \)-adrenoceptor mRNA was detected in infant brain (Rodriguez \textit{et al.}, 1995). Tissue distributions of the various subtypes of \( \beta \)-adrenoceptors are detailed in Table 1.6. All the \( \beta \)-adrenoceptor subtypes signal by coupling to the stimulatory G-protein \( G_\alpha_s \) leading to the activation of adenylyl cyclase and accumulation of the second messenger cAMP (Dixon \textit{et al.}, 1986; Emorine & Marullo, 1989; Frielle \textit{et al.}, 1987). However, it has also been demonstrated that \( \beta \)-adrenoceptor
subtypes particularly the $\beta_3$-adrenoceptor can also couple to $\mathrm{G}\alpha_i$ (Gauthier et al., 1996). Following agonist stimulation, the receptor is rapidly phosphorylated by GRKs (Section 1.3). The phosphorylated receptor binds arrestins that target these receptors for internalisation.

**Table 1.6. Tissue distribution of $\beta$-adrenoceptors based on mRNA distribution** (Nicholas et al., 1993; Krief et al., 1993; Rodriguez et al., 1995).

<table>
<thead>
<tr>
<th>Receptor Subtype</th>
<th>Tissue Distribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\beta_1$</td>
<td>Heart, lung cerebral cortex, anterior olfactory nucleus, pineal gland</td>
</tr>
<tr>
<td>$\beta_2$</td>
<td>Heart, lung, cerebellar cortex, olfactory bulb, hippocampal formation, skeletal muscle, platelets, smooth muscle of gastrointestinal tract, urinary bladder and liver</td>
</tr>
<tr>
<td>$\beta_3$</td>
<td>Brain, colon, adipose tissue and gall bladder</td>
</tr>
</tbody>
</table>

To understand the structural basis for the pharmacological difference between the $\beta_1$ and $\beta_2$-adrenoceptor, chimeric receptor constructs were made and expressed by injection of mRNA into Xenopus oocytes (Frielle et al., 1988). Transmembrane domain 4 was demonstrated to be largely responsible for agonist binding and efficacy, whilst transmembrane domains 6 and 7 played crucial roles in antagonist binding. A separate study attempted to dissect out receptor subtype-specific regulation using a chimeric construct between the $\beta_2$-adrenoceptor and $\beta_3$-adrenoceptor (Liggett et al., 1993). The $\beta_3$-adrenoceptor, in contrast to the $\beta_2$-adrenoceptor has only 3 serine residues in the carboxyl-terminal tail of the receptor. It also lacks the consensus site for PKA-mediated phosphorylation of the receptor and lacks sequence homology in the regions that are known to be crucial for agonist-mediated internalisation and down-regulation. The chimeric receptor comprised the $\beta_3$-adrenoceptor sequences up to proline 365 of the cytoplasmic tail and the carboxyl-tail of the $\beta_2$-adrenoceptor. In comparison to the wildtype $\beta_3$-adrenoceptors, the chimeric constructs displayed agonist-mediated phosphorylation, sequestration and down-regulation (Liggett et al., 1993).
β-Adrenoceptor agonists and antagonists have been used for therapeutic applications. Clinical uses of β₁-adrenoceptor antagonists include treatment for hypertension, angina pectoris following myocardial infarction and cardiac dysrhythmias. On the other hand, β₂-adrenoceptor agonists have been used extensively for treatment of airway diseases, particularly asthma and chronic obstructive pulmonary disorder. Selective β₃-adrenoceptor agonists are being developed for treating obesity.

1.6.1 The β₂-adrenoceptor

This receptor is primarily associated with its ability to mediate smooth muscle relaxation induced by the production of cAMP. The β₂-adrenoceptor forms the predominant subtype expressed in most vascular smooth muscles. The contractile machinery of the smooth muscle is activated when the myosin light chain undergoes phosphorylation, detaching from the actin filaments. This phosphorylation is catalysed by myosin-light-chain kinase, which is activated when it binds Ca²⁺-calmodulin. PKA phosphorylates the myosin-light-chain kinase, inactivating the enzyme, thereby causing the cell to relax. Smooth muscle contraction is driven by Ca²⁺ entry through voltage gated ion channels or by Ins(1,4,5)P₃-mediated Ca²⁺ release from the sarcocomplasmic reticulum. Regulation of β₂-adrenoceptor signalling is therefore of immense physiological relevance. Receptor knockout studies have attempted to address other aspects of the β₂-adrenoceptor pharmacology including contribution of this receptor subtype in the cardiovascular, metabolic and reproductive functions.

Knockout of β₂-adrenoceptors did not cause an upregulation of β₁-adrenoceptor numbers, as measured by saturation antagonist binding studies (Chruscinski et al., 1999). Furthermore, there was no embryonic or post-natal lethality associated with the gene knockout. The knockouts appeared ‘grossly’ normal and fertile in comparison to wildtypes (Chruscinski et al., 1999). The major effects of receptor knockouts were only observed during stress of exercise, in that they were able to exercise longer with a lower respiratory exchange ratio, implicating a role for changes in metabolism rates of the knockouts. They were also hypertensive during exercise suggesting an imbalance between vasoconstrictive and vasodilatory effects of catecholamines (Chruscinski et al., 1999). Knockout animals were obese in comparison to the wildtype controls, demonstrating an effect on metabolism in adulthood (Jimenez et al., 2002). Subtype
specific effect on the functioning of the cardiovascular system was examined by using neonatal cultures of cardiomyocytes from wildtype controls and knockout animals (Devic et al., 2001). These cells contracted spontaneously in culture and contraction rate was responsive to catecholamines. The results obtained from these cultures indicated that β2-adrenoceptors have the ability to couple to pertussis toxin sensitive G-proteins while mediating contractions of the myocytes. Isoproterenol-mediated contraction of these cells was not sensitive to PKA inhibitors implicating a direct interaction between the G-proteins and Ca^{2+} channels (Devic et al., 2001). These results highlight the combined role of the β₁-adrenoceptor and β₂-adrenoceptor in maintaining the cardiovascular tone. The coupling, signalling and regulation of β₂-adrenoceptors in vitro will be discussed in more detail below.

1.6.1.1 Adenylyl cyclases and coupling of the β₂-adrenoceptor

The β₂-adrenoceptor is perhaps one of the most extensively studied GPCRs. It is known primarily to couple to Gaₐ, activating adenylyl cyclases, which stimulates the hydrolysis of ATP to cAMP (Kobilka et al., 1987). Deletion of amino acids in the C-terminal of the 3i loop (267-273) indicate that these residues are crucial for agonist-mediated activation of Gaₐ and adenylyl cyclases, without affecting agonist binding properties (Hausdorff et al., 1990).

Currently 9 membrane-bound isoforms of mammalian adenylyl cyclases (1-9) have been cloned and characterised (Cooper, 2003; Hanoune & Defer, 2001). These isoforms are ubiquitously expressed and are regulated by multiple modes of action, allowing integration of various signalling pathways. Factors regulating adenylyl cyclases include Ca^{2+} concentration, Ga, and Gβγ subunits, calmodulin, PKA and PKC (Chabardes et al., 1999). Although most tissues co-express various isoforms of adenylyl cyclase, the type of tissue may also determine the adenylyl cyclase isoform expressed. For example, Ca^{2+}-calmodulin regulated adenylyl cyclases are restricted to neuronal and secretory tissues, while Ca^{2+}-inhibited adenylyl cyclases are abundantly expressed in striatum and cardiac tissues. Based on the stimulus, adenylyl cyclases can be broadly classified as Ca^{2+}-calmodulin regulated (adenylyl cyclase 1, 3 and 8), Ca^{2+}-inhibited (adenylyl cyclase 5 and 6), Ca^{2+}-insensitive but PKC-stimulated (adenylyl cyclase 2, 4 and 7) and calcineurin regulated adenylyl cyclase 9 (Cooper, 2003).
The source of Ca\(^{2+}\) also influences the type of adenylyl cyclases activated. For example, depletion of intracellular Ca\(^{2+}\) stores facilitates capacitative Ca\(^{2+}\) entry, which inhibits adenylyl cyclase 5 and 6 but activate adenylyl cyclase 1 and 8. However, Ca\(^{2+}\) release mediated by Ins(1,4,5)P\(_3\) or inhibition of the sarco-endoplasmic Ca\(^{2+}\)-ATPases has little or no effect on these adenylyl cyclase isoforms (Dyer et al., 2005). Recent work in a rat aortic smooth muscle cell line defined the physiological relevance of interaction between the Ca\(^{2+}\) and adenylyl cyclase signalling pathways. Stimulation of the vasopressin receptor (V\(_{1a}\)) by arginine\(^8\)-vasopressin (AVP) inhibited adenylyl cyclase activity by the Ins(1,4,5)P\(_3\)-mediated transient release of Ca\(^{2+}\) and this inhibition considerably outlasted the stimuli that initiated it. By contrast, the inhibition mediated by capacitative Ca\(^{2+}\) entry was sustained only as long as the Ca\(^{2+}\) signal prevailed (Dyer et al., 2005).

The \(\beta_2\)-adrenoceptor also couples to the cAMP inhibitory G-protein \(G_\alpha_i\), in recombinant and endogenous systems. This allows the \(\beta_2\)-adrenoceptor to mediate MAP kinase activation (Daaka et al., 1997). Phosphorylation of the receptor by PKA was demonstrated to be the crucial switch between \(G_\alpha_s\) and \(G_\alpha_i\) coupling (Zamah et al., 2002). Further evidence for alternate coupling was demonstrated in embryonic chick ventricular cardiomyocytes that express both functional \(\beta_1\) and \(\beta_2\)-adrenoceptors. Zinterol (a specific \(\beta_2\)-adrenoceptor agonist) triggered arachidonic acid (AA) release from \(^{3}\text{H}\)-AA-loaded cells by the activation of the cytosolic PLA\(_2\). This was abolished after treatment of the cardiomyocytes with pertussis toxin. These results demonstrate the involvement of the PLA\(_2\)/AA pathway in mediating positive ionotropic effects through pertussis toxin sensitive G-proteins (Pavoine et al., 1999). These results were confirmed in the human myocardium, demonstrating the ability of the \(\beta_2\)-adrenoceptor to couple to \(G_\alpha_i\) G-proteins (Pavoine et al., 2003).

1.6.1.2 Structural basis for \(\beta_2\)-adrenoceptor ligand binding

Several mutagenesis studies have attempted to identify specific residues in the \(\beta_2\)-adrenoceptor that are responsible for ligand binding and conformational changes. Using site directed mutagenesis, cysteine molecules on the extracellular loops were demonstrated to be crucial for ligand binding and functional expression of the receptor (Dohlman et al., 1990). To address the molecular basis of ligand binding, mutants
encoding for alanine in the place of cysteine in residues 106, 184, 190 and 191 were constructed (Noda et al., 1994). Replacement of Cys\textsuperscript{106} with alanine reduced the affinity of agonists and resulted in lower expression of the receptor. Replacement of the Cys\textsuperscript{184} with alanine did not affect antagonist binding to the receptor indicating that this residue is not crucial for ligand binding. On the contrary, double mutant constructs lacking Cys\textsuperscript{106} and Cys\textsuperscript{191} showed a low affinity phenotype similar to the Cys\textsuperscript{106} mutant. Using sensitivity to dithiothreitol (DTT) as a measure of formation of disulphide bonds and hence high affinity state of the receptor, the bonds between Cys\textsuperscript{106} and 191 and between Cys\textsuperscript{184} and 190 were shown to be crucial (Noda et al., 1994).

Another study demonstrated that Ser\textsuperscript{204} and Ser\textsuperscript{207} in transmembrane domain 5 interacts directly with the catechol hydroxyls of agonists (Strader et al., 1989). Mutation of Ser\textsuperscript{203} to alanine, valine or cysteine reduced the binding affinity and potency of agonists (Liapakis et al., 2000). The importance of serine residues in transmembrane domain 5 of the receptor was further highlighted by the use of single and multiple mutants (Del Carmine et al., 2004). Using these mutants, the binding affinity of various catechol ligands differing only in the presence of substituents in the ethanolamine tail of the ligand was measured. For all the mutants, there was a clear relationship between the loss of binding caused by receptor mutation and that caused by ligand modification.

Fluorescence spectroscopic studies have also been developed to study agonist-induced receptor conformation. One such study utilised a highly cysteine specific, environment-sensitive fluorescence tag N, N-dimethyl-N (iodoacetyl)-N'-(7-nitrobenz-2-oxa-1, 3-diazol-4-yl) ethylenediamine (IANBD; Gether et al., 1995). This fluorescent molecule exhibits an increase in fluorescence intensity with a decrease in polarity of the environment. Purified β\textsubscript{2}-adrenoceptor tagged with IANBD challenged with isoproterenol showed a stereoscopic, reversible decrease in fluorescence intensity on agonist binding. This loss of fluorescence intensity was mirrored with the challenge of partial agonists but to a lesser extent in comparison to full agonists. The magnitude of response correlated with agonist efficacy, suggesting that this change was due to conformational change in receptor configuration upon agonist binding.

A recent study used fluorescence lifetime imaging of site-specific labelled amino acid residues (fluorescein maleimide labelled Cys\textsuperscript{265} residue) in the 3i loop of the β\textsubscript{2}-
adrenoceptor to understand functional differences between agonist and antagonist binding and receptor coupling (Ghanouni et al., 2001). Agonist-induced movement was characterised by examining interactions between fluorescein maleimide labelled Cys265 residue and fluorescence quenching reagents localised to different molecular environments of the receptor. Binding of full agonist to the chimeric molecule reduced the fluorescence intensity by ~15%, whereas partial agonist binding resulted in smaller changes in fluorescence intensity. The binding of antagonist had no effect. These results are consistent with the notion that either a clockwise rotation of transmembrane domain 6 or a tilting of the cytoplasmic end of transmembrane domain 6 towards transmembrane domain 5 occurs on ligand binding. Based on their findings, a model with multiple agonist-specific receptor states was proposed, where activation occurs through a series of conformational changes (Ghanouni et al., 2001).

1.6.1.3 Regulation of β2-adrenoceptor signalling

Following agonist stimulation, the β2-adrenoceptor is rapidly desensitised by phosphorylation of the receptor by GRKs. A number of factors influence the receptor’s ability to desensitise and its subsequent down-regulation. For example in murine L-cells, receptor density influences desensitisation of the receptor (Rousseau et al., 1997). In cells with high receptor number, pre-treatment with isoproterenol induced a desensitisation pattern resulting in reduction of both agonist potency and efficacy. However, in cells with lower receptor expression, desensitisation resulted only in decrease of potency of isoproterenol (Rousseau et al., 1997). Moreover, desensitisation mediated by nanomolar concentrations of isoproterenol was mediated by PKA alone, while both PKA and GRKs mediated desensitisation of the β2-adrenoceptor when stimulated with higher concentrations of isoproterenol. This was confirmed in CHO-K1 cells recombinantly expressing β2-adrenoceptors (Pippig et al., 1993). Over expression of either GRKs or β-arrestin augments homologous (agonist-mediated) desensitisation in comparison to heterologous desensitisation (Pippig et al., 1993).

β-Arrestins are versatile in their action, acting as scaffolding proteins and recruiting several binding partners to aid with receptor down-regulation. In HEK 293 cells co-expressing β-arrestins and β2-adrenoceptors, stimulation with isoproterenol results in the time-dependent recruitment of phosphodiesterases 4 (PDE4) to the membrane, in a
manner similar to β-arrestin recruitment (Perry et al., 2002). Phosphodiesterases (PDE) plays a crucial role in downregulating Gaα-mediated signalling by degrading cAMP. Currently more than 11 families of PDEs are known, each family being distinguished by their unique enzymatic activity (Soderling & Beavo, 2000). Of these, PDE4, PDE7 and PDE8 are specific for cAMP (Baillie & Houslay, 2005; Conti et al., 2003; Xiang et al., 2005). Using a catalytically inactive mutant of PDE4, it was demonstrated that the ability to bind β-arrestin was retained but recruitment of endogenous PDE4 to the membrane in response to agonist stimulation of β2-adrenoceptors were inhibited (Perry et al., 2002). By recruiting PKA to the agonist-activated receptor, β-arrestins target cAMP degradation to sites of localised PKA activity at the plasma membrane co-ordinating receptor desensitisation and quenching of PKA activity (Perry et al., 2002).

Studies of β2-adrenoceptors expressed endogenously in mouse cardiomyocytes or recombinantly expressed in HEK 293 cells have shown that β-arrestin mediated recruitment of PDE4 is also crucial for Gaα, to Gaα, switching (Baillie et al., 2003; Xiang et al., 2005). In HEK 293 cells where β-arrestin expression was suppressed by RNA interference, stimulation of β2-adrenoceptors with agonists resulted in enhanced cAMP generation and marked reduction in the ability of the receptors to internalise (Ahn et al., 2003).

Following receptor desensitisation, the β2-adrenoceptor is internalised in a clathrin-dependent manner promoted by β-arrestin binding to the carboxyl-tail of the receptor (Laporte et al., 1999; Lin et al., 1997; Oakley et al., 1999). Although several studies have detailed β2-adrenoceptor internalisation patterns, very little is known about the ability of heterotrimeric G proteins to undergo agonist-induced endocytosis. One study attempted to address this by fluorescently tagging the Gβ and Gγ subunit with yellow fluorescence protein (YFP) and the Gaα subunit with cyan fluorescence protein (CFP; Hynes et al., 2004). This study used bimolecular fluorescence complementation, whereby a fluorescence signal is produced only when fragments of YFP are brought together by interactions between proteins fused to each fragment. Upon agonist stimulation, these fluorescent subunits co-localise in intracellular vesicles that are distinct from vesicles to which labelled β2-adrenoceptor internalise. A dynamin double-negative mutant blocked β2-adrenoceptor-mediated internalisation but had no effect on
the labelled G-protein subunits, demonstrating that the Ga\textsubscript{s} subunit and β\textsubscript{2}-adrenoceptor dissociate and utilise distinct mechanisms for internalisation.

More recently, to investigate the intracellular trafficking of Ga\textsubscript{s} a functional Ga\textsubscript{s}-green fluorescent protein (GFP) fusion protein was cloned (Allen et al., 2005). C6 and MCF-7 cells expressing Ga\textsubscript{s}-GFP were treated with 10µM isoproterenol, and G-protein trafficking assessed with fluorescence microscopy. Upon isoproterenol stimulation, Ga\textsubscript{s}-GFP dissociated from the plasma membrane and internalized into vesicles. Vesicles containing Ga\textsubscript{s}-GFP did not co-localise with markers for early endosomes or late endosomes/lysosomes, revealing that Ga\textsubscript{s} does not traffic through common endocytic pathways. Activated Ga\textsubscript{s}-GFP co-localised in vesicles labelled with fluorescent cholera toxin B, a lipid raft marker. Agonists significantly increased Ga\textsubscript{s} protein in Triton X-100 insoluble membrane fractions, suggesting that Ga\textsubscript{s} moves into lipid rafts after activation. Disruption of rafts by treatment with cyclodextrin prevented agonist-induced internalization of Ga\textsubscript{s}-GFP, as did over expression of a dominant-negative dynamin. Taken together, these results suggest that receptor-activated Ga\textsubscript{s} moves into lipid rafts and is internalized from these membrane microdomains.

Following internalisation, receptors are either recycled to the surface after dephosphorylation or are degraded. Ubiquitination marks proteins for degradation by the 26S proteosome, through the enzymatic activity of ubiquitin activating, ubiquitin carrying enzyme and ubiquitin ligase. Until recently, there has been little or no evidence connecting ubiquitination and mammalian GPCR regulation. Shenoy et al., (2001) demonstrated that in Chinese hamster fibroblast cells recombinantly expressing the β\textsubscript{2}-adrenoceptor, agonist activation promotes the ubiquitination of both receptors and β-arrestins. Proteosome inhibition reduced receptor internalisation and degradation as measured by ligand binding, implicating a role for ubiquitination in receptor regulation.

1.7 Thesis aims

All cells express a variety of cell surface receptors. Although each of these receptor types generally displays ligand selectivity, there are instances in which different receptors are activated simultaneously. The cellular responses are, therefore, a spatial integration of these signals. Crosstalk exists in which the activation of one signalling
pathway can influence the activity of another in either a positive or negative fashion. This study investigates in HEK 293 cells, the possibility of crosstalk between receptors coupling preferentially to $\text{G}_\alpha_s$ (β-adrenoceptors) and those coupling to $\text{G}_\alpha_{q/11}$ (muscarinic receptors). In particular, I wish to determine if any such crosstalk is mediated by similar or distinct mechanisms to those by which activation of $\text{G}_\alpha_{q/11}$-coupled receptors facilitates release of intracellular $\text{Ca}^{2+}$ by $\text{G}_\alpha_i$-coupled receptors, as has been described previously (Werry et al., 2003). This Thesis also aims to characterise full and partial agonists of the muscarinic receptor and to examine their ability, if any, to mediate crosstalk to the subsequent stimulation of β$_2$-adrenoceptors. Furthermore, crosstalk is often described in recombinant cells with fewer demonstrations in native cell systems. This study therefore also attempts to investigate if crosstalk between muscarinic receptors and β$_2$-adrenoceptors are mediated in primary cells.
Chapter 2: Materials and Methodology

2.1 Materials

All standard laboratory reagents were obtained from Sigma Aldrich (Poole, U.K.) or Fisher Scientific (Loughborough, U.K.) unless mentioned otherwise. Cell culture reagents were from Invitrogen (Inchinnam, Scotland, U.K.) including various culture media, serum (foetal calf and goat), poly-D-lysine and phosphate buffered saline (PBS). Smooth muscle cells were maintained in medium 231 supplemented with smooth muscle growth supplement (consisting of hormones, growth factors and proteins; Cascade Biologies, Nottingham, U.K.). Cell culture plastics were obtained from Nalgene (Hereford, U.K.). Poly-D-lysine coated 96-well plates used in FLIPR assays were from BD Biocoat Cell Environment (Oxford, U.K.). 96 well white Costar plate used in cAMP assays were from Fisher Scientific (Loughborough, U.K.).

Protein kinase inhibitors H-89, KT5720 and CMIQ were from Sigma Aldrich. Myristoylated peptide PKA 14-22 amide inhibitor and myristoylated peptide PKC 20-28 inhibitor were from Merck Bioscience (Nottingham, U.K.). Adenyl cyclase inhibitor SQ22,536, cholera toxin, cAMP, inositol 1,4,5-trisphosphate and pertussis toxin were from Sigma Aldrich (Poole, U.K.). Transfection reagent Genejuice was obtained from Merck Bioscience (Nottingham, U.K.). Pluronic F-127 was obtained from Molecular Probes (Eugene, U.S.A.). 25mm diameter borosilicate glass coverslips (thickness number: 1.5) for confocal imaging were purchased from VWR International (Poole, U.K.). Radiochemicals myo-[3H]-inositol with PT6-271 (81Ci/mmmole) and [35S]-GTPγS (1250Ci/mmmole) were from Amersham (Little Chalfont, U.K.), while [32P]-orthophosphate (10mCi/ml), [45Ca2+]-radionuclide and the muscarinic receptor antagonist N-methyl scopolamine ([3H]-NMS, 81Ci/mmmole) were from Perkin Elmer (Boston, U.S.A.). All other materials for radioactive liquid scintillation determination, including 20ml scintillation vials (Milli-20 PE), 6 ml scintillation vials (Pico-vials/6) and Emulsifier-safe scintillant were obtained from Perkin Elmer (Bucks, U.K.). 1,1,2-trichlorofluoroethane was purchased from Merck Bioscience (Nottingham, U.K.). Dowex 1 X8 (chloride form) for anion exchange columns was from Sigma Aldrich. Protein-A Sepharose beads (30% (W/V)) suspension in TE buffer) were purchased from Amersham Biosciences (Uppsla, Sweden).
Anti-sera to Gaq/11 were generated by Genosys Biotechnologies (Pampisford, U.K.) by inoculation of rabbits with a decapeptide corresponding to 344-353 residues of the Gaq sequence, which is identical to Ga11 sequence (Bundey and Nahorski, 2001). Anti-sera against the muscarinic M3 receptor was raised in rabbits against a fusion protein representing a portion of the third intracellular loop of the muscarinic M3 receptor previously described and characterised (Tobin et al., 1993). Gaq(1-3) and Ga(1-3) polyclonal antibodies were purchased from Santa Cruz (Heidelberg, Germany). All other antibodies including the mouse α-actin antibody, FITC-conjugated goat anti-mouse antibody, mouse γ-tubulin antibody and HRP-conjugated secondary antibodies were from Sigma Aldrich (Poole, U.K.). Pre-stained molecular weight markers used for western blot (10-250kDa range) were from BioRad (CA, U.S.A.). Protran nitrocellulose transfer membrane was from Schleider & Schuell BioScience (Dassel, Germany). ECL+ reagents and hyperfilm were from Amersham Biosciences (Little Chalfont, U.K.). For immunocytochemistry, coverslips were mounted onto glass slides with Dako Fluorescence protecting mounting medium (DAKO, Glostrup, Denmark).

2.2 Cell culture and transfection procedures

2.2.1 Cell Culture

HEK 293 cells were routinely cultured in Minimum essential medium-alpha (MEM-α) supplemented with foetal calf serum (FCS, 10% (V/V)), non-essential amino acids (1%) and L-glutamine (2mM). HEK 293 cells stably expressing the muscarinic M3 receptor (HEK-M3) was maintained in MEM-α supplemented with foetal calf serum (FCS, 10% (V/V)), non-essential amino acids (1%), L-glutamine (2mM) and 500μg/ml Geneticin. Two CHO-K1 cell lines, stably transfected with the muscarinic M3 receptor varying only in levels of receptor expression (Buckley-M3; 1550fmol/mg protein (Budd et al., 1999) and VT-31; 194fmol/mg protein, as determined by saturation antagonist binding; refer to section 2.10) were used to characterise partial agonists of the muscarinic receptor. These cell lines were maintained in MEM-α media supplemented with FCS (10% (V/V)), penicillin (50 units/ml), Funigizone (amphotericin-B, 25μg/ml) and Geneticin (500μg/ml). Airway epithelial cells, NCI H292 (generously provided by Mandy Lawson, AstraZeneca R&D, Charnwood obtained from American Type Culture Collections, Rockville, U.S.A.) were routinely cultured in RPMI-1640 media supplemented with FCS (10% (V/V)) and L-glutamine.
(2mM). The cells were passaged by detaching the cells with trypsin (0.5g/L)-EDTA (0.2g/L). The trypsin-EDTA solution was added to the flask, the cells briefly rinsed and the flask incubated at 37°C for 2-5min. The cells were then resuspended in fresh MEM-α media and passaged into new flasks as 1:10-1:50 dilutions. Cells were maintained at 37°C in a 5% CO₂ humidified environment.

2.2.2 Transfection

Transfections of HEK 293 cells were carried out either in 6-well plates or 24 well plates using Genejuice transfection reagent. Cells were transfected when 50-70% confluent. For HEK 293 cells, multi-well dishes were treated with poly-D-lysine (0.01% solution was added to plasticware or coverslips for 10-15min. Solution was then removed and allowed to dry, prior to cell plating) to enhance cell adhesion to the surface. In addition to transfections with the cDNA encoding the muscarinic M₃ receptor tagged with cyan fluorescence protein (CFP, M₃-CFP), a plasmid encoding the green fluorescent protein-tagged PH domain of PLCδ₁ (e-GFP-PH PLCδ₁; see section 2.8) was also used. M₃-CFP transfected into HEK 293 cells were used for spectroscopic studies looking at receptor internalisation upon agonist stimulation by confocal microscopy. For transfection of a six well dish, 1μg of plasmid DNA was used with 3μl of Genejuice per well, according to the manufacturer’s protocol.

2.3 Measuring elevations of intracellular Ca²⁺

2.3.1 Measurement of intracellular Ca²⁺ using a fluorometric imaging plate reader (FLIPR)

2.3.1.1 Cell preparation and loading

Elevations in intracellular Ca²⁺ ([Ca²⁺]) were measured using fluo-3-acetoxymethyl ester (fluo-3AM) loaded cells in a FLIPR (Molecular Devices, Wokingham, U.K.) For the assay, 100μl of HEK 293, Buckley-M₃, VT-31 or NCI H292 cells at a density of 500,000 cell/ml were added to each well of a poly-D-lysine coated 96-well plate. Cells were incubated at 37°C in a 5% CO₂ humidified environment, overnight. Where required, cells were plated in media with either 2μg/ml of cholera toxin or 100ng/ml of pertussis toxin and incubated for 18-20h. The cells were loaded in Hank’s balanced salt solution (HBSS; Heps 10mM, NaCl 136mM, KCl 5.3mM, D-glucose 5mM,
MgSO₄·7H₂O 0.8mM, CaCl₂ 1.2mM and NaHCO₃ 4.1mM, pH 7.4) containing 5μM fluo-3AM and pluronic F-127 (0.044%) for 1h at 37°C. Prior to the assay the cells were washed twice with 100μl of HBSS to remove any excess dye. The cells were finally resuspended in 100μl of HBSS (± CaCl₂, for some studies) and assayed.

2.3.1.2 FLIPR assay

For examining crosstalk in HEK 293 and NCI H292 cells, fluorescence (λ₂₅ = 488nm and λₜₐₚ = 540nm) was initially measured for 5-10 seconds, to establish a baseline for recording. Following this, a muscarinic receptor agonist or vehicle control (HBSS) was added (30-50μl; speed of addition, 30-40 μl s⁻¹) and fluorescence recorded for 130-180s. In the continued presence of muscarinic receptor agonists or buffer control, the cells were stimulated with noradrenaline (30-50μl; speed of addition, 30-40 μl s⁻¹) and fluorescence recorded for a further 130-180s. Similarly, to examine the efficacy of partial agonists in Buckley-M₃ or VT-31 cells, a baseline for recording was measured for 5-10s prior to agonist addition (50μl; speed of addition, 40 μl s⁻¹). Fluorescence was recorded for 180s after agonist addition. When compounds were dissolved in DMSO were used, DMSO was included in control experiments at the appropriate concentrations and shown to be without any effect, at the highest concentration tested.

2.3.1.2 Data analysis

Responses to agonists were plotted either as a measure of fluorescence units against time or as a difference between peak and baseline fluorescence units (change in fluorescence units) against agonist concentrations. Concentration response curves were fitted using non-linear regression with a four-parameter logistic equation with equal weighting to all points using Graphpad Prism software (San Diego, U.S.A.). From these graphs, the Eₘₐₓ (maximal responses) and pEC₅₀ (negative logarithm of the concentration required to bring about 50% of maximal response) were determined. “N numbers” refer to the number of independent experiments with each condition performed in duplicate.
2.3.2 Measurement of intracellular Ca\textsuperscript{2+} using confocal microscopy

HEK 293 cells were plated onto 25mm sterile borosilicate glass coverslips coated with 0.01% poly-D-lysine. Cells were loaded with 5\textmu M fluo-3AM and pluronic F-127 (0.044%) in Krebs-Hepes buffer for 1h at 37°C (KHB; NaCl 118mM, KCl 4.7mM, CaCl\textsubscript{2} 1.3mM, KH\textsubscript{2}PO\textsubscript{4} 1.2mM, MgSO\textsubscript{4} 1.2mM, NaHCO\textsubscript{3} 25mM, Hapes 5mM, D-glucose 10mM, pH 7.4). Cells were washed twice with KHB at 37°C and imaged on an Olympus laser-scanning microscope using an open perfusion microincubator (Harvard Applications Inc, Kent, U.K.). Temperature of the perfusion chamber was maintained at 37°C using a Harvard Apparatus temperature controller (Harvard Applications Inc, U.K.). Agonists or buffer were continuously perfused over the cells at a rate of 5ml/min maintaining a chamber volume of approximately 1ml (Gilson Miniplus 2, Anachem, Luton, U.K.). Fluorescence (\textlambda_{ex}=488nm and \textlambda_{em}=540nm) prior to agonist addition was regarded as baseline fluorescence. Data was captured using the Olympus Fluoview software (version 4.3).

For imaging of primary cell cultures, cells were plated on sterile borosilicate glass coverslips and allowed to incubate at 37°C in a 5% CO\textsubscript{2} humidified environment for 1-7 days. Cells were loaded with KHB supplemented with 5\textmu M fluo-3AM and pluronic F-127 (0.044%) for 30min at room temperature. Following loading, cells were washed twice with KHB at 37°C and imaged as detailed above.

2.3.2.1 Data analysis

From a field of view, a random number of cells (10-16) were analysed. A region of interest was chosen within the cytoplasm of each cell, using a purpose written software (Olympus Fluoview software). Changes in cytosolic fluorescence provided an index of changes in [Ca\textsuperscript{2+}]. The data obtained from each coverslip was averaged and expressed as fold change in cytosolic fluorescence (F/F\textsubscript{0}) relative to basal levels. "N numbers" refer to the number of independent experiments with each condition performed in duplicate on each coverslip.
2.4 Measurement of total inositol phosphates accumulation

2.4.1 Radiolabelling and sample generation

HEK 293 cells, Buckley-M3 or VT-31 cells were plated onto 24 well dishes in media supplemented with 3μCi/ml of myo-[\(^3\)H]-inositol with PT6-271 for 48h prior to the assay. Cells were washed with KHB at 37°C and incubated with 250μl of KHB containing lithium chloride (10mM) for 20min. Lithium blocks the metabolism of inositol-1 monophosphate to inositol by inositol monophosphatases, causing the accumulation of inositol phosphates species ([\(^3\)H]-InsP\(_x\), Berridge et al., 1982). For time-course experiments, cells were challenged with 100μM methacholine. For investigating crosstalk, cells were challenged with methacholine or partial agonists for 12 or 20min in the presence or absence of 10μM noradrenaline. The reactions were terminated using 0.5ml of ice-cold 1M-trichloroacetic acid (TCA) and cells allowed to stand on ice for 10-15min. To 800μl of cell lysate in 5ml Sarstedt polypropylene tubes, 200μl of 10mM EDTA and 1ml of Freon (1:1, (V/V) of 1,1,2-trichlorofluoroethane and tri-N-octyl amine) was added. The tubes were vortexed thoroughly and 750μl of supernatant added to clean 1.5ml micro-centrifuge tubes. Finally, 50μl of 250mM NaHCO\(_3\) solution was added and the samples vortexed. [\(^3\)H]-InsP\(_x\) was recovered by ion exchange chromatography on Dowex 1 X 8 200-400 (chloride form) columns as described below.

2.4.2 \([\(^3\)H]-InsP\(_x\), sample separation

Inositol phosphates were separated according to their charge by anion exchange chromatography, over a 3ml-depth of dowex 1 X 8 as described previously (Challiss et al., 1992). Dowex columns were washed with 10ml of distilled water and allowed to drain completely. The samples were added, followed by 10ml of distilled water and 12ml of ammonium formate (25mM) and allowed to drain after each addition. Hydrochloric acid (1M, 10ml) was added to the column and the flow-through collected in 20ml scintillation vials. Of the flow-through, 3ml was kept for analysis and the rest discarded. 15ml of Emulsifier-safe scintillant was added to the vials and radioactive counts obtained using the Packard Tri-Carb liquid scintillation analyzer.
2.4.3 Data Analysis

All data points obtained were expressed as fold increase in $[^3]H$-InsP$_x$ relative to basal levels of $[^3]H$-InsP$_x$. For this, the cells were challenged with agonist-free buffer for the longest time point of the experiment. Samples were extracted and processed as described above. N numbers refer to the number of independent experiments with each condition performed in duplicate.

2.5 Measurement of cyclic AMP accumulation

cAMP generated in airway epithelial NCI H292 cells was determined using the AlphaScreen kit (amplified luminescent proximity homogenous assay; Perkin Elmer). Detection of cAMP generated in response to agonist stimulation is based on competition between endogenous cAMP and exogenously added biotinylated cAMP. Capture of cAMP is achieved using a specific antibody conjugated to acceptor beads. The streptavadin donor beads contain a photosensitiser phathocyanine, which converts ambient oxygen to an excited form of oxygen upon illumination at 680nm. This species of oxygen can diffuse approximately 200nm in solution within its half-life of 0.4$\mu$s. If an acceptor bead is within that proximity, energy is transferred from the singlet oxygen to thioxene derivatives within the acceptor bead, culminating in light production at 520-620nm, which is measured.

2.5.1 Cell preparation

NCI H292 cells maintained in RPMI-1640 media were plated in 96 well plates at a density of 5000 cells/well and incubated overnight. Following removal of media, cells were washed twice with 100μl of incubation buffer (per 500ml, HBSS 497.5ml, Hepes 5mM and BSA 0.1%) and incubated in 50μl of incubation buffer for a minimum of 20min at room temperature.

2.5.2 Sample Generation

To prevent breakdown of cAMP by phosphodiesterases, 25μl of incubation buffer containing 300μM rolipram was added to NCI H292 cells and incubated for a further 10min at room temperature. Rolipram is a phosphodiesterase 4 selective inhibitor and
used in this assay as cAMP hydrolysis in human bronchial tissue is attributed mainly to phosphodiesterase 4 (de Boer et al., 1992). Cells were stimulated by 25μl of stated concentrations of either formoterol or methacholine for intervals indicated. Plates were rapidly washed with 100μl of incubation buffer and cells lysed with addition 50μl of lysis buffer (Hepes 5mM, Tween-20 0.3%, BSA 0.1% and 100μM rolipram). Cell samples were snap frozen at -80°C for 20-30min, following which they were thawed rapidly at room temperature on a plate shaker, to allow uniform thawing. Plates were assayed for cAMP generation using the Perkin Elmer Alphascreen kit as described briefly below.

2.5.2 cAMP determination

Samples generated were analysed for cAMP content using the Alphascreen cAMP assay kit as described by the manufacturer. Briefly, 10μl of the sample or standard cAMP (1μM-0.1pM diluted in incubation buffer) were added to 96 well white Costar plates containing 40μl of streptavadin donor and acceptor beads (prepared in the dark). For determining non-specific binding, buffer control was used. The plates were incubated in the dark for a minimum of 5h prior to analysing for cAMP generation by determining the emitted light at 520-620nm on the AlphaQuest (Perkin Elmer).

2.5.3 Data Analysis

Standard curves were fitted using a four-parameter logistic equation in Graphpad Prism. cAMP content of the samples was obtained by interpolation of the standard curve. All dilutions performed were taken into consideration and cAMP content was related to content of each well. N numbers refer to the number of independent experiments with each condition performed in duplicate.

2.6 $^{35}$S-Guanosine5'-[y-thio] triphosphate determination of G-protein activation

GTP binding to an activated G-protein is a crucial moment in the initiation of signal transduction. Binding of the radiolabelled GTP-analogue $^{35}$S-GTPyS to activated G-protein in membrane preparation allows for the quantification of G-protein activation by agonists (Sim et al., 1997).
2.6.1 Membrane preparation

HEK 293 cells were grown to confluence in 175cm² flasks. Media was removed from flasks and washed once with PBS. Cells were harvested by incubation with 5ml of lifting buffer for 5-10min (Hepes 10mM, NaCl 0.9% and EDTA 0.2%, pH 7.4). The flasks were washed with 5ml of lifting buffer and cells thoroughly resuspended. All subsequent steps were performed at 4°C. The cell suspension was centrifuged at 350g for 5min and the pellet resuspended in 10ml of lysis buffer (Hepes 10mM and EDTA 10mM, pH 7.4). Using a Polytron homogeniser (large probe; setting 30), cells were homogenised for 20s. This suspension was subject to centrifugation at 14,000g for 20min at 4°C. The pellet obtained was resuspended in 10ml of freezing buffer (Hepes 10mM and EDTA 0.1mM) and homogenised as above. The cell lysate was centrifuged for a further 20min at 14,000g. The pellet obtained was resuspended in 1ml of freezing buffer per flask of cells. Estimation of protein content was carried out using Lowry's protein estimation method (Lowry et al., 1951). Aliquots of the membrane were stored at −20°C until required.

2.6.2 [35S]GTPγS assay

The assay was carried out as described by Akam et al., (2001). Briefly, frozen membrane aliquots were diluted in assay buffer (Hepes 10mM, NaCl 10mM, and MgCl₂ 10mM, pH 7.4) to obtain a final protein concentration of 1μg/μl. Membrane aliquots were added to microfuge tubes containing 1nM [35S]GTPγS (1250Ci/mmol), 1μM GDP (Ga(q11 and Ga(b)), 1μM GTPγS for non-specific binding and where appropriate muscarinic receptor agonists or noradrenaline. The volume was adjusted to 75μl using assay buffer. The tubes were incubated at 37°C for time points as indicated. The reactions were rapidly terminated by adding 1ml of ice-cold assay buffer and transferred immediately to an ice-bath. All subsequent steps were carried out at 4°C.

Membrane was recovered from the reactions by centrifugation at 14,000g for 6min and the resulting supernatant removed. Membrane pellets were solubilised by the addition of ice-cold solubilisation buffer (Tris-HCl 100mM, NaCl 100mM, EDTA 1mM, Igepal 1.25%, pH 7.4) with 0.2% sodium-dodecyl sulphate (SDS) and vortexed. To the solubilised membrane preparation, 50μl of solubilisation buffer (no SDS) was added.
Rabbit serum (1:100 dilution) and 30μl of Protein-A Sepharose beads (30% (W/V) suspension in TE buffer) was added to pre-clear the solubilised protein for 60min at 4°C, with rolling. Beads and insoluble material were removed from the suspension by centrifugation at 14,000g for 6min at 4°C. 100μl of the supernatant was transferred to a fresh tube containing appropriate G-protein anti-sera (Gαq,11 monoclonal antibody or Gαi(1-3)) at a final dilution of 1:100, vortexed and rolled overnight at 4°C. The following day, 70μl of Protein-A Sepharose beads were added to the samples and rolled for a further 90min at 4°C. Complexes containing the Protein-A Sepharose beads and [35S]-GTPγS were collected by centrifugation at 14,000g for 6min at 4°C. The supernatant was carefully removed by aspiration and the beads washed thrice with 1ml of solubilisation buffer (no SDS). Following the final wash, the beads were re-suspended in 1ml of scintillation cocktail and vortexed. After 30min, the tubes were vortexed again and radioactivity determined by liquid scintillation counting. For data analysis, the radioactivity associated with agonist stimulation was expressed as the difference between radioactive counts per minute (CPM) bound in membrane preparations under basal or stimulated conditions and the non-specific binding for each respective condition.

2.7 Smooth muscle cell culture

2.7.1 Smooth muscle culture and dissociation

Male Wistar rats (<300gm) were culled by concussion followed by cervical dislocation in accordance with Schedule 1 of the Animals (Scientific Procedures) Act 1986. A mid-line ventral incision was made and the trachea isolated. The body cavity was also dissected and bladder removed. The isolated tissue was placed immediately in dissociation buffer (NaCl 137mM, KCl 5.4mM, Hepes 10mM, Na2HPO4 0.4mM, NaHCO3 4.2mM, pH 7.4) at 35°C and dissected free of any fat or connective tissue. The tissue was diced into 1-2mm pieces and subject to enzymatic digestion as detailed. Tissue was digested initially with papain (1mg/ml) in the presence of 1,4-dithiothreitol (DTT; 0.75mg/ml) for 30min. This was followed by digestion with collagenase (type F; 1mg/ml) and hyaluronidase (0.75mg/ml) for 45-60min. Smooth muscle cells was dissociated by mechanical sheer by repeated triturations through a fire-polished Pasteur pipette. After digestion, the cells were collected by centrifugation at 200g for 5min.
Cells were resuspended in medium 231, supplemented with smooth muscle growth supplement. Cell suspensions were plated on 25mm diameter sterile coverslips and incubated at 37°C in a 5% CO₂ humidified environment.

2.7.2 Immunocytochemical staining of α-actin

Rat tracheal smooth muscle cells cultured on glass coverslips for up to 7 days were washed twice in PBS (without Ca²⁺ or Mg²⁺) permeabilised and fixed using 100% methanol incubation for 10min at -20°C. Cells were washed a further 3 times with PBS. The α-actin primary antibody diluted in 10% goat serum at 1:500 was added to cells and incubated overnight at 4°C. The following day, cells were washed and incubated with FITC-conjugated goat anti-mouse antibody (diluted 1:200 in 10% goat serum) for 2h at room temperature in the dark. The coverslips were washed and mounted onto glass slides with Dako Fluorescence protecting mounting medium. These were left to dry before being sealed with nail-varnish and stored at 4°C until imaging.

The glass slides were mounted onto the stage of an UltraVIEW confocal microscope (Perkin Elmer Life Sciences, Cambridge, U.K.) with a 40X oil-immersion objective lens. Cells were excited with the 568nm line of a Krypton/Argon laser and the emitted light collected with a broad band RGB emission filter. Images captured with the purpose written software were compared to the phase images of the same cells. Human airway epithelial NCI H292 cells were used as negative controls for α-actin staining. At the time of the assay, the cells were plated onto 25mm coverslips, permeabilised and stained with the α-actin antibody as described above.

2.8 Agonist-mediated phosphorylation of the muscarinic M₃ receptor

2.8.1 Cell preparation

CHO-K1 cells stably expressing the muscarinic M₃ receptor at 1550fmole/mg protein (Buckley-M₃; Budd et al., 1999) were plated in 6-well dishes overnight to obtain 70-80% confluence.
2.8.2 Sample preparation

Cells were washed twice with phosphate free-KHB (KHB without added \( \text{KH}_2\text{PO}_4 \)) and incubated with 1ml of phosphate free buffer containing 50\( \mu \text{Ci/ml} \) of \( ^{32}\text{P} \)-orthophosphate for 1h at 37\(^\circ\)C. Muscarinic receptor agonists were added for 10min following which, the media was rapidly aspirated and 2ml of ice-cold radioactive immunoprecipitation assay buffer added (RIPA; Tris 10mM, EDTA 2mM, NaCl 4mM, deoxycholic acid 12mM, NP-40 1%). Samples were left on ice for 15min and cleared by centrifugation for 3min at 10,000g. Anti-sera (0.2\( \mu \text{g} \)) were added and samples left on ice for 60-90min. Immunocomplexes were isolated on Protein A-Sepharose beads and washed thrice with ice-cold TE buffer (Tris 10mM and EDTA 10mM, pH 7.4). Isolated immunocomplexes were extracted in 20\( \mu \text{l} \) of 2x sample buffer (Tris-HCl (pH 6.8) 100mM, DTT 200mM, SDS 2% (W/V), bromophenol blue 0.1% (W/V) and glycerol 10% (V/V) by standing in water for 2min at 65\(^\circ\)C. Proteins were resolved on a 10% SDS-PAGE gel (Molecular Cloning: A Laboratory Manual. Sambrook, J., Fritsch, E.F., Maniatis, T.). The gels were subsequently dried and subject to autoradiography for 1-3 days.

2.8.3 Data Analysis

Receptor phosphorylation obtained by autoradiography was quantified by densitometric analysis using a BioRad GS710 imaging densitometer. Areas of interest of equivalent size were placed over each of the bands representing the phosphorylated receptors. Optical density values were then determined as the sum of the intensities of pixels inside the area of interest times the area of a single pixel by the Quantity One densitometric analysis software (BioRad). The optical density values obtained from unstimulated cells (basal) were subtracted from values obtained for agonist-stimulated cells. Statistical analyses were carried out on these values. For data representation, phosphorylation mediated by partial agonists (above basal) was represented as a percentage of methacholine-mediated receptor phosphorylation (above basal). N numbers refer to the number of independent experiments, with each condition performed in duplicate.
2.9 Single cell imaging of phospholipase-C activity

2.9.1 Transfection of eGFP-PH<sub>PLCδ1</sub>

The eGFP vector fused to the pleckstrin homology domain of phospholipase-Cδ1 (PLC-δ1) was generously donated by Professor T. Meyer (Stanford University, CA, U.S.A.). The fusion construct (eGFP-PH<sub>PLCδ1</sub>) was transiently transfected into 50-60% confluent HEK 293 cells as detailed in section 2.1.2.

2.9.2 Imaging of eGFP-PH<sub>PLCδ1</sub>

Cells were washed twice with KHB at 37°C and imaged on an Olympus laser-scanning microscope using an open perfusion microincubator (Harvard Applications Inc, Kent, U.K.). Temperature of the perfusion chamber was maintained at 37°C using a Harvard Apparatus temperature controller (Harvard Applications Inc, U.K.). Agonists or buffer were continuously perfused over the cells at a rate of 5ml/min maintaining a chamber volume of approximately 1ml (Gilson Miniplus 2, Anachem, Luton, U.K.). PLC activity at the single cell level was assessed as previously described by Nash <i>et al.</i>, (2001). Briefly, cells were stimulated with muscarinic agonists alone or in the presence of noradrenaline (10μM), following a basal recording of 20s. Under basal conditions, the eGFP-PH<sub>PLCδ1</sub> is localised at the plasma membrane because of its relatively high affinity for phosphatidylinositol 4,5-phosphate (PtdIns(4,5)P<sub>2</sub>). On agonist stimulation, the increase in phospholipase C activity and production of Ins(1,4,5)P<sub>3</sub> causes the PH-domain to translocate to the cytosol because of its higher affinity for Ins(1,4,5)P<sub>3</sub>.

2.9.3 Data Analysis

Change in cytosolic fluorescence was taken as an index of Ins(1,4,5)P<sub>3</sub> production, hence phospholipase C activity. A random number of transfected cells (10-12) from the field of view were chosen for analysis. In these cells, a region of interest was chosen in the cytoplasm, using a purpose written software (Olympus Fluoview software). Changes in cytosolic fluorescence in regions of interest were represented as a fold increase over basal levels. N numbers refers to the number of independent experiments with each condition performed on duplicate coverslips.
2.10 Saturation antagonist binding

The radiolabelled muscarinic receptor antagonist N-methyl scopolamine ($[^3]H$-NMS, 81Ci/mmole) was used for binding studies on both transient and stably transfected muscarinic M₃ receptor expressing cell lines or endogenously expressed muscarinic receptors (Willars et al., 1999). Cells were plated in a 24-well dish to achieve 50-80% confluence. The cells were washed twice with 1ml KHB at 37°C and incubated for 60-75min with 1ml of $[^3]H$-NMS containing a range of concentrations of $[^3]H$-NMS (0.1nM–6nM; total binding). Non-specific binding (NSB) was determined in the presence of 2µM atropine. To terminate binding, cells were briskly washed twice with 1ml of ice-cold KHB. For cell digestion, 0.5ml of NaOH (0.1M) was added and incubated at room temperature for 15-20min. The cell lysate was transferred into scintillation vials (Pico-vials/6). Wells were washed with 0.5ml of HCl (0.1M) and this was added to the scintillation vials. To this, 5ml of scintillant was added and thoroughly mixed. A Packard Tri-Carb liquid scintillation analyzer was used to determine radioactivity. For determination of protein content, cells were lysed in 1ml 0.1M NaOH and protein content estimated by the method of Lowry (Lowry et al., 1951; Section 2.14).

2.10.1 Data Analysis

Specific binding of the antagonist to the receptor was plotted as the difference between total binding and NSB. Analysis of saturation antagonist binding was achieved using non-linear regression either with a one site binding hyperbola equation or a four-parameter logistic equation in Graphpad Prism. The $B_{max}$ (density of receptors in membrane expressed as fmol/mg of protein) and $K_d$ (concentration of the drug that would occupy 50% of receptors at equilibrium) were also obtained from these graphs. N numbers refer to the number of independent experiments with each condition performed in duplicate.

2.11 Internalisation of muscarinic M₃ receptors

2.11.1 Cell plating and sample generation

HEK 293 or Buckley-M₃ cells were plated overnight in 24-well plates, to obtain 70-80% confluence. HEK 293 cells were transiently transfected with the fluorescently
tagged muscarinic M3 receptor cDNA, 48h prior to the assay. Cells were washed with 1ml of KHB at 37°C thrice and incubated in 1ml KHB for 10min at 37°C. Cells were stimulated with 100μl of methacholine or partial agonists of the muscarinic receptor for time-periods indicated and reactions terminated by aspirating off the drug-buffer mixture. Cells were washed rapidly with 1ml KHB at 4°C thrice. All subsequent steps were carried out at 4°C to prevent receptor trafficking (Thompson & Fisher, 1990).

2.11.1 [3H]-NMS binding

A saturating concentration of [3H]-NMS (6nM; as determined by saturation antagonist binding, described previously) was used to determine receptor density following agonist stimulation. Cells were incubated with 300μl of radiolabelled antagonist for a minimum of 4h at 4°C to achieve binding equilibrium (Budd et al., 1999). Cells were subsequently washed thrice, with 1ml KHB at 4°C and lysed with 0.5ml of 0.1M NaOH. The cell lysate was transferred into scintillation vials (Pico-vials/6). Wells were washed with 0.5ml of 0.1M HCl and this was added to the scintillation vials. To this, 5ml of scintillant was added and thoroughly mixed. A Packard Tri-Carb liquid scintillation analyzer was used to obtain the radioactivity. Protein concentrations were determined by lysing cells with 1ml of 0.1M NaOH and analysing by the method of Lowry (Lowry et al., 1951; Section 2.14).

2.11.2 Data analysis

Data were plotted in Graphpad Prism as a percentage of [3H]-NMS bound to muscarinic receptors in un-stimulated cells. N numbers refer to the number of independent experiments with each condition performed in duplicate.

2.12 Western Blotting for Gaε expression

To determine the effect of cholera toxin (CTX) on Gaε protein expression, HEK 293 cells were treated with 2μg/ml of the cholera toxin, lysed and immunoblotted for Gaε. Cells were treated with cholera toxin for 20, 4, 2, 1 and 0.5h. After the indicated times, cells were solubilised in 300μl of RIPA buffer and left on ice for 10min. The cell extract was transferred into microfuge tubes and spun at 16,000g for 10min at 4°C. Avoiding aggregated DNA, 250μl of supernatant was removed into a fresh tube and
stored at -20°C. The protein concentration was assessed using the method of Lowry (Lowry et al., 1951; Section 2.14).

Samples were equalised for protein content and approximately 30µg of protein loaded onto each lane of a 10% SDS-Page gel (Molecular Cloning: A Laboratory Manual. Sambrook, J., Fritsch, E.F., Maniatis, T.) after being mixed 1:1 (V/V) with sample buffer. Samples were boiled for 5min at 100°C prior to loading. Samples and pre-stained molecular weight markers (10-250kDa range) were electrophoresed at a constant voltage of 200V for 40min. After electrophoresis, the gel was equilibrated in transfer buffer (methanol 20% (V/V), Trisbase 25mM, glycine 192mM and SDS 0.037%) for 15min. The proteins were electrotransfered at 0.65mA/cm² for 1h onto Protran nitrocellulose transfer membrane pre-soaked in transfer buffer.

The nitrocellulose was incubated in Tris-buffered saline (TBS±T; NaCl 300mM and Tris-HCl 25mM ± Tween 20 0.05%) containing 5% (W/V) powdered low fat skimmed milk (blocking buffer), to prevent non-specific binding for 1-2h. After washing with TBST, the nitrocellulose was incubated overnight at 4°C with Ga rabbit polyclonal antibody at a final dilution of 1:1000. After copious washing with TBST, the nitrocellulose was incubated for 1-2h with horseradish peroxidase conjugated mouse anti-rabbit secondary antibody (1:1000 dilution). The nitrocellulose was washed with TBST, dried and developed with ECL+ reagents, according to the manufacturer’s instruction and exposed to hyperfilm.

To determine equivalent protein loading the nitrocellulose membranes were routinely stripped using stripping buffer (2-βmercaptoethanol 0.7%, SDS 2% and Tris-HCl (pH 6.8) 62.5mM). Membranes were incubated with stripping buffer at 50°C for 30min with constant agitation. Membranes were washed with copious amounts of TBST for 30min, the nitrocellulose membrane blocked and re-probed for γ-tubulin. The antibody was used at dilution of 1:10,000 in TBST and processed as described above.
2.13 Ins(1,4,5)P₃-mediated release of ⁴⁵Ca²⁺ from intracellular stores of permeabilised cells

⁴⁵Ca²⁺ release assays were performed in cytosol-like buffer (CLB; KCl 120mM, KH₂PO₄ 2mM, (CH₂COONa)₂ 5mM, MgCl₂ 2.4mM, Hapes 20mM and ATP 2mM, pH 7.2). The [Ca²⁺] of CLB was determined using fura-2 and buffered to 120-190nM with EGTA. Media from HEK 293 cells grown to confluency in 175cm² flasks were aspirated and 10ml of Hapes/EDTA/NaCl solution (lifting buffer) added and incubated for 5min at 37°C. Detached cells were transferred to a centrifuge tube and flask washed with an additional 10ml of lifting buffer. Cells were centrifuged for 3min at 500g. The pellet obtained was washed twice with 3ml of CLB. Cells were centrifuged for 1min at 500g following each wash. For saponin permeabilisation, cells were resuspended in 2ml of CLB and 200µl of 1mg/ml saponin added and incubated for 1min. Following this, cells were centrifuged at 500g for 2min. The supernatant was removed and cells resuspended in required final volume of CLB. Intracellular Ca²⁺ stores were loaded with ⁴⁵Ca²⁺ by the addition of 2.3mCi/ml of ⁴⁵CaCl₂ and cells gently vortexed. Cells were allowed to incubate for 15-20min at room temperature. To initiate the release of ⁴⁵Ca²⁺, 50µl of cells were added to 50µl of Ins(1,4,5)P₃ prepared at 2x concentration. After 60s, 500µl of silicon oil was added and the cells centrifuged at 16,000g for 2min. The aqueous phase and most of the silicon oil-phase was aspirated, the tubes were inverted and the remaining oil drained. The pellet was solubilised in scintillant and the unreleased ⁴⁵Ca²⁺ was determined. ⁴⁵Ca²⁺ retained in the stores were calculated as a percentage of the total ⁴⁵Ca²⁺ loaded. The size of the rapidly releasable pool was also determined using 10µM ionomycin to indicate the amount of ⁴⁵Ca²⁺ loaded into the intracellular store. For determining any potentiation of Ca²⁺ release by cAMP or noradrenaline, cells were prestimulated with varying concentrations of cAMP or 10µM noradrenaline for 10min prior to stimulation with methacholine or exogenous Ins(1,4,5)P₃. Neil Johnston (Research Technician, Department of Cell Physiology and Pharmacology, University of Leicester) carried out these experiments.

2.14 Protein determination by the method of Lowry

Protein estimations of cell samples were carried out by the method of Lowry (Lowry et al., 1951). The cells were lysed with 1ml of 0.1M NaOH. Protein standards were
prepared by diluting bovine serum albumin (BSA, 1mg/ml) in 0.1M NaOH. Standard concentrations of 0, 25, 50, 100, 200, 250 and 400μg/ml were used. To standards and samples, 1ml of the solution containing NaHCO₃ 2%, CuSO₄ 1% and NaKC₄H₄O₆.4H₂O 2% was added. The tubes were vortexed and allowed to incubate at room temperature for 10min. To this, 100μl of Folin’s reagent was added (1:2 dilution in water) and vortexed. The tubes were incubated for 20min at room temperature. Finally, 1ml of water was added to the samples, vortexed and the absorbance measured at 750nm in the Beckman Du 65 spectrophotometer. Standard curves were fitted using GraphPad Prism using a third order polynomial equation. The unknown protein concentrations were calculated by interpolation of the standard curve.

2.15 Data analysis

All data presented are mean of at least 3 experiments, unless otherwise stated. The standard error of the mean (S.E.M.) for all experiments, were determined from the mean of the values from each individual experiment. Where representative data are presented, experiments were performed to n of at least 3 or more. Where statistical analyses were required, the format of the data was taken into account in the selection of appropriate test. An Unpaired Student’s t-test was used for direct comparison of a test value with a control. Statistical significance of differences between multiple data sets was determined using either one-way analysis of variance (ANOVA) or two-way analysis of variance (ANOVA) with an appropriate post test, for multiple comparisons. Statistical significance in either cases was considered for p values less than 0.05 and is indicated by * throughout.
Chapter 3: Comparison of the role for partial agonists of the muscarinic receptor in second messenger accumulation, receptor phosphorylation and internalisation

3.1 Introduction

The focus of this thesis was to examine and characterise crosstalk in HEK 293 cells and to examine the ability of muscarinic receptor agonists, varying in intrinsic activity to mediate crosstalk. This chapter aims to characterise signalling, phosphorylation and internalisation mediated by a range of muscarinic receptor agonists in a recombinant system with either high or low muscarinic M₃ receptor expression. These recombinant systems were used to explore the concept of partial agonism. Agonists bind to a receptor (affinity) producing a biologic response (efficacy). Efficacy is a molecule-related property with different molecules having different abilities to induce a physiological response. Therefore, it is essential to describe the response-inducing ability of a molecule as its intrinsic efficacy. Each molecule has a unique value for its intrinsic efficacy and the response evoked is a function of receptor number and intrinsic efficacy of the agonist. For any given receptor subtype, the coupling efficiency of the recombinant host often determines the characteristics of agonism. For example, a given agonist could produce no response, a partial response or maximal response in the system. Agonists that produce submaximal responses are termed partial agonists while agonists that mediate a maximal response via the same receptors are called full agonists. In principle, partial agonists can activate receptors at desired submaximal points when inadequate amounts of the endogenous ligand are present or they can act as antagonists and turn off receptors when excess amounts of the endogenous ligand are present (Zhu, 2005). These effects of partial agonists have been exploited in drug therapy as they help to avoid the development of adverse effects such as desensitisation, tolerance or dependence associated with over stimulation of the receptors by full-agonists (Zhu, 2005). However, partial agonism is a context dependent phenomenon. The intrinsic activity of agonists is influenced by the coupling efficiency of the system in which the responses are tested as well as a range of other factors including the biological response evaluated and receptor expression levels.

Classically, an agonist is thought to induce active receptor conformations causing a response. Several theories have been put forward to explain agonist binding and
subsequent receptor signalling (refer to Section 1.2). Full and partial agonists were classically thought only to differ in intrinsic activity, a term coined by Ariëns to explain the inability of certain drugs to elicit maximal responses, despite maximal receptor occupancy (Clark & Bond, 1998). However, evidence is beginning to emerge about agonist-specific receptor configurations. For example, fluorescence lifetime imaging of site-specific labelled amino acid residues in the 3i loop of the β2-adrenoceptor was used to understand functional difference between agonist binding and receptor coupling (Ghanouni et al., 2001). Agonist induced movement was characterised by examining interactions between fluorescein maleimide labelled Cys265 residue and fluorescence quenching reagents localised to different molecular environments of the receptor. Binding of full agonist to the chimeric molecule reduced the fluorescence intensity by ~15%, whereas the binding of partial agonists resulted in smaller changes in fluorescence intensity. These results are consistent with the notion that either a clockwise rotation of transmembrane domain 6 or a tilting of the cytoplasmic end of transmembrane domain 6 towards transmembrane domain 5 occurs on ligand binding. Furthermore, they demonstrated that different agonists of the β2-adrenoceptor induced distinct conformations in the G-protein coupling domain of the receptor. Based on their findings they proposed a model with multiple agonist-specific receptor states, where activation occurs through a series of conformational changes (Ghanouni et al., 2001).

To examine the hypothesis that functional properties of partial agonists are due to their ability to stabilise distinct conformational states of GPCRs, Seifert et al., (2001) used fusion proteins either between the β2-adrenoceptor and Ga4 (β2ARGα4) or a constitutively active mutant of the β2-adrenoceptor and Ga4 (β2ARCAMGa4) in Sf9 insect cells. The use of fusion proteins has several advantages in that it eliminates the bias in the analysis of agonist potencies and efficacies caused by varying ratios of GPCR and G-proteins and allows accurate determination of agonist efficacy in an expression level-independent manner by measurement of steady state GTP hydrolysis and ternary complex formations (Seifert et al., 2001). This study demonstrated that ligands stabilise distinct configurations that differs from each other in their ability to interact with G-proteins. The full agonist isoproterenol and salbutamol induced conformations in wildtype and constitutively active mutants that were functionally distinguishable from those induced by the partial agonist dobutamine, ephedrine and dichloroisoproterenol.
(Seifert et al., 2001). These studies highlight the importance of exploring various aspects of receptor function whilst studying partial agonism.

This chapter details the initial characterisation of partial agonists of the muscarinic receptors, in cell systems expressing either high or low receptor number and defines the pharmacological parameters describing them (maximal responses and pEC$_{50}$ values). Properties of the agonists in mediating second messenger responses, receptor phosphorylation and internalisation were used as a basis for comparison for different agonists.
3.2 Results

3.2.1 Saturation antagonist binding in cells stably expressing the muscarinic M₃ receptor

CHO-K1 cells recombinantly expressing human muscarinic M₃ receptors were used to characterise the signalling and down-regulation of receptors following agonist stimulation. Partial agonism is a context dependent phenomenon, with levels of receptor expression influencing the subsequent signalling mediated by the agonist (January et al., 1998). To determine the levels of muscarinic M₃ receptor expression, saturation antagonist binding with the non-selective muscarinic receptor antagonist N-methyl scopolamine ([³H]-NMS) was carried out as described in Section 2.10. Specific binding of the antagonist to the receptor was plotted as the difference between total binding and non-specific binding (NSB; Figure 3.2.1a). The Bₘₐₓ was calculated as 194 ± 25 fmol/mg protein and Kᵩ as 0.25nM (-log₁₀ (M) Kᵩ, 9.6 ± 0.30; n=3). This cell line is referred to as VT-31 henceforth. A CHO-K1 cell line stably expressing higher levels of muscarinic M₃ receptors (Buckley-M₃) was also used to compare the properties of arecoline, pilocarpine, oxotremorine and methacholine (Bₘₐₓ 1550 fmol/mg protein; Budd et al., 1999; Buckley et al., 1988).
Fig. 3.2.1a. $[^3]H$-NMS binding on CHO-K1 cells recombinantly expressing muscarinic $M_3$ receptors (VT-31 cells). VT-31 cells were incubated for 60-75 min with a range of concentrations of $[^3]H$-NMS. Non-specific binding was determined in the presence of 2μM atropine. Data are mean±S.E.M. (n=3).
3.2.2 **Muscarinic receptor agonist-mediated Ca\(^{2+}\) responses in VT-31 and Buckley-M\(_3\) cells**

To assess if arecoline, pilocarpine and oxotremorine were partial agonists in respect of Ca\(^{2+}\) responses, VT-31 cells were stimulated with a range of concentrations of these agonists in a FLIPR. The responses evoked by these agonists were compared to the response to methacholine in these cells (Figure 3.2.2a, Panel A). The rank order of efficacy based on the magnitude of Ca\(^{2+}\) responses in VT-31 cells was methacholine>arecoline>oxotremorine>pilocarpine. The experiments were also repeated in Buckley-M\(_3\) cells with higher receptor expression. In these cells, responses mediated by arecoline, pilocarpine and oxotremorine were not significantly different to the methacholine-mediated Ca\(^{2+}\) responses (Figure 3.2.2a; Panel B).

The concentration response curves to arecoline, pilocarpine, methacholine and oxotremorine obtained in each cell type are represented in Figure 3.2.2b. The pEC\(_{50}\) of muscarinic receptor agonists in mediating Ca\(^{2+}\) responses varied significantly in each cell line with the responses being more potent in the Buckley-M\(_3\) cells in comparison to VT-31 cells with lower receptor expression (Table 3.2.2a). The rank order of agonist potency was methacholine>oxotremorine>arecoline>pilocarpine in both VT-31 and Buckley-M\(_3\) cells.
Fig. 3.2.2a. Muscarinic receptor agonist-mediated $E_{\text{max}} \text{Ca}^{2+}$ responses in VT-31 cells and Buckley-M$_3$ cells. Cells were stimulated in a FLIPR with a range of muscarinic receptor agonists (t=10s) and fluorescence recorded for 180s. Change in fluorescence on agonist stimulation was quantified and taken as an index of change in the $[\text{Ca}^{2+}]_i$. Histogram represents $E_{\text{max}}$ responses to muscarinic receptor agonists in VT-31 cells (Panel A) and Buckley-M$_3$ cells (Panel B). Data are mean $\pm$ S.E.M. (n=3).
Fig. 3.2.2b. Muscarinic receptor agonist-mediated \( \text{Ca}^{2+} \) responses in VT-31 cells and Buckley-M3 cells. Cells were stimulated with a range of concentrations of muscarinic receptor agonists in a FLIPR. Change in fluorescence on agonist stimulation was quantified and taken as an index of change in the \([\text{Ca}^{2+}]_i\). Panel A represents concentration-dependent responses to the muscarinic receptor agonists in VT-31 cells. Panel B represents concentration-dependent responses to the muscarinic receptor agonists in Buckley-M3 cells. Data are mean \( \pm S.E.M. \). (n=3).
Table 3.2.2. The pEC\textsubscript{50} values of muscarinic receptor agonist-mediated Ca\textsuperscript{2+} responses in VT-31 cells and Buckley-M\textsubscript{3} cells (relate to Figure 3.2.2b). Data are mean ± S.E.M. (n=3). **, p<0.01; ***, p<0.001, by one-way ANOVA with Bonferroni’s post tests.

<table>
<thead>
<tr>
<th>Muscarinic receptor agonists</th>
<th>pEC\textsubscript{50} of muscarinic receptor agonist-mediated Ca\textsuperscript{2+} responses in VT-31 cells</th>
<th>pEC\textsubscript{50} of muscarinic receptor agonist-mediated Ca\textsuperscript{2+} responses in Buckley-M\textsubscript{3} cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arecoline</td>
<td>*** 5.41±0.11</td>
<td>** 6.17±0.09</td>
</tr>
<tr>
<td>Pilocarpine</td>
<td>4.94±0.19 **</td>
<td>5.36±0.12 ***</td>
</tr>
<tr>
<td>Methacholine</td>
<td>6.84±0.12</td>
<td>7.46±0.12</td>
</tr>
<tr>
<td>Oxotremorine</td>
<td>*** 6.40±0.09</td>
<td>*** 6.78±0.10</td>
</tr>
</tbody>
</table>

3.2.3 Muscarinic receptor agonist-mediated total inositol phosphates accumulation in VT-31 cells and Buckley-M\textsubscript{3} cells

To assess whether the muscarinic receptor agonists had a similar profile in their ability to mediate accumulation of total inositol phosphates ([\textsuperscript{3}H]-InsP\textsubscript{x}) as the Ca\textsuperscript{2+} responses, cells were stimulated with 1mM concentrations of agonists for 20min. The assay was carried out as described in Section 2.4.1. Data obtained were expressed as the fold increase in [\textsuperscript{3}H]-InsP\textsubscript{x} relative to basal levels of [\textsuperscript{3}H]-InsP\textsubscript{x} in unstimulated cells.

In VT-31 cells, the responses evoked by arecoline, pilocarpine and oxotremorine were significantly different to methacholine-mediated accumulation of [\textsuperscript{3}H]-InsP\textsubscript{x}. Methacholine stimulation resulted in a 10-fold increase in accumulation of [\textsuperscript{3}H]-InsP\textsubscript{x} while arecoline, pilocarpine and oxotremorine mediated a 8-fold, 5-fold and 7-fold accumulation of [\textsuperscript{3}H]-InsP\textsubscript{x} respectively (Figure 3.2.3a; Panel A). In Buckley-M\textsubscript{3} cells, methacholine evoked a 13-fold increase in accumulation of [\textsuperscript{3}H]-InsP\textsubscript{x} in comparison to arecoline (9-fold), pilocarpine (6-fold) and oxotremorine (12-fold; Figure 3.2.3a; Panel B). The responses evoked by pilocarpine were significantly different to the accumulation of [\textsuperscript{3}H]-InsP\textsubscript{x} mediated by methacholine and oxotremorine. Furthermore, in these cells the responses mediated by oxotremorine and methacholine were similar.
The maximal accumulation of $[^3\text{H}]-\text{InsP}_x$ in VT-31 cells were of the order methacholine$>$arecoline$>$oxotremorine$>$pilocarpine which was similar to the rank order for agonist-mediated Ca$^{2+}$ responses. In Buckley-M$_3$ cells, arecoline and pilocarpine mediated comparable maximal Ca$^{2+}$ responses in these cells (87% of the maximal methacholine response). However, for the accumulation of $[^3\text{H}]-\text{InsP}_x$, pilocarpine behaved as a partial agonist, mediating only 56% of the methacholine-mediated response, while arecoline mediated 84% of the response to methacholine. A comparison between the maximal responses evoked by muscarinic receptor agonists in the two cell-types is represented in Table 3.2.3.
Fig. 3.2.3a. Muscarinic receptor agonist-mediated accumulation of [3H]-InsP₃ in VT-31 and Buckley-M₃ cells. Cells were stimulated with 1mM concentrations of muscarinic receptor agonists for 20min. Panel A and B represents [3H]-InsP₃ accumulation in VT-31 and Buckley-M₃ cells stimulated with muscarinic receptor agonists. Basal values at t=0 were 5663±589 and 6587±947 DPM/well in VT-31 and Buckley-M₃ cells respectively. Data are mean ± S.E.M. (n=3). ***, p<0.001; **, p<0.01; *, p<0.05, by one-way ANOVA with Bonferroni's post tests.
Table 3.2.3. The $E_{\text{max}}$ value of muscarinic receptor agonist-mediated $\text{Ca}^{2+}$ responses and accumulation of $[^{3}\text{H}]$-InsP$_{x}$ in VT-31 and Buckley-M$_{3}$ cells (relate to Figure 3.2.3a; Figure 3.2.2a). Data are represented as a percentage of methacholine-mediated responses. Data are mean ± S.E.M. (n=3).

<table>
<thead>
<tr>
<th>Muscarinic receptor agonist</th>
<th>VT-31 cells</th>
<th>Buckley-M$_{3}$ cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\text{Ca}^{2+}$</td>
<td>$[^{3}\text{H}]$-InsP$_{x}$</td>
</tr>
<tr>
<td>Arecoline</td>
<td>74±32</td>
<td>79±3</td>
</tr>
<tr>
<td>Pilocarpine</td>
<td>42±18</td>
<td>49±8</td>
</tr>
<tr>
<td>Methacholine</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Oxotremorine</td>
<td>46±19</td>
<td>74±3</td>
</tr>
</tbody>
</table>

3.2.4 Agonist-mediated phosphorylation of muscarinic receptors in Buckley-M$_{3}$ cells

GPCRs, despite their structural and functional diversity, often share common mechanisms of desensitisation. Typically, uncoupling of the G-protein from the receptor is dependent on receptor phosphorylation mediated by intracellular second messenger kinases or GRKs. A 1 mM concentration of carbachol has been previously demonstrated to mediate a robust phosphorylation of muscarinic receptors in Buckley-M$_{3}$ cells (Tobin et al., 1995; Tobin & Nahorski, 1993). To assess if there was a positive correlation between agonist-mediated second messenger responses and receptor phosphorylation, Buckley-M$_{3}$ cells were stimulated with maximally effective concentrations of agonists (1 mM, based on previous results) for 2 min. Unstimulated cells were used to determine basal phosphorylation. Following a 2 min stimulation, 1 mM methacholine-mediated a significant increase in receptor phosphorylation (p<0.001 unpaired Student’s t-test). Although there was some indication of differences between the ability of partial agonists and methacholine to mediate phosphorylation, these were not statistically significant (all statistical analyses were carried out on optical density values in agonist stimulated cells after basal subtraction).
Fig. 3.2.4a. Agonist-mediated phosphorylation of muscarinic $M_3$ receptors in Buckley-$M_3$ cells. Cells loaded with [$^{32}$P]orthophosphate for 60 min were stimulated with muscarinic receptor agonists for 2 min. Panel A shows representative blots for receptor phosphorylation mediated by muscarinic receptor agonists. Data were quantified by densitometric analysis. Optical density values obtained from unstimulated cells (basal) were subtracted from values obtained in agonist stimulated cells. Histogram represents the receptor phosphorylation expressed as a percentage of methacholine-mediated receptor phosphorylation (Panel B). Data are mean ± S.E.M. (n=3).
3.2.5 **Agonist-mediated internalisation of muscarinic receptors in Buckley-M₃ cells**

Following phosphorylation of GPCRs by serine-threonine kinases, these receptors are sequestered into clathrin-coated or uncoated pits for subsequent receptor resensitisation or down-regulation. To determine the ability of muscarinic receptor agonists to mediate receptor internalisation, a radiolabelled antagonist ([^3]H-NMS) was used to determine plasma membrane receptor density following agonist stimulation. This allows calculation of the percentage of receptors that have internalised following agonist stimulation (defined here as a reduction in the number of receptors at the cell surface). To determine the time-course for agonist-mediated muscarinic M₃ receptor internalisation, Buckley-M₃ cells were stimulated with 1mM methacholine for up to 60min. This concentration of the agonist was demonstrated to cause robust agonist-mediated second messenger responses and receptor phosphorylation in these cells. In cells stimulated with 1 mM methacholine, there was a gradual loss in the receptor number for up to 20min. The percentage of internalised receptors reached a plateau after 30min stimulation with methacholine (Figure 3.2.5a). To assess if other muscarinic receptor agonist-mediate internalisation of the receptor to the same extent as methacholine, Buckley-M₃ cells were stimulated with 1mM concentrations of arecoline, pilocarpine or oxotremorine for 30 or 60min (Figure 3.2.5b).

Arecoline and pilocarpine mediated internalisation of only a small proportion of receptors in comparison to methacholine (48% and 30% of methacholine response respectively; Figure 3.2.5b; Table 3.2.5). Oxotremorine on the other hand, mediated internalisation of receptors comparable to methacholine-mediated receptor internalisation (Figure 3.2.5b). This apparent increase in the proportion of internalised receptors could also result from residual agonist competing with[^3]H-NMS for cell surface receptor binding. This could be determined by using[^3]H-QNB binding. QNB is lipophilic and will label both cell surface and internalised receptors. Total receptor number should remain largely unaffected over the period of 60 min if internalisation is occurring. On the other hand, if there is a decrease in[^3]H-QNB binding, particularly at early time points when receptor degradation is likely to be minimal, this would suggest that residual agonist may be competing for binding with[^3]H-NMS.
Fig. 3.2.5a. Time-course for internalisation of muscarinic M₃ receptors in methacholine stimulated Buckley-M₃ cells. Cells were stimulated with 1mM methacholine for times indicated before determination of receptor density. Data are represented as a percentage of receptor density in unstimulated cells. Data are mean±S.E.M. (n=3). ***, p<0.001, by one-way ANOVA with Dunnett's post tests.

Fig. 3.2.5b. Internalisation of muscarinic M₃ receptors mediated by agonists in Buckley-M₃ cells. Cells were stimulated with 1mM concentration of muscarinic receptor agonists for 30 or 60 min before determination of receptor density. Data are represented as a percentage of receptor density in unstimulated cells. Data are mean±S.E.M. (n=3). ***, p<0.001; **, p<0.01, by one-way ANOVA with Dunnett's post tests.
Table 3.2.5. Comparison of muscarinic receptor agonist-mediated $Ca^{2+}$ responses, accumulation of $[^3H]$-InsP$_x$, receptor phosphorylation and internalisation in Buckley-$M_3$ cells (relate to Figure 3.2.2a; Figure 3.2.3a; Figure 3.2.4a; Figure 3.2.5b). $Ca^{2+}$ responses, accumulation of $[^3H]$-InsP$_x$, receptor phosphorylation and internalisation are represented as percentage of methacholine-mediated responses. Data are mean ± S.E.M. (n≥3).

<table>
<thead>
<tr>
<th>Muscarinic receptor agonists</th>
<th>Ca$^{2+}$ responses</th>
<th>$[^3H]$-InsP$_x$ responses</th>
<th>Receptor phosphorylation</th>
<th>Receptor internalisation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arecoline</td>
<td>73±25</td>
<td>69±7</td>
<td>59±15</td>
<td>48±9</td>
</tr>
<tr>
<td>Pilocarpine</td>
<td>60±16</td>
<td>44±2</td>
<td>36±6</td>
<td>30±6</td>
</tr>
<tr>
<td>Methacholine</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Oxotremorine</td>
<td>67±21</td>
<td>92±0.13</td>
<td>61±21</td>
<td>101±8</td>
</tr>
</tbody>
</table>
3.3 Discussion

3.3.1 Summary of data

This chapter examines a range of muscarinic receptor agonists in their ability to mediate second messenger responses, receptor phosphorylation and internalisation of muscarinic M₃ receptors stably expressed in CHO-K1 cells. It was demonstrated that partial agonism is a context dependent phenomenon with levels of receptor expression affecting the degree of agonism, as expected. This chapter also provides the direct comparison of the ability of muscarinic receptor agonists to mediate second messenger responses, phosphorylation and internalisation. Of the agonists tested, oxotremorine, although mediating smaller second messenger responses and receptor phosphorylation in comparison to arecoline was able to cause internalisation of the receptor in a manner equivalent to the full agonist methacholine.

3.3.2 Partial agonists and second messenger responses

A number of different muscarinic receptor agonists were used in this study. Arecoline, pilocarpine and oxotremorine have been shown to be partial agonists at the muscarinic receptor in a range of cell systems (Sharif et al., 1995; Szekeres et al., 1998; Mistry et al., 2005). In agonist-mediated Ca²⁺ responses in VT-31 cells with lower receptor expression, pilocarpine and oxotremorine were partial agonists in comparison to methacholine. However, in Buckley-M₃ cells (with higher receptor expression) arecoline, pilocarpine and oxotremorine all mediated comparable maximal responses. The difference in the intrinsic activity of pilocarpine and oxotremorine is expected due to the large receptor reserve in Buckley-M₃ cells allowing enough G-proteins to be activated to produce a full response over a longer time-period. All the agonists mediated a more potent response in the Buckley-M₃ cells in comparison to VT-31 cells. However, the agonist potency order of methacholine>oxotremorine>arecoline>pilocarpine was similar in both cell-lines. This is in agreement with the rank order of agonist potency in mediating Ins(1,4,5)P₃ response in SH-SY5Y neuroblastoma cells, which endogenously expressed muscarinic M₃ receptors (Szekeres et al., 1998). For agonist-mediated accumulation of [³H]-InsPₓ, in Buckley-M₃ cells, oxotremorine-mediated a response equivalent to methacholine, while pilocarpine mediated a partial response. The maximal accumulation of [³H]-InsPₓ was of the order methacholine>oxotremorine>arecoline>
pilocarpine. In VT-31 cells, pilocarpine was a partial agonist in mediating accumulation of $[^3\text{H}]$-InsP$_x$ and the maximal responses were of the order methacholine$>$arecoline$>$oxotremorine$>$pilocarpine. This indicates that accumulation of $[^3\text{H}]$-InsP$_x$ is a more robust assay in comparison to Ca$^{2+}$ responses to determine varying degrees of agonism of the ligands. The discrepancy in Buckley-M$_3$ cells between pilocarpine-mediated Ca$^{2+}$ responses and accumulation of $[^3\text{H}]$-InsP$_x$ could be explained on the basis that if one second messenger, Ins(1,4,5)P$_3$ (measured here as accumulation of $[^3\text{H}]$-InsP$_x$) triggers the activation of a second measurable response (Ca$^{2+}$) the magnitude of the second response will be more sensitive the strength of the signal (Kenakin, 1995).

Studies in endogenous muscarinic receptors in the human neuroblastoma SH-SY5Y cell line have demonstrated that measurement of $[^3\text{H}]$-InsP$_x$ accumulation for a longer time period does not reflect the initial levels of PLC activity, which drive the peak Ca$^{2+}$ responses (Willars & Nahorski, 1995). In these cells there was a biphasic accumulation of $[^3\text{H}]$-InsP$_x$, being more rapid in initial minute of agonist stimulation in comparison to the slower linear accumulation for the subsequent 29min of agonist stimulation. This change in rate may reflect the biphasic nature of Ins(1,4,5)P$_3$ responses (Willars & Nahorski, 1995). Therefore, measurement of $[^3\text{H}]$-InsP$_x$ accumulation at extremely short time-periods may reflect the initial level of PLC activity with measurement for prolonged time-periods reflecting PLC activity during the sustained phase of Ins(1,4,5)P$_3$ generation (Willars & Nahorski, 1995). Similarly in Buckley-M$_3$ cells, accumulation of $[^3\text{H}]$-InsP$_x$ may be biphasic, which may not be reflected in measurement of $[^3\text{H}]$-InsP$_x$ for longer time-periods.

### 3.3.3 Muscarinic receptor agonist-mediated receptor phosphorylation and internalisation

This study characterised the ability of the muscarinic receptor agonists not only to mediate second messenger responses but also to mediate phosphorylation of the receptor by endogenous serine-threonine kinases in Buckley-M$_3$ cells. The ability of methacholine to mediate phosphorylation of the muscarinic M$_3$ receptor in this cell line has been previously characterised (Tobin et al., 1995; Tobin & Nahorski, 1993). To determine if there is a good correlation between the efficacies of agonists to mediate
second messenger responses and receptor phosphorylation, Buckely-M₃ cells were stimulated with arecoline, pilocarpine, oxotremorine or methacholine (all at 1 mM). Although there were suggestions of an ability of arecoline, pilocarpine and oxotremorine to phosphorylate the muscarinic M₃ receptors partially in comparison to methacholine, statistically significant differences were not found. However, the rank order of efficacy of these agonists to phosphorylate the receptor was similar to their ability to mediate total inositol phosphate accumulation. To further characterise the ability of pilocarpine, oxotremorine and arecoline to mediate receptor phosphorylation, time-course and concentration-response experiments could be carried out.

The results obtained in this study are in agreement with literature evidence of a correlation between the ability of agonist efficacy to mediate second messenger responses and receptor phosphorylation (January et al., 1997; January et al., 1998; Yu et al., 1997). For example, a study examining the ability of a range of β₂-adrenoceptor agonists to mediate second messenger responses and receptor phosphorylation has demonstrated that at a concentration of the agonist yielding >90% occupancy of the receptor, the rate of agonist-induced phosphorylation exhibited a dependence on the agonist strength (January et al., 1997). However, previous studies in Buckely-M₃ cells revealed that receptor phosphorylation is closely linked with receptor-mediated PLC activation but not with agonist-mediated increase in 

$$\left[\text{Ca}^{2+}\right]_i$$ (Tobin et al., 1995). It was demonstrated that the concentration response curves for elevation of 

$$\left[\text{Ca}^{2+}\right]$$ lie to the left of Ins(1,4,5)P₃ generation and receptor phosphorylation, while agonist binding curve lie to the right of agonist-mediated receptor phosphorylation, Ins(1,4,5)P₃ generation and Ca²⁺ mobilisation (Tobin et al., 1995). This indicates a receptor reserve for phosphorylation, probably due to the amplification of the receptor signal (Tobin et al., 1995). Furthermore, studies in constitutive active mutants of angiotensin II (AT₁₆) receptors have shown that despite the ability of these mutants to mediate [³H]InsPₓ accumulation, there was no increase either the basal levels of receptor phosphorylation or enhanced phosphorylation on angiotensin stimulation (Thomas et al., 2000). An inactive analogue of angiotensin paradoxically enhanced phosphorylation of the wild-type receptor but failed to mediate phosphorylation in the constitutive mutants. This was despite the angiotensin analogue having a higher affinity for the mutant receptor, indicating that the molecular switches required for phosphorylation are distinct from those required for G protein-mediated signalling (Thomas et al., 2000). Based on their
observations, the authors suggest that AT1a receptors can attain a conformation for phosphorylation without going through the conformation required for [3H]-InsP3 accumulation providing evidence for transition of the receptor through multiple states each associated with a separate stage for receptor activation (Thomas et al., 2000). This provides evidence that a perfect correlation between agonist-mediated second messenger responses and receptor phosphorylation may not exist.

To determine if there is a correlation between agonist-mediated receptor phosphorylation and internalisation, plasma membrane receptor density was quantified by binding of the hydrophilic muscarinic receptor antagonist, [3H]-NMS. These studies revealed that in Buckley-M3 cells, pilocarpine and arecoline although very effective in mediating second messenger responses and receptor phosphorylation in comparison to methacholine, mediated internalisation of only 30 and 48% of receptors, respectively. This was in contrast to oxotremorine, which mediated internalisation comparable to full agonist methacholine. The rank order of the agonists ability to mediate receptor internalisation was methacholine ≈ oxotremorine > arecoline > pilocarpine. However, these results should be interpreted with caution as similar results could be attained if residual agonist was competing for receptor binding with [3H]-NMS. To confirm receptor internalisation, total receptor number could be estimated using [3H]-QNB binding. If there were a significant loss of receptor binding as determined using QNB this would suggest that the observed decrease in [3H]-NMS binding is likely due to the presence of residual agonist. There were also differences in the internalisation of muscarinic M3 receptors endogenously expressed in HEK 293 cells versus Buckley-M3 cells. In HEK 293 cells, stimulation with 1 mM methacholine resulted in the internalisation of only 22% of total plasma membrane receptors. Although it is difficult to explain such differences in internalisation of receptors between cell lines, factors such as rate constants for endocytosis and recycling may influence internalisation (Edwardson & Szekeres, 1999).

Szekeres et al., (1998) demonstrated that there is a linear relationship between the increase in the rate constant for muscarinic M3 receptor endocytosis and the intrinsic activity of ligands. They demonstrated that the full agonist methacholine caused internalisation of nearly 90% of the muscarinic M3 receptors in SHY5Y cells, in comparison to pilocarpine and arecoline that exhibited partial agonism. Furthermore,
they demonstrated that the intrinsic activity of muscarinic agonists followed the same rank order as receptor internalisation. Similarly, the rank order of opiate ligand efficacies in producing μ receptor-mediated functional desensitisation were shown to be similar to their rank order of efficacy in mediating receptor phosphorylation (Yu et al., 1997). This is in contrast to the lack of correlation for agonist-mediated receptor phosphorylation and internalisation observed in this study. This is further highlighted in the study examining the endocytotic trafficking of opioid receptors in HEK 293 cells (Keith et al., 1996). In cells stably expressing δ and μ-opioid receptors stimulation with morphine did not induce endocytosis despite both the receptor subtypes being activated (Keith et al., 1996). On the other hand, etorphine that exhibits a similar structure to morphine induced internalisation of both receptor subtypes. The authors propose that different agonists can induce different conformations of the receptor that similarly activate G-proteins, but differ significantly in their intracellular trafficking (Keith et al., 1996). However other studies have reported that morphine is not as potent an agonist as described previously, thereby accounting for its reduced ability to induce receptor internalisation (Yu et al., 1997; Kovoor et al., 1998). In summary, literature evidence suggests that despite some deviations, there exists a good correlation between agonist-coupling efficiency with desensitisation, phosphorylation and internalisation (Clark et al., 1999).

The following Chapters examine the ability of partial agonists of the muscarinic receptor to mediate crosstalk and to determine if a correlation between intrinsic activity of the agonists and crosstalk exists. This Chapter provides a platform for the continuation of this research, aimed at the elucidation of the ability of partial agonists of the muscarinic receptor to mediate crosstalk in HEK 293 cells.
Chapter 4: Crosstalk between differentially coupled GPCRs that result in enhanced intracellular calcium signalling.

4.1 Introduction

Activation of a GPCR does not always lead to a direct effect on a particular signalling pathway, but an augmentation of responses by synchronized signalling by distinct pathways (Selbie & Hill, 1998). The phenomenon of crosstalk has been described extensively with numerous receptor combinations resulting in the enhancement or loss (desensitisation) of function. The work detailed in this thesis describes crosstalk resulting in enhanced \([\text{Ca}^{2+}]\). Cytosolic \(\text{Ca}^{2+}\) is a versatile cellular signal that is involved in the control of numerous cellular functions ranging from fertilisation, proliferation, differentiation, secretion and contraction (Rottingen & Iversen, 2000). Any changes in cytosolic \(\text{Ca}^{2+}\) therefore can influence a plethora of cellular functions.

Enhanced mobilisation of \([\text{Ca}^{2+}]\), resulting from crosstalk between different GPCRs can be illustrated by the demonstration that the recombinant \(\text{G}_{\alpha_i}\)-coupled chemokine receptor CXCR\(_2\) only elevates \([\text{Ca}^{2+}]\), in HEK 293 cells in the presence of activated endogenously expressed \(\text{G}_{\alpha_i}\)-coupled purinergic receptors (Werry et al., 2003). This signalling is dependent on the continued presence of the purinergic receptor agonist but independent of extracellular \(\text{Ca}^{2+}\), implicating that crosstalk results from enhanced \(\text{Ca}^{2+}\) release from an intracellular store. In another example of crosstalk between endogenously expressed receptor systems, the parathyroid hormone receptor (\(\text{G}_{\alpha_s}\)-coupled) potentiates \(\text{Ca}^{2+}\) release mediated by the activation of \(\text{G}_{\alpha_{q11}}\)-coupled purinergic receptors in rat osteoblasts, whereas parathyroid hormone (PTH) alone is unable to elevate \([\text{Ca}^{2+}]\) (Buckley et al., 2001). PTH is the systemic regulator of bone and mineral homeostasis, resulting in reabsorption and new bone formation. The potentiated \(\text{Ca}^{2+}\) response results in enhancement of transcription factor activation and gene expression. The transcription factor cAMP response element binding-protein (CREB) bind the cAMP response element and initiates transcription in response to a number of extracellular factors including elevated \([\text{Ca}^{2+}]\). The phosphorylation of CREB (phospho-CREB) is also affected by the potentiation of \(\text{Ca}^{2+}\) responses to PTH. Phospho-CREB in turn activates the proto-oncogene \(c-fos\) that has been implicated in...
regulation of osteoblast functions including proliferation and differentiation (Buckley et al., 2001; Lee et al., 1995).

Crosstalk can also occur between endogenously expressed $\alpha_{q11}$-coupled purinergic receptors and $\alpha_{q}$-coupled $\beta$-adrenoceptors in HEK 293 cells (Werry et al., 2002). Noradrenaline was able to elicit $Ca^{2+}$ signalling only in the presence of an activated $\alpha_{q11}$-coupled purinergic receptor. In this study, we used the HEK 293 cell system to characterise crosstalk and to explore the mechanism by which this occurs between the $\alpha_{q11}$-coupled muscarinic M$_3$ receptors and $\beta_2$-adrenoceptors, expressed endogenously in these cells (Ancellin et al., 1999; Freidman et al., 2002).

Characterisation of crosstalk between these receptors are important as they are physiologically co-expressed in a multitude of cell types including smooth muscle cells (refer to Sections 1.4 and 1.6). Furthermore, altered responsiveness of airway smooth muscle cells to contractile or relaxing agonists has been implicated in the pathogenesis of bronchial hyperreactivity (Amrani et al., 2004). Although these receptors mediate their signalling through distinct pathways, evidence for the ability of the two signalling pathways to interact is emerging (Bruce et al., 2003). The muscarinic receptors activate membrane-bound PLC-β that accelerates the hydrolysis of PtdIns(4,5)P$_2$ into DAG and Ins(1,4,5)P$_3$. These second messengers activate PKC and elicit the release of $Ca^{2+}$ from intracellular stores. The $\beta_2$-adrenoceptor on the other hand, activates adenylyl cyclases that in turn generate intracellular cAMP, by hydrolysing ATP to cAMP.

This chapter details the initial characterisation of crosstalk between muscarinic M$_3$ receptors and $\beta_2$-adrenoceptors, endogenously expressed in HEK 293 cells. Furthermore, partial agonists of the muscarinic receptor were also used to assess if they behave differently from full agonists in mediating crosstalk. This chapter defines the pharmacological parameters describing them (maximal responses and $pEC_{50}$ values) and some of the basic properties of crosstalk that suggest a potential mechanism(s). The data from this chapter provide a platform for the continuation of this research aimed at the elucidation of the exact mechanism(s) underlying this crosstalk.
4.2 Results

4.2.1 Demonstration of crosstalk between the muscarinic M₃ receptor and β₂-adrenoceptor

Initial studies using the FLIPR demonstrated that stimulation of HEK 293 cells with the muscarinic agonist methacholine (1 mM) resulted in an increase in \([\text{Ca}^{2+}]\), consisting of a rapid transient peak, followed by a slower declining phase. On the other hand, stimulation with 10 µM noradrenaline failed to elevate \([\text{Ca}^{2+}]\), (Figure 4.2.1a). However, stimulation of HEK 293 cells with 10 µM noradrenaline, in the continued presence of 1 mM methacholine, enabled these cells to elicit a rise in \([\text{Ca}^{2+}]\), with similar kinetics to the methacholine-mediated response (Figure 4.2.1a).

In cells prestimulated with 1 mM methacholine, 10 µM noradrenaline evoked a 7-fold change in fluorescence in comparison to cells stimulated with buffer (change in fluorescence of 2106±352 with noradrenaline in the continued presence of methacholine Vs 310±233 with buffer in the continued presence of methacholine, n=7; p<0.001; Figure 4.2.1b). Similarly, in cells challenged with buffer and subsequently stimulated with noradrenaline little or no elevation of \([\text{Ca}^{2+}]\) was observed (change in fluorescence of 355±90 with noradrenaline in buffer challenged cells, n=7; p<0.001; Figure 4.2.1a, 4.2.1b). The change in fluorescence observed induced by the addition of noradrenaline in buffer pre-treated cells were identical to the change in fluorescence observed when methacholine pre-treated cells were stimulated with buffer. These results demonstrate the ability of noradrenaline to reveal \(\text{Ca}^{2+}\) signalling following and in the continued presence of methacholine.

Experiments were repeated in a single-cell based assay to rule out artefacts from the mechanised addition of the FLIPR such as addition rate or height from which agonists are dispensed into the well. Noradrenaline did not cause an elevation of \([\text{Ca}^{2+}]\), (Figure 4.2.1c). However, in the presence of muscarinic receptor agonists, noradrenaline induced an increase in \([\text{Ca}^{2+}]\), (Figure 4.2.2d).
Fig. 4.2.1a. Demonstration of crosstalk in HEK 293 cells. Representative FLIPR traces of cells challenged with 1mM methacholine or buffer, followed by addition of 10μM noradrenaline or buffer at t=180s. Buffer addition following methacholine, failed to elevate [Ca^{2+}]_i. Challenge of cells with buffer also failed to reveal an elevation of [Ca^{2+}]_i to the addition of noradrenaline. However, in the continued presence of methacholine, noradrenaline caused an elevation in [Ca^{2+}]_i. Data are representative of 7 or more experiments. Mean data are represented in Figure 4.2.1b.
Fig. 4.2.1b. Average responses to 10μM noradrenaline or buffer in HEK 293 cells prestimulated with either 1mM methacholine or buffer. Using the FLIPR, cells were initially challenged with buffer or methacholine (1mM). In its continued presence, cells were subsequently stimulated (t=180s) with 10μM noradrenaline or buffer (relate to Figure 4.2.1a). Change in fluorescence on agonist stimulation was quantified and taken as an index of changes in the level of [Ca$^{2+}$]$_i$. Histogram represents the maximal changes in fluorescence on noradrenaline or buffer challenge, 180s following addition of methacholine or buffer. Data are mean ± S.E.M. (n=7). ***, p<0.001, by one-way ANOVA with Bonferroni's post tests.
Fig. 4.2.1c. Effect of noradrenaline addition on $[Ca^{2+}]_i$ in a single HEK 293 cell. Representative traces showing the lack of response to 10μM noradrenaline in the absence of methacholine challenge. Cells were grown on coverslips, loaded with fluo-3AM and imaged by confocal microscopy. Data are represented as the fold increase in fluorescence over the basal fluorescence. Data are representative of 3 or more experiments.
4.2.2 Demonstration of crosstalk with partial agonists of the muscarinic receptor

A range of muscarinic receptor agonists that have been previously described (refer to Chapter 3) was also used to examine crosstalk. Given that partial agonism is context dependent (January et al., 1998), the efficacies of these agonists were examined in HEK 293 cells. The level of receptor expression of endogenously expressed muscarinic M3 receptor is 40 fmol/mg protein (Tovey & Willars, 2004). These agonists exhibited a range of efficacies and potencies in these cells (Figure 4.2.2a). Methacholine evoked a change in fluorescence units of 20199±5786 (n=7) relative to basal in comparison to arecoline (11081±3948; n=7), pilocarpine (347±290; n=7) and oxotremorine (2786±1383; n=7). From the data obtained, it is apparent that arecoline, pilocarpine and oxotremorine act as partial agonists in comparison to methacholine in this system. However, a potency value for pilocarpine could not be derived as a concentration-dependent effect was not obtained (Figure 4.2.2a, Panel B; Table 4.2.2a).

To assess whether partial agonists at muscarinic receptors behave differently from full agonists in revealing noradrenaline-mediated Ca²⁺ responses, cells were stimulated with 10μM noradrenaline subsequent to stimulation with either partial or full agonists. Priming of cells with partial agonists led to a robust elevation of [Ca²⁺], to 10μM noradrenaline (Figure 4.2.2b). In the continued presence of 1mM concentration of muscarinic receptor agonists, noradrenaline induced a change in fluorescence units of 2106±352 when cells were primed with methacholine, in comparison to 6580±1901 with arecoline, 4718±2032 with pilocarpine and 8285±2325 with oxotremorine (n=7; Figure 4.2.2d). These results indicate that despite the apparent inability of some agonists to mediate a Ca²⁺ response on their own, they revealed Ca²⁺ signalling to noradrenaline, comparable to methacholine or better. For example, 1mM pilocarpine, which evoked a Ca²⁺ response corresponding to 2.3% of the response to 1mM methacholine, induced a noradrenaline response corresponding to 96% of the noradrenaline response in the presence of methacholine. Noradrenaline mediated elevation of [Ca²⁺], was also dependent on the concentrations of the prestimulating muscarinic agonists (Figure 4.2.2c). However, the response to 10 μM noradrenaline was “bell-shaped” with respect of its dependence on the concentration of methacholine. The difference in potencies of
muscarinic receptor agonists (oxotremorine and arecoline) and the potencies of these agonists to reveal a subsequent Ca\textsuperscript{2+} response to 10\mu M noradrenaline were not significantly different (Table 4.2.2b). The potency of methacholine to facilitate a subsequent Ca\textsuperscript{2+} response to 10\mu M noradrenaline could not be derived due to the bell-shaped nature of the concentration response. To confirm this crosstalk and allow more detailed consideration of the responses of individual cells (see also Section 4.2.7), single-cell Ca\textsuperscript{2+} imaging was performed. These experiments demonstrated that in the presence of muscarinic receptor agonists, noradrenaline induced an increase in [Ca\textsuperscript{2+}]\textsubscript{i} (Figure 4.2.2d).
Fig. 4.2.2a. Representation of responses to muscarinic receptor agonists in HEK 293 cells. Using the FLIPR, cells were stimulated (t=10s) with muscarinic receptor agonists. Change in fluorescence on agonist stimulation was quantified and taken as an index of changes in the level of [Ca$^{2+}$]. Panel A represents concentration-dependent responses to the muscarinic receptor agonists. Bar chart (Panel B) represents the $E_{max}$ responses to 1mM concentration of muscarinic receptor agonists. Data are mean ± S.E.M. (n=7). ***, p<0.001, by one-way ANOVA with Bonferroni’s post tests.
Table 4.2.2a. The $E_{\text{max}}$ values of responses to muscarinic receptor agonists in a FLIPR assay represented as a percentage of the maximal methacholine response (relate to Figure 4.2.2a). Data are mean ± S.E.M. (n=7).

<table>
<thead>
<tr>
<th>Muscarinic receptor agonist</th>
<th>$E_{\text{max}}$ values (as a percentage of the methacholine-mediated response)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arecoline</td>
<td>47.3 ± 6.0</td>
</tr>
<tr>
<td>Pilocarpine</td>
<td>2.3 ± 0.6</td>
</tr>
<tr>
<td>Methacholine</td>
<td>100</td>
</tr>
<tr>
<td>Oxotremorine</td>
<td>13.0 ± 4.0</td>
</tr>
</tbody>
</table>

Fig. 4.2.2b. Demonstration of crosstalk in HEK 293 cells. Representative FLIPR traces of cells challenged with 1mM muscarinic receptor agonists, followed by addition of 10µM noradrenaline at t=180s. In the continued presence of muscarinic receptor agonists, noradrenaline caused an elevation in $[Ca^{2+}]_i$. Data are representative of 7 or more experiments.
Fig. 4.2.2c. Responses to 10μM noradrenaline in the continued presence of varying concentrations of muscarinic receptor agonists in HEK 293 cells. Cells were stimulated in a FLIPR with a range of muscarinic receptor agonists (t=10s) and fluorescence recorded for 180s. 10μM noradrenaline was subsequently added fluorescence recorded for a further 180s (relate to Figure 4.2.2b). Change in fluorescence on agonist stimulation were quantified and taken as an index of changes in the level of [Ca\textsuperscript{2+}]\textsubscript{i}. Data are mean ± S.E.M. (n=7).

Table 4.2.2b. The pEC\textsubscript{50} values of muscarinic receptor agonist-mediated Ca\textsuperscript{2+} responses and their ability to reveal Ca\textsuperscript{2+} signalling by 10μM noradrenaline (relate to Figure 4.2.2a [Panel A] and 4.2.2c). Data are mean ± S.E.M. (n=7).

<table>
<thead>
<tr>
<th>Muscarinic receptor agonists</th>
<th>pEC\textsubscript{50} of muscarinic receptor agonist-mediated Ca\textsuperscript{2+} responses</th>
<th>pEC\textsubscript{50} of muscarinic receptor-agonists ability to mediate Ca\textsuperscript{2+} responses to 10μM noradrenaline</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arecoline</td>
<td>4.37± 0.21</td>
<td>5.03± 0.15</td>
</tr>
<tr>
<td>Pilocarpine</td>
<td>-</td>
<td>4.39± 0.16</td>
</tr>
<tr>
<td>Methacholine</td>
<td>6.26± 0.52</td>
<td>-</td>
</tr>
<tr>
<td>Oxotremorine</td>
<td>5.54± 0.23</td>
<td>5.94± 0.13</td>
</tr>
</tbody>
</table>
Fig. 4.2.2d. Representative traces of responses to 1mM muscarinic receptor agonists and 10μM noradrenaline in HEK 293 cells measured by confocal microscopy. Cells were stimulated with oxotremorine or pilocarpine (1mM) and fluorescence recorded for 180s. 10μM noradrenaline was subsequently added and fluorescence recorded for a further 180s. Data are representative of 3 or more experiments.
4.2.3 **Crosstalk is mediated by the β-adrenoceptor with little or no contribution from α-adrenoceptors**

To determine the subtype of adrenoceptor expressed endogenously expressed in HEK 293 cells that mediate crosstalk an array of antagonists were used. A range of concentrations of the β₂-adrenoceptor selective antagonist ICI-118,551 (Hoffmann *et al.*, 2004; Baker, 2005), the non-selective α-adrenoceptor antagonist phentolamine (Cox *et al.*, 2000) and the β₁-adrenoceptor selective atenolol (Hoffmann *et al.*, 2004; Baker, 2005) were used. Cells were incubated with the compounds for 5-10 min, prior to stimulation with 100 μM methacholine and subsequently with a range of noradrenaline concentrations. The responses to methacholine was unaffected by the presence of the antagonists (Figure 4.2.3a).

The ability of noradrenaline to provoke Ca²⁺ signalling in the presence of methacholine was unaffected by phentolamine by concentrations up to 0.1 μM, suggesting that α-adrenoceptors play no role in mediating crosstalk (Figure 4.2.3; Panel A). Atenolol caused a concentration-dependent rightward-shift of the noradrenaline response curve, but this reached statistical significance only at concentrations ≥1 μM (Figure 4.2.3; Table 4.2.3). Using the lowest concentration of atenolol to give a significant curve-shift (1 μM) an apparent pA₂ value of 6.26±0.01 (n=3) was derived using the equation: apparent pA₂ = -log([antagonist]/concentration ratio-1). ICI 118,551 at 0.1 and 0.01 μM inhibited the maximal responses to noradrenaline in the presence of 100 μM methacholine by 52 and 65% respectively, whilst a 1 μM concentration of ICI 118,551 abolished the response (Figure 4.2.3b).
**Fig. 4.2.3a.** 
**Ca^{2+}** responses to 100μM methacholine in the presence of adrenoceptor antagonists. Cells were incubated with a range of concentrations of adrenoceptor antagonists for 5-10 min prior to stimulation with 100μM methacholine. Bar chart represents the increase in fluorescence units when stimulated with 100μM methacholine in the presence of the highest concentration of each antagonist (10μM atenolol, 1μM ICI-118,551 and 0.1μM phentolamine). Data are representative of three or more experiments performed in duplicates.
Fig. 4.2.3b. Responses to noradrenaline in cells prestimulated with 100μM methacholine in the presence of ICI-118,551. Cells were incubated with a range of concentrations (1, 0.1 and 0.01μM) of ICI-118,551 for 5-10min, prior to stimulation with 100μM methacholine. These cells were subsequently stimulated with a range of concentrations of noradrenaline. Fluorescence was recorded for a further 180s using the FLIPR. Graph represents the normalised responses in fluorescence units when stimulated with noradrenaline. Data are mean ± S.E.M. (n=3).

Table 4.2.3. The pEC₅₀ values of noradrenaline responses in cells prestimulated with 100μM concentration of methacholine, in a FLIPR assay (relate to Figure 4.2.3c). Data are mean ± S.E.M. (n=3). **, p<0.01; *, p<0.05 by one-way ANOVA with Bonferroni’s post tests.

<table>
<thead>
<tr>
<th>[Atenolol] μM</th>
<th>pEC₅₀ of noradrenaline’s ability to mediate a Ca²⁺ responses in the continued presence of 100μM methacholine</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>6.67± 0.06</td>
</tr>
<tr>
<td>10</td>
<td>5.79± 0.08 **</td>
</tr>
<tr>
<td>0.1</td>
<td>6.34± 0.03</td>
</tr>
<tr>
<td>0.01</td>
<td>6.54± 0.18</td>
</tr>
</tbody>
</table>
A responses to noradrenaline
in the presence of methacholine ± phentolamine
- Control (no antagonist)
- 0.001μM Phentolamine
- 0.01μM Phentolamine
- 0.1μM Phentolamine

B responses to noradrenaline
in the presence of methacholine ± atenolol
- Control (no antagonist)
- 0.01μM Atenolol
- 0.1μM Atenolol
- 1μM Atenolol
- 10μM Atenolol

Fig. 4.2.3c. Responses to noradrenaline in cells prestimulated with 100μM methacholine in the presence of phentolamine [Panel A; (0.1, 0.01 and 0.001)] or atenolol [Panel B; (10, 1, 0.1 and 0.01μM)]. Cells were incubated with a range of concentrations of adrenoceptor antagonists for 5-10min prior to stimulation with 100μM methacholine. These cells were subsequently stimulated range of concentrations of noradrenaline. Fluorescence was recorded for a further 180s using the FLIPR. Graphs represent the normalised responses in fluorescence units when stimulated with noradrenaline. Data are mean ± S.E.M. (n=3).
**4.2.4 Crosstalk is mediated by selective β2-adrenoceptor agonists**

Noradrenaline is an endogenous non-selective adrenoceptor agonist. We investigated the effect of a range of commercially available β2-adrenoceptor-selective agonists. HEK 293 cells were stimulated initially with a range of concentrations of either methacholine or oxotremorine and subsequently challenged with fenoterol, procaterol-HCl, salbutamol, terbutaline or noradrenaline (all at 10 μM; Figure 4.2.4). Evidence from the literature demonstrate that the ability of these agonists to mediate accumulation of cAMP in recombinant cells, or relax bovine tracheal smooth muscle strips precontracted with potassium chloride, occurs at a concentration of 10 μM (Scott *et al.*, 1999; De Vries *et al.*, 2001; Viard *et al.*, 2001; Viard *et al.*, 2000).

In cells prestimulated with methacholine, sigmoidal concentration-effect curves were obtained contrasting with the bell-shaped concentration-effect curves obtained previously. The $E_{\text{max}}$ responses to β2-adrenoceptor agonists in the continued presence of muscarinic receptor agonists were determined as a percentage of the noradrenaline-response. In cells challenged with either methacholine or oxotremorine, salbutamol evoked a maximum Ca$^{2+}$ responses of 65±3.1% (n=3) and 81±9.6% (n=3) respectively of the noradrenaline-mediated response. A reduced maximal response to terbutaline was also observed in methacholine challenged cells (75.1±14%; n=3) in comparison to oxotremorine pre-challenged cells (85.6±4.4%; n=3). Procaterol-HCl and fenoterol evoked comparable maximal responses in the presence of methacholine and oxotremorine. Table 4.2.4 summarises the maximal responses to the β2-adrenoceptor agonists in the presence of either methacholine or oxotremorine. These differences in maximal responses in cells challenged with β2-adrenoceptor agonists in the continued presence of either methacholine or oxotremorine were not significant.

The pEC$_{50}$ values of the muscarinic receptor agonists to reveal Ca$^{2+}$ signalling by the β2-adrenoceptor agonists are summarised in Table 4.2.4. In summary, prestimulation of cells with the partial agonist oxotremorine led to a more potent and efficacious response to β2-adrenoceptor agonists in comparison to the full agonist methacholine (p value 0.004; one-way ANOVA).
Fig. 4.2.4. Responses to 10μM β₂-adrenoceptor agonists in the continued presence of varying concentrations of muscarinic receptor agonists in HEK 293 cells. Cells were stimulated in the FLIPR with a range of concentrations of methacholine (Panel A) or oxotremorine (Panel B; t=10s) and fluorescence recorded for 180s. 10μM β₂-adrenoceptor agonists were subsequently added and the cells fluorescence recorded for a further 180s. Changes in fluorescence on β₂-adrenoceptor agonists stimulation was quantified and taken as an index of [Ca²⁺]ᵢ. Data are mean ±S.E.M. (n=3)
Table 4.2.4. The pEC\textsubscript{50} and $E_{\text{max}}$ values determined from concentration response curves to 10\mu M $\beta_2$-adrenoceptor agonists in the presence of a range of concentrations of methacholine or oxotremorine, in a FLIPR assay (relate to Figure 4.2.4). $E_{\text{max}}$ values are represented as a percentage of response to 10\mu M noradrenaline in the presence of 1mM methacholine or oxotremorine. Data are mean ± S.E.M. (n=3).

<table>
<thead>
<tr>
<th>Methacholine + $\beta_2$-adrenoceptor agonists</th>
<th>pEC\textsubscript{50} of methacholine's ability to mediate a Ca\textsuperscript{2+} responses to 10\mu M $\beta_2$-adrenoceptor agonists</th>
<th>$E_{\text{max}}$ (as a % of noradrenaline-mediated Ca\textsuperscript{2+} responses)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Salbutamol</td>
<td>4.55± 0.04</td>
<td>65.1± 3.17</td>
</tr>
<tr>
<td>Terbutaline</td>
<td>4.60± 0.16</td>
<td>75.7± 14.2</td>
</tr>
<tr>
<td>Procaterol-HCl</td>
<td>4.65± 0.12</td>
<td>95.5± 3.9</td>
</tr>
<tr>
<td>Fenoterol</td>
<td>4.58± 0.08</td>
<td>100.3± 1.1</td>
</tr>
<tr>
<td>Noradrenaline</td>
<td>4.82± 0.11</td>
<td>100</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Oxotremorine + $\beta_2$-adrenoceptor agonists</th>
<th>pEC\textsubscript{50} of oxotremorine's ability to mediate a Ca\textsuperscript{2+} responses to 10\mu M $\beta_2$-adrenoceptor agonists</th>
<th>$E_{\text{max}}$ (as a % of noradrenaline-mediated Ca\textsuperscript{2+} responses)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Salbutamol</td>
<td>5.40± 0.46</td>
<td>81.3± 9.6</td>
</tr>
<tr>
<td>Terbutaline</td>
<td>5.40± 0.50</td>
<td>85.6± 4.4</td>
</tr>
<tr>
<td>Procaterol-HCl</td>
<td>5.55± 0.36</td>
<td>98.0± 18.4</td>
</tr>
<tr>
<td>Fenoterol</td>
<td>5.61± 0.18</td>
<td>103± 9.6</td>
</tr>
<tr>
<td>Noradrenaline</td>
<td>5.56± 0.09</td>
<td>100</td>
</tr>
</tbody>
</table>
4.2.5 Partial agonists of the muscarinic receptor allow a concentration dependent Ca\(^{2+}\) response to noradrenaline

In previous studies, a fixed concentration (10 µM) of noradrenaline was used to explore crosstalk. To examine if varying concentrations of noradrenaline could evoke a concentration-dependent response in the presence of muscarinic receptor agonists, HEK 293 cells were initially stimulated with maximal concentrations of muscarinic receptor agonists and fluorescence recorded for 180 sec. These cells were subsequently stimulated with a range of concentrations of noradrenaline and fluorescence recorded for a further 180 sec. In the continued presence of a 1 mM concentration of muscarinic receptor agonists, noradrenaline mediated a concentration-dependent Ca\(^{2+}\) response (Figure 4.2.5a). The response evoked by noradrenaline varied depending on the prestimulating muscarinic receptor agonist. For example, partial agonists at the muscarinic receptor mediated the most efficacious response to noradrenaline, in comparison to methacholine. This indicates that the size of the intracellular Ca\(^{2+}\) store accessible to the subsequent noradrenaline stimulation might be a major determinant of the magnitude of the Ca\(^{2+}\) response to noradrenaline. However, the potency of the noradrenaline-mediated Ca\(^{2+}\) response did not vary significantly between cells prestimulated with partial agonists (Table 4.2.5). The potency of the noradrenaline response in the presence of 1mM methacholine could not be derived as a sigmoidal concentration dependent effect was not observed.
response to noradrenaline in the presence of 1mM

- Pilocarpine
- Oxotremorine
- Arecoline
- Methacholine

Fig. 4.2.5a. Responses to noradrenaline in cells prestimulated with 1mM muscarinic receptor agonists in HEK 293 cells. Cells prestimulated with 1mM concentration of muscarinic receptor agonists were subsequently challenged with a range of concentrations of noradrenaline. Change in fluorescence on agonists stimulation was quantified and taken as an index of change in the level of $[Ca^{2+}]_i$. Data are mean ± S.E.M. (n=4).

Table 4.2.5. The pEC$_{50}$ values of noradrenaline responses in cells prestimulated with 1mM concentration of muscarinic receptor agonists, in a FLIPR assay (relate to Figure 4.2.5a). Data are mean ± S.E.M. (n=4).

<table>
<thead>
<tr>
<th>Prestimulating muscarinic receptor agonist</th>
<th>pEC$_{50}$ of noradrenaline-mediated Ca$^{2+}$ responses in the presence of 1mM muscarinic receptor agonists</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arecoline</td>
<td>6.85± 0.27</td>
</tr>
<tr>
<td>Pilocarpine</td>
<td>7.10± 0.06</td>
</tr>
<tr>
<td>Methacholine</td>
<td>-</td>
</tr>
<tr>
<td>Oxotremorine</td>
<td>7.34± 0.05</td>
</tr>
</tbody>
</table>
Noradrenaline causes potentiation of methacholine-induced $Ca^{2+}$ responses

The effects of noradrenaline pre-stimulation on muscarinic receptor agonists mediated $Ca^{2+}$ responses were tested to assess whether the relationship between muscarinic receptor agonists and noradrenaline was a cooperative one in which total $Ca^{2+}$ signalling was potentiated in the presence of the two agonists, regardless of the order of addition. Using the FLIPR, cells were initially stimulated with either 10µM noradrenaline or buffer and subsequently, 180s later, with a range of concentrations of methacholine or oxotremorine. In cells stimulated with methacholine, a $Ca^{2+}$ response that consists of a transient peak and plateau response kinetics was obtained. However, in cells pre-stimulated with noradrenaline, methacholine mediated $Ca^{2+}$ responses consistently resulted in an enhanced plateau phase (Figure 4.2.6a). At EC$_{50}$ concentration of methacholine (1µM), the maximal responses were also enhanced in cells pre-stimulated with noradrenaline in comparison to buffer control (Figure 4.2.6a; Panel B). However, at higher concentration of methacholine (1mM), this difference was not observed (Figure 4.2.6a; Panel A). For the partial agonist oxotremorine, in cells prestimulated with noradrenaline, the maximal responses were enhanced (for both maximal and EC$_{50}$ concentrations) in comparison to buffer pre-treated cells (Figure 4.2.6b; 4.2.6c). The maximal responses to 1µM concentration of muscarinic receptor agonists were significantly increased in the cells challenged with noradrenaline in comparison to cells challenged with buffer (p<0.01 for methacholine response, p<0.05 for oxotremorine response; Figure 4.2.6.c).

The plateau phase of the muscarinic receptor agonists-mediated $Ca^{2+}$ responses was significantly different at higher concentrations of agonists (Figure 4.2.6a-c, 4.2.6e). As an index of this, changes in fluorescence 49 sec after agonist addition was calculated (Figure 4.2.6d). The magnitude of responses at this time-point was approx. 50% of the peak response. Following noradrenaline pre-stimulation, methacholine mediated a more potent elevation of $[Ca^{2+}]_{i}$ in comparison to the pre-addition of buffer control (pEC$_{50}$ value 6.73±0.01, (n=3) vs 6.24±0.05, (n=3); p<0.01). However, when taking into account the enhanced plateau phase of the $Ca^{2+}$ responses, the pEC$_{50}$ value of the methacholine response was significantly different 6.48±0.07, (n=3) in comparison to buffer control (5.72±0.04, n=3; p<0.001). Oxotremorine-mediated a more potent $Ca^{2+}$
response when cells were pre-stimulated by noradrenaline compared to buffer (pEC$_{50}$ value 6.59±0.03, (n=3) vs 6.03±0.06, (n=3); p<0.05). When taking into account the enhanced plateau phase of the Ca$^{2+}$ responses, the pEC$_{50}$ value of the oxotremorine response was not significantly different 6.35±0.01, (n=3) in comparison to buffer control (6.03±0.19, n=3). Changes in potency and $E_{\text{max}}$ values obtained by the two analyses are summarised in Table 4.2.6
Fig. 4.2.6a. Effect of pre-stimulating HEK 293 cells with 10μM noradrenaline or buffer, prior to methacholine stimulation. Representative FLIPR traces of cells challenged with 1mM (Panel A) or 1μM (Panel B) methacholine in the continued presence of 10μM noradrenaline or buffer. Data are representative of 3 experiments. Mean data are represented in Figure 4.2.6c.
Fig. 4.2.6b. Effect of pre-stimulating HEK 293 cells with 10μM noradrenaline or buffer, prior to oxotremorine stimulation. Representative FLIPR traces of cells challenged with 1mM (Panel A) or 1μM (Panel B) oxotremorine in the continued presence of 10μM noradrenaline or buffer. Data are representative of 3 experiments. Mean data are represented in Figure 4.2.6c.
Fig. 4.2.6c. Effect of pre-stimulating HEK 293 cells with 10μM noradrenaline or buffer prior to muscarinic agonist stimulation. Cells challenged with noradrenaline or buffer was subsequently stimulated with muscarinic receptor agonists. Change in fluorescence on agonist stimulation was quantified and taken as an index of changes in the level of [Ca^{2+}]. Data are mean ± S.E.M. (n=3). **, p<0.01; *, p<0.05 by one-way ANOVA with Bonferroni’s post tests.
Fig. 4.2.6d. Responses to muscarinic receptor agonists in cells following a pre-addition of either 10μM noradrenaline or buffer. Cells were stimulated in a FLIPR with a range of muscarinic receptor agonists following challenge with 10μM noradrenaline or buffer. Panel A represents the responses to agonists calculated as a difference between peak and baseline fluorescence units. Panel B is agonist responses represented as a difference between fluorescence units 49s after agonist addition and baseline. Data are mean±S.E.M. (n=3).
Fig. 4.2.6e. Responses to methacholine or oxotremorine in the continued presence of 10μM noradrenaline or buffer. The data represent E_max values of the peak and plateau responses to methacholine (Panel A) or oxotremorine (Panel B) in cells prestimulated with noradrenaline, represented as a percentage of E_max responses in buffer pre-treated cells. Data are mean + S.E.M. (n=3). ***, p<0.001, by one-way ANOVA with Bonferroni's post tests.
Table 4.2.6. The pEC$_{50}$ (and corresponding statistical significance for agonist-induced Ca$^{2+}$ responses) and $E_{\text{max}}$ values (represented as a % of maximal methacholine or oxotremorine responses) determined from concentrations responses to muscarinic receptor agonists prestimulated with either 10$\mu$M noradrenaline or buffer, in a FLIPR assay (relate to Figure 4.2.6d, 4.2.6e). Data are mean ± S.E.M. (n=3). **, $p<0.01$, ***, $p<0.001$, by one-way ANOVA with Bonferroni’s post tests.

<table>
<thead>
<tr>
<th>Response to agonist measured as</th>
<th>Difference between peak fluorescence and baseline (peak response)</th>
<th>Difference between fluorescence 49s after peak response and baseline (plateau response)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$E_{\text{max}}$</td>
<td>pEC$_{50}$</td>
</tr>
<tr>
<td>Buffer+ Methacholine</td>
<td>100</td>
<td>6.24±0.05$^\star\star$</td>
</tr>
<tr>
<td>Noradrenaline+ Methacholine</td>
<td>102±1.3</td>
<td>6.73±0.01$^\star\star$</td>
</tr>
<tr>
<td>Buffer+ Oxotremorine</td>
<td>100</td>
<td>6.03±0.06$^\star$</td>
</tr>
<tr>
<td>Noradrenaline+ Oxotremorine</td>
<td>144±7.0</td>
<td>6.59±0.03$^\star\star$</td>
</tr>
</tbody>
</table>

4.2.7 Noradrenaline causes changes in the oscillatory pattern of Ca$^{2+}$ signalling induced by submaximal concentrations of methacholine and oxotremorine

The data generated by the FLIPR represent the average changes in fluorescence intensity measured from a population of cells. In contrast, single cell imaging offers more flexibility in investigating the variability of the responses and the patterns of Ca$^{2+}$ signalling (e.g. oscillations). This was explored here using Ca$^{2+}$ imaging with a confocal microscope as detailed in section 2.3.2. Cells were initially stimulated with submaximal concentrations of methacholine or oxotremorine (1$\mu$M; bath addition) and imaged for 360s. They were subsequently stimulated with 10$\mu$M noradrenaline or buffer (bath addition) and imaged for a further 180s. When it became clear that noradrenaline was affecting oscillation patterns, cells were imaged for longer (up to 720s) to allow quantification of aspects such as oscillation frequency. For data analysis, 16 cells in
each coverslip were analysed and changes in fluorescence represented as fold increase
ever baseline. A minimum of two coverslips was used per experiment for each setup,
and experiment repeated at least three times. n numbers quoted refer to the total number
of experiments carried out.

For the control experiments, cells were stimulated with muscarinic receptor agonists
followed by buffer or alternatively, buffer stimulated cells were subsequently stimulated
with noradrenaline. In cells stimulated with methacholine and imaged for 360s prior to
the addition of buffer or noradrenaline, any transient increase in [Ca^{2+}], 60s after
muscarinic receptor agonist stimulation (fluorescence F/F_0 > 1.5; t=60-360s) was
counted as an oscillation. Following subsequent stimulation with noradrenaline or
buffer, any transient increase in [Ca^{2+}], 30s after stimulation (fluorescence F/F_0 > 1.5;
t=390-720s) was considered as an oscillation. Challenge with 1 µM methacholine or 1
µM oxotremorine induced oscillations in 82% and 33% of cells respectively.
Noradrenaline stimulation following buffer addition did not induce any elevation of
[Ca^{2+}], (Figure 4.2.7a). However, 10µM noradrenaline mediated Ca^{2+} oscillations in the
continued presence of methacholine and oxotremorine, in comparison to buffer-
challenged cells. Representative traces of responses to methacholine and oxotremorine
in the presence of buffer or noradrenaline is shown in Figure 4.2.7b and 4.2.7c.

One parameter analysed in this experiment was the percentage of cells oscillating to
noradrenaline in the presence of muscarinic receptor agonists. The percentage of cells
oscillating in response to the addition of 10µM noradrenaline in comparison to buffer,
in the continued presence of 1µM oxotremorine was significantly increased (91.0±3.0,
(n=3) Vs 33.3±4.7, (n=3); p<0.001; Figure 4.2.7d). In contrast, the percentage of cells
responding to noradrenaline in 1µM methacholine pre-stimulated cells was not
significantly different in comparison to buffer (92.7±4.9, (n=3) Vs 82.5±4.1, (n=3);
Figure 4.2.7d).

Oscillatory behaviour can be defined as either baseline (a transient increase in [Ca^{2+}],
that rise from a baseline and which can be maintained over relatively long periods
between individual spikes) or sinusoidal (symmetrical oscillations superimposed on a
sustained level of [Ca^{2+}], above the prestimulus baseline) (Thomas et al., 1996). In cells
stimulated with methacholine, 56.2±6.2%, (n=3) oscillated in a baseline manner while 32.2±5.9% (n=3) oscillated in a sinusoidal manner (Figure 4.2.7e). In the presence of 10μM noradrenaline, the number of baseline oscillations decreased to 46.8±10.0% (n=3), whilst the number of sinusoidal oscillations increased to 45.8±8.1% (n=3). The oscillations in cells induced by oxotremorine were mainly of baseline nature with 32.14±6.74%, (n=3) of them oscillating in this manner and <1% of cells having sinusoidal oscillations. The addition of 10μM noradrenaline increased baseline oscillations by 2.1 fold to 66.9±7.0% (n=3; p<0.001) while the sinusoidal oscillations were increased by ~27 fold to 24.1±12.8% (n=3; p<0.001).

Stimulation of cells with noradrenaline following muscarinic receptor activation induced oscillations of greater frequency than addition of buffer. For calculating oscillation frequencies, the number of transient increases in [Ca\textsuperscript{2+}]\textsubscript{i} induced in each 360s imaging period in each cell was estimated and expressed as frequency of oscillation per minute (Fmin\textsuperscript{-1}). From each coverslip, 16 cells were analysed and the total number of oscillations averaged for each 360s recording and frequency calculated. In methacholine pre-stimulated cells, noradrenaline stimulation significantly increased oscillation frequency (1.13±0.10 Fmin\textsuperscript{-1}, (n=3) Vs 0.61±0.10 Fmin\textsuperscript{-1}, (n=3); p<0.01; Figure 4.2.7f). Similarly, in cells pre-treated with oxotremorine, noradrenaline also significantly increased oscillation frequency in comparison to buffer (0.84±0.11 Fmin\textsuperscript{-1}; (n=3) Vs 0.10±0.01 Fmin\textsuperscript{-1}; (n=3); p<0.001; Figure 4.2.7f).
Fig. 4.2.7a. Effect of noradrenaline addition in cells challenged with buffer. Cells were stimulated with 10μM noradrenaline, subsequent to buffer challenge. Data are represented as the fold increase over basal fluorescence and representative of three different experiments with each experiment consisting of at least 2 coverslips, with 16 cells being analysed from each coverslip.
Fig. 4.2.7b. Responses to 10μM noradrenaline or buffer in methacholine pre-stimulated HEK 293 cells. Cells were challenged (t=360s) with buffer (Panel A) or noradrenaline (Panel B), subsequent to methacholine stimulation. Data are represented as fold increase over basal fluorescence and representative of three different experiments with each experiment consisting of at least 2 coverslips, with 16 cells being analysed from each coverslip.
Fig. 4.2.7c. Responses to 10μM noradrenaline or buffer in oxotremorine pre-stimulated HEK 293 cells. Cells were challenged with buffer (Panel A) or noradrenaline (Panel B), subsequent to oxotremorine stimulation. Data are represented as fold increase over basal fluorescence and representative of three different experiments with each experiment consisting of at least 2 coverslips, with 16 cells being analysed from each coverslip.
Fig. 4.2.7d. Percentage of cells oscillating in response to noradrenaline in cells pre-stimulated with muscarinic receptor agonists. Cells were stimulated with methacholine (Panel A) or oxotremorine (Panel B) and in its continued presence subsequently challenged with either buffer or noradrenaline. Data are represented as fold increase in fluorescence over basal. Data are mean ± S.E.M. (n=3). *** p<0.001, by Unpaired Student's t-test.
Fig. 4.2.7e. Percentage of cells oscillating in response to muscarinic receptor agonists and muscarinic receptor agonist in the presence of noradrenaline. Cells were stimulated with either methacholine (Panel A) or oxotremorine (Panel B) and subsequently challenged with noradrenaline. Data are mean ± S.E.M. (n=3). ***, p<0.001, by Unpaired Student’s t-test.
Fig. 4.2.6f. Increase in frequency of oscillations in cells challenged with noradrenaline in the continued presence of muscarinic receptor agonists. Panel A represents the changes in oscillatory frequency in cells stimulated with noradrenaline versus buffer in the continued presence of methacholine. Panel B represents changes in oscillatory frequency in cells challenged with noradrenaline versus buffer in the continued presence of oxotremorine. Data are mean ± S.E.M. (n=3). *** p<0.001, ** p<0.01, by one-way ANOVA with Bonferroni’s post tests.
4.3 Discussion

4.3.1 Summary

This chapter provides evidence of crosstalk between the muscarinic M₃ receptor and the β₂-adrenoceptor, expressed endogenously in HEK 293 cells. It was shown in this study that noradrenaline could only induce Ca²⁺ signalling in cells primed with methacholine. This chapter also provides the first demonstration of the ability of partial agonists of the muscarinic receptor to mediate Ca²⁺ signalling to noradrenaline with similar or greater magnitude than full agonists. Using selective adrenoceptor agonists and antagonists, crosstalk was demonstrated to be mediated by β₂-adrenoceptors. Examination of crosstalk at the single cell level revealed that noradrenaline not only affects the number of cells oscillating but also the frequency and types of Ca²⁺ oscillations in the presence of submaximal concentrations of muscarinic receptor agonists.

4.3.2 Noradrenaline reveals Ca²⁺ signalling

An important observation in this study was that noradrenaline, over a range of concentrations, was unable to elevate [Ca²⁺]ᵢ in HEK 293 cells. Noradrenaline could only elevate [Ca²⁺]ᵢ when cells were pre-stimulated with methacholine. This is similar to the study by Werry et al. (2002), where they demonstrated that stimulation of the endogenous purinergic receptors in HEK 293 cells also led to elevation of [Ca²⁺]ᵢ in response to noradrenaline. In cells prestimulated with varying concentrations of methacholine, stimulation with 10 μM noradrenaline resulted in a ‘bell-shaped’ curve with respect to the concentration of methacholine (Figure 4.2.2b). One possible cause of this is depletion of the intracellular Ca²⁺ stores accessible to noradrenaline by the initial muscarinic receptor stimulation, which is increasingly evident at higher methacholine concentrations. However, over the course of the study, the bell-shaped concentration response-curve obtained turned out to be the exception rather than the rule, as in the majority of subsequent experiments sigmoidal concentration-effect curves (with respect to the concentration of methacholine) were observed. The reasons for this are unclear, but could include differences in passage number of cells, initial store loading and the degree of cell confluency.
The greater ability of the full agonist methacholine to deplete the intracellular Ca\(^{2+}\) store compared to the partial agonists may also cause or contribute to the greater Ca\(^{2+}\) responses to noradrenaline in the presence of partial compared to full muscarinic receptor agonists. However, even when concentrations of full and partial muscarinic receptor agonists were used that cause approximately equivalent Ca\(^{2+}\) responses (e.g. 1 µM methacholine increased fluorescence by 7365±3170 (n=6) units and 100 µM arecoline increased fluorescence by 7421±2952 (n=6) units), the subsequent Ca\(^{2+}\) response to 10 µM noradrenaline in the presence of arecoline was still greater than the response in the presence of methacholine. This suggests that factors other than the extent of store depletion account or contribute to the different abilities of full and partial muscarinic agonists to mediate crosstalk. However, it is possible that monitoring the extent of Ca\(^{2+}\) elevation in response to different muscarinic receptor agonists does not accurately reflect the extent of store depletion. The kinetics and extent of Ca\(^{2+}\) release and subsequent removal from the cytosol could, for example, differ between full and partial agonists. Monitoring the filling state of the intracellular Ca\(^{2+}\) store following activation of the muscarinic receptors by the different agonists would be one way experimentally to address this issue. Similarly it is possible that muscarinic receptor activation of, for example, Ca\(^{2+}\) extrusion mechanisms is greater in the presence of full compared to partial agonists and that this contributes to the apparently greater Ca\(^{2+}\) responses to noradrenaline in the presence of a partial rather than a full muscarinic receptor agonist.

A series of muscarinic receptor agonists were used to examine crosstalk. Arecoline, pilocarpine and oxotremorine have been shown to be partial agonists at the muscarinic receptor (Sharif et al., 1995; Szekeres et al., 1998; Mistry et al., 2005). This study has confirmed partial agonistic properties of these compounds in elevating [Ca\(^{2+}\)]\(_i\) in HEK 293 cells with a rank order of agonist efficacy of pilocarpine<oxotremorine<arecoline<methacholine. This corresponds to the efficacy of these agonists to stimulate production of Ins(1,4,5)P\(_3\) in human neuroblastoma SH-SY5Y cells and human bladder detrusor smooth muscle cells (Szekeres et al., 1998; Harris et al., 1995). The rank order of agonist potency in elevating [Ca\(^{2+}\)]\(_i\) in this study was arecoline<oxotremorine<methacholine. Although this rank order of agonist potency is comparable to the data obtained by Szekeres et al., (1998), it differs from the study by Harris et al., (1995) in human bladder detrusor smooth muscle cells. Bladder detrusor smooth muscle cells
were stimulated with agonists for 40min prior to measurement of accumulation of total inositol phosphates. This is in contrast to the assays measuring elevation of \([\text{Ca}^{2+}]_i\), or accumulation of \(\text{Ins}(1,4,5)P_3\) where responses are measured seconds to minutes after agonist stimulation. The rank order of potency of muscarinic receptor agonists obtained in bladder detrusor smooth muscle cells were pilocarpine<\text{methacholine}<\text{oxotremorine}. This difference could be an indication for different populations of muscarinic receptors mediating responses or due to the differences in the time-period during which the assay was carried out. In SHY-5Y cells, stimulation of the endogenously expressed muscarinic M₃ receptors with carbachol results in a bi-phasic accumulation of \(\text{Ins}(1,4,5)P_3\) consisting of a rapid peak response and a slower plateau response. The plateau phase of the response is an indication that the receptors have partially desensitised (Willars & Nahorski, 1995). The \(\text{pEC}_{50}\) values determined for the peak and plateau phases of the \(\text{Ins}(1,4,5)P_3\) responses varied significantly. Moreover, the \(\text{pEC}_{50}\) of carbachol determined from the accumulation of total inositol phosphates over 30min was comparable to the \(\text{pEC}_{50}\) value determined from plateau phase of the \(\text{Ins}(1,4,5)P_3\) responses (\(t=5\text{min}\)). This indicates that measurement of PLC activity by total inositol phosphates accumulation assays over relatively long time frames correspond to the ability of the agonists to drive PLC activation potentially through a partially desensitised receptor as opposed to \(\text{Ca}^{2+}\) or \(\text{Ins}(1,4,5)P_3\) responses driven through fully sensitised receptors (Willars & Nahorski, 1995).

To assess whether partial muscarinic receptor agonists behave differently in potentiating the noradrenaline-mediated \(\text{Ca}^{2+}\) responses, cells were stimulated with 10\(\mu\)M noradrenaline subsequent to stimulation with either partial or full muscarinic receptor agonists (Figure 4.2.2b). The partial agonist pilocarpine and oxotremorine mediated a \(\text{Ca}^{2+}\) response to noradrenaline that was comparable to the full agonist methacholine. The rank order potency of muscarinic receptor agonists in their ability to reveal noradrenaline-mediated \(\text{Ca}^{2+}\) signalling was pilocarpine<\text{arecoline}<\text{oxotremorine}. An accurate \(\text{pEC}_{50}\) value for the ability of methacholine to mediate a \(\text{Ca}^{2+}\) response to subsequent stimulation with 10\(\mu\)M noradrenaline could not be derived due to bell-shaped concentration effect, suggesting a depletion of the accessible \(\text{Ca}^{2+}\) store. The potency by which the muscarinic receptor agonists (arecoline and oxotremorine) mediated a \(\text{Ca}^{2+}\) response was not significantly different to the potency by which these agonists reveal \(\text{Ca}^{2+}\) signalling to noradrenaline.
This study has successfully demonstrated the ability of the $\mathrm{G}_\alpha_\mathrm{s}$-coupled $\beta_2$-adrenoceptor to mediate elevation of $[\mathrm{Ca}^{2+}]_i$, only in the presence of an activated muscarinic receptor. However, other studies have demonstrated the ability of $\mathrm{G}_\alpha_\mathrm{s}$-coupled receptors to mediate elevation of $[\mathrm{Ca}^{2+}]_i$ in the absence of priming stimulus (Schmidt et al., 2001). A role for cAMP and Rap GTPases was implicated in $\mathrm{Ca}^{2+}$ signalling mediated by $\beta_2$-adrenoceptors. Any contribution from $\mathrm{G}_\alpha_\mathrm{i}$-coupled receptors was ruled out using pertussis toxin. Moreover, transient transfection of PLC-ε enhanced the ability of the $\mathrm{G}_\alpha_\mathrm{s}$-coupled receptors to stimulate PLC. The authors propose on the basis of their observations, a PLC and $\mathrm{Ca}^{2+}$ signalling pathway that are triggered by the activation of $\mathrm{G}_\alpha_\mathrm{s}$ and adenyl cyclase that is dependent on cAMP production. However, this model is not applicable in our system, since the $\beta_2$-adrenoceptor cannot elevate $[\mathrm{Ca}^{2+}]_i$ without an activated muscarinic receptor. However, any role for enhanced PLC activity in mediating $\mathrm{Ca}^{2+}$ responses to noradrenaline will be investigated in Chapter 5.

4.3.3 $\mathrm{Ca}^{2+}$ signalling is mediated via $\beta_2$-adrenoceptors

To confirm that noradrenaline-mediated $\mathrm{Ca}^{2+}$ responses are mediated via the endogenous $\beta_2$-adrenoceptors, a range of commercially available adrenoceptor selective antagonists were used. To rule out any involvement of $\alpha$-adrenoceptors, a non-selective $\alpha$-adrenoceptor antagonist phentolamine was used (Ambler & Taylor, 1986; Cox et al., 2000). A range of concentrations of phentolamine was chosen based on the literature evidence that 1μM phentolamine inhibits $\alpha_1$-adrenoceptor mediated $\mathrm{Ca}^{2+}$ responses. At phentolamine concentrations greater than 1μM, methacholine-mediated $\mathrm{Ca}^{2+}$ responses were inhibited, indicating the non-selective nature of this antagonist at high concentrations. Pre-incubation of HEK 293 cells with 0.1, 0.01 and 0.001μM phentolamine did not affect either the muscarinic agonist-mediated $\mathrm{Ca}^{2+}$ response or the ability of noradrenaline to reveal $\mathrm{Ca}^{2+}$ signalling, indicating $\alpha$-adrenoceptors were not involved in mediating crosstalk.

To differentiate the $\beta$-adrenoceptor subtype mediating crosstalk, atenolol and ICI-118,551 were used. In $^{125}$I-iodocyanopindolol ($^{125}$I-CYP) competition binding assay in CHO-K1 cells recombinantly expressing the $\beta$-adrenoceptor subtypes at comparable receptor expressions, atenolol was up to-63 fold selective for $\beta_1$-adrenoceptor over $\beta_2$-adrenoceptor (Hoffmann et al., 2004). In the same study, ICI 118,551 was demonstrated
to be most selective for $\beta_2$-adrenoceptor in comparison to other $\beta$-adrenoceptor subtypes. Other studies have also demonstrated the highly selective nature of atenolol at the $\beta_1$-adrenoceptor and ICI 118,551 at the $\beta_2$-adrenoceptor (Baker, 2005). Based on these reports, a range of concentrations of these antagonists was chosen. HEK 293 cells pre-incubated with atenolol or ICI 118,551 were stimulated with a single concentration of either methacholine or oxotremorine and subsequently challenged with a range of concentrations of noradrenaline. These antagonists had no effect on muscarinic receptor-mediated $\text{Ca}^{2+}$ responses. Atenolol inhibited the noradrenaline-mediated $\text{Ca}^{2+}$ response with an apparent $\text{pA}_2$ value of 6.26±0.01 (n=3). Literature evidence suggests that the $\text{pA}_2$ value for atenolol at the $\beta_1$-adrenoceptor is ~7.01, while at the $\beta_2$-adrenoceptor is ~5.77 (Tanaka et al., 2005; Tanaka et al., 2004; Hillman et al., 2005; Suh et al., 1999). Furthermore, noradrenaline-mediated $\text{Ca}^{2+}$ responses were concentration-dependently inhibited in an apparently non-competitive manner in cells pre-incubated with varying concentrations of ICI 118,551. Although generally considered a competitive antagonist (Skeberdis et al., 1997; Jurgens et al., 2005), such apparent non-competitive inhibition by ICI 118,551 has also been previously reported in BC3H1 cells which endogenously express $\beta_2$-adrenoceptors (Hopkinson et al., 2001). The authors suggested that in low receptor reserve systems, the affinity of ICI 118,551 is greater a receptor conformation that does not couple to the $G_s$ protein, effectively reducing the proportion of receptors in the pool leading to a decrease in maximal responses (Hopkinson et al., 2001). HEK 293 cells too are a low receptor reserve system. A potentially major drawback of determining the effects of antagonists on agonist-mediated $\text{Ca}^{2+}$ responses is the transient nature of the responses measured. Thus, the non-equilibrium agonist-antagonist binding competition could give rise to false estimates of antagonist activity. Irresepctive of these asides, the whole dataset strongly suggests that HEK 293 cells possess a small endogenous population of $\beta_2$-adrenoceptors which participate in the crosstalk described in this study.

4.3.4 $\beta_2$-adrenoceptor selective partial and full agonist elevate $[\text{Ca}^{2+}]$.

$\beta_2$-adrenoceptor agonists are frequently used as bronchodilators in several respiratory diseases. An array of $\beta_2$-adrenoceptor selective agonists have been developed which are either long-acting (e.g. formoterol, salmeterol) or short-acting (e.g. salbutamol, fenoterol). Some of these agonists like salbutamol and terbutaline can act as partial
agonists at the $\beta_2$-adrenoceptor (Swaminath et al., 2005; Hoffmann et al., 2004; Hopkinson et al., 2000). Levels of receptor expression are one of the crucial factors determining the ability of an agonist to induce a full or partial response. A dramatic difference in the efficacy of salmeterol and adrenaline in the activation adenylyl cyclases in HEK 293 cells was demonstrated in high versus low $\beta_2$-adrenoceptor numbers (January et al., 1998). The efficacy for the stimulation of adenylyl cyclase by salbutamol in cells expressing lower receptor numbers was $\sim$10% of the ability of adrenaline to stimulate adenylyl cyclases in the same cells, consistent with the partial agonistic nature of salbutamol. However, in cells expressing higher receptor numbers adrenaline and salbutamol evoked maximal responses that were identical, indicating that receptor expression levels affect the degree of agonism of a ligand (January et al., 1998). To assess whether $\beta_2$-adrenoceptors agonists demonstrated to be partial in other systems, would behave differently in revealing Ca$^{2+}$ responses, cells were stimulated with either methacholine or oxotremorine. They were subsequently exposed to maximal concentrations of the $\beta_2$-adrenoceptor agonists (10$\mu$M). In methacholine pre-treated cells, fenoterol and procaterol-HCl mediated a Ca$^{2+}$ response comparable to noradrenaline (Figure 4.2.4). Salbutamol and terbutaline, which have been shown to be partial in other systems, mediated 65% and 75% of the noradrenaline-mediated Ca$^{2+}$ response, effectively acting as partial agonists in mediating crosstalk. On the other hand, in cells pre-treated with oxotremorine, all the $\beta_2$-adrenoceptor agonists mediated comparable maximal responses. This may be due to the ability of the partial agonists to affect receptor coupling, thereby increasing the intrinsic efficacy and resulting in an increased Ca$^{2+}$ response to noradrenaline. The EC$_{50}$ concentrations of the $\beta_2$-adrenoceptor agonists to reveal Ca$^{2+}$ signalling varied, depending on the pre-stimulating muscarinic receptor agonists. These results indicate a crucial role for the pre-stimulating muscarinic receptor agonists in mediating the subsequent Ca$^{2+}$ response to the $\beta_2$-adrenoceptor agonists.

4.3.5 Noradrenaline potentiates muscarinic agonist mediated elevation of [Ca$^{2+}$]

This study has demonstrated that pre-incubation of cells with noradrenaline potentiates the subsequent Ca$^{2+}$ responses to full and partial agonists of the muscarinic receptor (Figure 4.2.6a and 4.2.6b). The plateau phase of the Ca$^{2+}$ responses to muscarinic receptor agonists were more enhanced in cells pre-stimulated with noradrenaline in
comparison to buffer pre-stimulated cells. To quantify differences in the enhanced plateau phase, the rate of decline of responses was derived. In cells pre-stimulated with noradrenaline, the rates of decline of responses were significantly lower in comparison to buffer pre-treated cells. In the presence of noradrenaline, the intracellular Ca\textsuperscript{2+} stores may be depleted to a greater extent, driving capacitative calcium entry, which may account for the enhanced plateau phase. Another explanation could be that the enhanced plateau response of muscarinic response is driven by contributions from Ca\textsuperscript{2+} stores distinct from those driven by Ins(1,4,5)P\textsubscript{3} receptors in the endoplasmic reticulum, like the ryanodine sensitive stores. There is conflicting evidence for the presence of functional ryanodine receptors in HEK 293 cells (Querfurth et al., 1998; Tong et al., 1999). This will be addressed in detail in Chapter 6. In addition to the effect on maximal responses to agonists, the potency of the muscarinic receptor agonists on Ca\textsuperscript{2+} responses are also affected. In cells pre-stimulated with noradrenaline, the muscarinic receptor agonists mediated a significantly more potent response. This implicates that noradrenaline influences the ability of the muscarinic receptors to activate Ga\textsubscript{q/11} and stimulate PLC-\(\beta\).

Potentiation of Ca\textsuperscript{2+} responses to oxotremorine in the presence of noradrenaline was more pronounced in comparison to methacholine, with the maximal responses being enhanced by 2 fold or more (Table 4.2.6). This may be due to the ability of noradrenaline to enhance the partial agonist-occupied muscarinic M\textsubscript{3} receptor to couple more efficiently and activate Ga\textsubscript{q/11} in comparison to full agonists, increasing its intrinsic efficacy. These results implicate a role for extracellular Ca\textsuperscript{2+} influx through Ca\textsuperscript{2+} channels, a role for Ca\textsuperscript{2+} stores distinct from those activated by Ins(1,4,5)P\textsubscript{3} receptors and perhaps a role for enhanced PLC activity. These may be indications for the probable mechanism(s) by which crosstalk occurs and will be examined in detail in Chapters 5 and 6.

### 4.3.6 Noradrenaline influences muscarinic agonist mediated oscillations

Ca\textsuperscript{2+} oscillations in cells is one mode by which Ca\textsuperscript{2+} signals over an extended period of time without the toxicity associated with persistently elevated [Ca\textsuperscript{2+}]\textsubscript{i} (Wayman et al., 1995). These oscillations depend on a mechanism of repetitive intracellular signalling induced either at levels of PLC or at the levels of Ins(1,4,5)P\textsubscript{3} on intracellular Ca\textsuperscript{2+}.
stores or a combination of Ca\(^{2+}\) release and Ca\(^{2+}\) influx at the plasma membrane (Bird & Putney, 2005; Rooney & Thomas, 1993). In this study, stimulation of HEK 293 cells with submaximal concentrations of muscarinic receptor agonists induced oscillations that were mainly of baseline or sinusoidal nature. When stimulated by 10\(\mu\)M noradrenaline in the continued presence of muscarinic receptor agonists there was an increase in the number of cells oscillating and frequency.

Ca\(^{2+}\) oscillations induced by agonists are influenced by many factors and are often driven by receptor specific messenger oscillations. Nash et al., (2001) demonstrated that two PLC-linked receptors the muscarinic M\(_3\) receptor and the mGluR5a receptors induced distinct oscillatory patterns when expressed recombinantly in CHO-K1 cells. Stimulation through mGluR5a receptors resulted in oscillatory patterns of Ins(1,4,5)P\(_3\), which were paralleled by changes in \([\text{Ca}^{2+}]_i\). In contrast, the oscillations induced by stimulation of the muscarinic M\(_3\) receptors were independent of Ins(1,4,5)P\(_3\) oscillations, indicating that \([\text{Ca}^{2+}]_i\) alone does not significantly activate Ins(1,4,5)P\(_3\) production. Based on their observations, it was proposed that the muscarinic receptor agonists induced Ca\(^{2+}\) oscillations results from Ca\(^{2+}\) induced Ca\(^{2+}\) release (CICR) after an initial threshold stimulus of Ins(1,4,5)P\(_3\).

In a study by Bird and Putney, (2005) stimulation of endogenous muscarinic receptor in HEK 293 cells with 5\(\mu\)M methacholine induced oscillations that were sustained in the presence of Gd\(^{3+}\) (which prevents flux of Ca\(^{2+}\) ions across the plasma membrane). This indicates that extracellular Ca\(^{2+}\) was not a pre-requisite to initiate methacholine-mediated Ca\(^{2+}\) responses and to maintain Ca\(^{2+}\) oscillations (if the ions were prevented from being depleted from the cells). Similarly, in this study, it could be that extracellular Ca\(^{2+}\) may not be a prerequisite for sustaining crosstalk/oscillations induced by the muscarinic receptor agonists and noradrenaline. This will be addressed in Chapter 5. Stimulation with noradrenaline in muscarinic receptor agonist pre-treated cells also increased oscillatory frequency in comparison to buffer stimulated cells. Changes in oscillatory frequency are influenced by CICR or enhanced Ca\(^{2+}\) flux from the extracellular medium or both. For changes in oscillatory frequency influenced by CICR, Ca\(^{2+}\) released from Ins(1,4,5)P\(_3\) sensitive stores reach a cytosolic concentration which triggers the release of Ca\(^{2+}\) through Ins(1,4,5)P\(_3\) insensitive pools (e.g. ryanodine sensitive Ca\(^{2+}\) stores). The levels of Ins(1,4,5)P\(_3\) remain constant as determined by the
initial agonist stimulation. This controls the rate of $\text{Ca}^{2+}$ transfer from \(\text{Ins}(1,4,5)\text{P}_3\) sensitive or insensitive pools, influencing the oscillation frequency (Rooney & Thomas, 1993). The potential role for ryanodine sensitive $\text{Ca}^{2+}$ stores to drive crosstalk and ultimately $\text{Ca}^{2+}$ oscillations will be addressed in detail in Chapter 6. Increased $\text{Ca}^{2+}$ influx has also been known to effect oscillation frequency and wave velocity through a rise in the basal level of $[\text{Ca}^{2+}]_i$. Despite this important role for extracellular $\text{Ca}^{2+}$ influx, multiple $\text{Ca}^{2+}$ oscillations can occur after the chelation of extracellular $\text{Ca}^{2+}$ (Thomas et al., 1996; Bird & Putney 2005), although the oscillatory spiking terminates in other instances (Samogyi & Stucki, 1991). Any role for $\text{Ca}^{2+}$ flux through the plasma membrane in sustaining crosstalk will be addressed in Chapter 5.

Based on these preliminary findings there are a number of early mechanistic indications for crosstalk. It was therefore considered necessary to assess the relative affects of a variety of intracellular factors and the effect of crosstalk on signalling pathways to determine some of the components that characterise this crosstalk.
Chapter 5: Characterisation of crosstalk between muscarinic M₃ receptors and β₂-adrenoceptors.

5.1 Introduction

In the previous Chapter, it was demonstrated that interactions between receptors that couple preferentially to different signalling pathways could communicate to positively influence Ca²⁺ signalling. Pre-stimulation of muscarinic M₃ receptors with full or partial agonists facilitates Ca²⁺ release in response to a subsequent addition of noradrenaline. Using selective agonists and antagonists, noradrenaline-mediated Ca²⁺ signalling was confirmed to be mediated through activation of β₂-adrenoceptors. This chapter describes the characterisation of crosstalk and attempts to address the potential mechanisms by which crosstalk may occur, using a range of pharmacological inhibitors.

The β₂-adrenoceptor is preferentially coupled to the Ga₅ G-protein. However, it can switch coupling preference to the cAMP inhibitory G-protein, Gaᵢᵢ, in recombinant and endogenous systems (Zamah et al., 2002; Section 1.6.1.1). Gaᵢᵢ-coupled receptors are known to influence a range of cellular effectors including the inhibition of G-protein inwardly rectifying potassium channels, inhibition of adenylyl cyclase activity and mobilisation of Ca²⁺. Furthermore, there are numerous examples of crosstalk between Gaq/₁₁-coupled receptors and Gaᵢᵢ-coupled receptors resulting in potentiation of Ca²⁺ signalling (Werry et al., 2002; Werry et al., 2003; Rosethorne et al., 2004; Connor et al., 1997). For example, in CHO-K1 cells recombinantly expressing the Gaᵢᵢ-coupled chemokine receptor CCR4, prestimulation of endogenous Gaq/₁₁-coupled purinergic receptors with ATP, elevates [Ca²⁺]ᵢ in response to the CCR4 ligand, macrophage-derived chemokine (Rosethorne et al., 2004). This potentiation is independent of elevation of [Ca²⁺]ᵢ alone, as ionomycin and thapsigargin that elevate [Ca²⁺]ᵢ independent of G-protein activation, failed to mimic the potentiation. Similarly, the ability of recombinantly expressed neuropeptide Y₂ receptors and somatostatin sst₂ receptors to mobilise Ca²⁺ were mediated by crosstalk with Gaq/₁₁-coupled muscarinic M₃ receptors, expressed endogenously in SH-SY5Y cells (Connor et al., 1997). Although the exact mechanisms by which these crosstalk occur is unclear these examples highlight the possibility that ‘class switching’ of the β₂-adrenoceptor to Gaᵢ G-proteins may mediate Ca²⁺ responses to noradrenaline in this study.
Regulation of PLC-β is one potential mechanism for potentiating Ca$^{2+}$ signalling. Hydrolysis of PtdIns(4,5)P$_2$ by PLC results in the generation of Ins(1,4,5)P$_3$ and subsequent release of Ca$^{2+}$ from intracellular stores. Increased production of this second messenger could enhance Ca$^{2+}$ signalling. This is supported by various examples of crosstalk in which phosphoinositide hydrolysis is enhanced. In CHO-K1 cells, recombinantly expressing human 5-HT$_{1B}$ receptors, its activation can potentiate G$\alpha_{q/11}$-coupled receptor stimulated inositol phospholipid hydrolysis (Dickenson & Hill, 1998). In another study in COS-7 cells, recombinantly expressed G$\alpha_i$-coupled δ-opioid receptors could not stimulate PLC-β activation unless the endogenously expressed G$\alpha_{q/11}$-coupled receptors were co-activated. The enhanced stimulation of PLC-β obtained on co-stimulation was blocked by PTX treatment. These results implicate a role for G$\alpha_i$-coupled receptors in enhancing G$\alpha_{q/11}$ signalling by regulation of the PLC subunit (Chan et al., 2000). Phosphorylation by PKA or PKC plays an important role in the regulation of PLC-β isoforms (Section 1.4.1.1). Crosstalk involving G$\alpha_i$-coupled receptors suggests a possible role for PKA in mediating crosstalk. Generally, actions of PKA at PLC-β are inhibitory, but there are certain instances where they have been demonstrated to increase the activity of PLC-β. An emerging regulatory role of PKA is in the phosphorylation of Ins(1,4,5)P$_3$ receptors with subsequent alteration in their activity. For example, in mouse and human parotid acinar cells, PKA-mediated phosphorylation of Ins(1,4,5)P$_3$ receptors is responsible for potentiating Ca$^{2+}$ signalling in response to parathyroid hormone (Bruce et al., 2002; Brown et al., 2004). Similarly, the crosstalk between the muscarinic M$_3$ receptor and the β$_2$-adrenoceptor demonstrated in this study could be mediated by regulating PLC activity or through PKA-mediated regulation.

Based on the findings detailed in Chapter 4 and evidence from the literature, this chapter aims to characterise crosstalk by examining a range of factors such as second messenger kinases, using pharmacological inhibitors and by addressing whether regulation of PLC activity and/or β$_2$-adrenoceptor-mediated 'class switching' is responsible for mediating Ca$^{2+}$ responses to noradrenaline.
5.2 Results

5.2.1 Noradrenaline-mediated Ca\(^{2+}\) release requires ongoing muscarinic receptor activation

To assess the requirement for the continued presence of the pre-stimulating agonist, experiments were carried out in the presence of the muscarinic receptor antagonist-atropine. A concentration of the antagonist (10µM) previously demonstrated to be effective in inhibiting muscarinic receptor-mediated responses was used (Willars et al., 1999). Cells were stimulated with muscarinic receptor agonists (t=10s) and fluorescence recorded for 150s. These cells were subsequently treated with 10µM atropine for 5mins, following which they were stimulated with 10µM noradrenaline and fluorescence recorded for a further 150s (Figure 5.2.1a, 5.2.1b).

The pEC\(_{50}\) values for methacholine- and oxotremorine-mediated facilitation of Ca\(^{2+}\) responses to 10 µM noradrenaline were 4.85±0.32, (n=3) and 5.49±0.26, (n=3) respectively. Atropine abolished the ability of these muscarinic receptor agonists to facilitate Ca\(^{2+}\) responses to noradrenaline (Figure 5.2.1c, Panel B). These results indicate that noradrenaline can mediate Ca\(^{2+}\) responses only in the presence of activated muscarinic receptors.
Fig. 5.2.1a. Demonstration of the requirement for activated muscarinic receptors for crosstalk in HEK 293 cells. Representative FLIPR traces of HEK 293 cells challenged with 1mM methacholine (Panel A). Cells represented in Panel A were incubated for 5min with 10μM atropine following challenge with methacholine. These cells were subsequently challenged with 10μM noradrenaline (Panel B). Data are representative of 3 or more experiments. Mean data are represented in Figure 5.2.1b.
Fig. 5.2.1b. Effects of atropine on noradrenaline responses in cells prestimulated with either 1mM methacholine or oxotremorine. Using the FLIPR, cells were initially challenged with oxotremorine or methacholine (1mM) and fluorescence recorded for 150s. 10μM atropine or buffer was added to cells and incubated for 5min. In its continued presence, cells were subsequently stimulated with 10μM noradrenaline and fluorescence recorded for 150s (relate to Figure 5.2.1a). Change in fluorescence on noradrenaline stimulation was quantified and taken as an index of change in the level of 
\([Ca^{2+}]_{i}\). Data are mean ± S.E.M. (n=3). *** p<0.001, by unpaired Student’s t-tests.
Fig. 5.2.1c. Responses to muscarinic receptor agonists and noradrenaline in HEK 293 cells. Cells were stimulated in a FLIPR with a range of muscarinic receptor agonists (t=10s) and fluorescence recorded for 150s (Panel A). 10μM atropine was added and incubated for 5min. Noradrenaline was subsequently added and fluorescence recorded for a further 150s (Panel B). Change in maximal fluorescence on agonist stimulation was quantified and taken as an index of change in the [Ca^{2+}]. Data are mean ± S.E.M. (n=3).
5.2.2 Lack of dependence of noradrenaline-mediated Ca²⁺ signalling on extracellular Ca²⁺

To determine whether a flux of extracellular Ca²⁺ (Ca²⁺ₑ) across the plasma membrane was required for noradrenaline to mediate Ca²⁺ signalling in the continued presence of muscarinic receptor agonists, FLIPR experiments were performed in assay buffer either in the presence of 1.3 mM extracellular Ca²⁺ (+Ca²⁺ₑ) or under nominally Ca²⁺-free conditions (-Ca²⁺ₑ; i.e. buffer prepared without the addition of CaCl₂). Under these conditions, the magnitude of the peak response to the muscarinic receptor full agonist methacholine was unaffected (Figure 5.2.2a; Panel A, 5.2.2b; Panel A). The plateau phase of the response was less sustained in the absence of Ca²⁺ₑ (Figure 5.2.2a; Panel A). For the partial agonist oxotremorine, the Ca²⁺ response to 1mM concentration of the agonist was abolished in the absence of Ca²⁺ₑ (Figure 5.2.2a; Panel B). The peak response to arecoline was not significantly different in the presence or absence of Ca²⁺ₑ, while pilocarpine mediated little or no responses (Figure 5.2.2b; Panel A).

The cells challenged with muscarinic receptor agonists were subsequently challenged with 10μM noradrenaline and the Ca²⁺ responses quantified. In the absence of Ca²⁺ₑ, the addition of noradrenaline in the presence of the partial muscarinic receptor agonists still evoked an elevation of [Ca²⁺]ᵢ that was not significantly different from the response in the presence of Ca²⁺ₑ (Figure 5.2.2b; Panel B). However, noradrenaline-mediated Ca²⁺ responses were of greater magnitude in the absence of Ca²⁺ₑ in cells pre-stimulated with 1mM methacholine (p<0.002). These results indicate that noradrenaline-mediated Ca²⁺ release in the continued presence of muscarinic receptor agonists is independent of influx of Ca²⁺ from the extracellular media.
Fig. 5.2.2a. Effects of removal of \( \text{Ca}^{2+}_e \) on \( \text{Ca}^{2+} \) signalling by 1mM muscarinic receptor agonists. Using the FLIPR, cells were challenged with muscarinic receptor agonists (1mM) in the presence of 1.3mM extracellular \( \text{Ca}^{2+} (+\text{Ca}^{2+}_e) \) or in nominally \( \text{Ca}^{2+} \)-free conditions (\( -\text{Ca}^{2+}_e \)). Panel A represents responses to 1mM methacholine and Panel B represents responses to 1mM oxotremorine. Data are representative of 3 or more experiments. Mean data are represented in Figure 5.2.2b, Panel A.
Fig. 5.2.2b. Effects of $Ca^{2+}_{e}$ on $Ca^{2+}$ responses to 1mM muscarinic receptor agonist and the subsequent addition of noradrenaline. Using the FLIPR, cells were challenged with muscarinic receptor agonists (1mM) in the presence of 1.3mM extracellular $Ca^{2+}$ ($+Ca^{2+}_{e}$) or in nominally $Ca^{2+}$-free conditions ($-Ca^{2+}_{e}$; Figure 5.2.2a). In its continued presence, cells were subsequently stimulated with 10μM noradrenaline (Panel B). Change in maximal fluorescence on agonist stimulation was quantified and taken as an index of change in the $[Ca^{2+}]_{i}$. Data are mean + S.E.M. (n=3). **, p<0.002, by unpaired Student's t-tests.
5.2.3 Depletion of thapsigargin-sensitive Ca\textsuperscript{2+} stores abolishes noradrenaline-mediated Ca\textsuperscript{2+} signalling

To determine the source of Ca\textsuperscript{2+} that is released by noradrenaline in the presence of muscarinic receptor agonists, cells were treated for 5min with the sarco-endoplasmic reticulum Ca\textsuperscript{2+}-ATPase (SERCA) pump inhibitor, thapsigargin (2\mu M) before stimulating with muscarinic receptor agonists and noradrenaline. Responses to muscarinic receptor agonist were significantly inhibited by pre-incubation of cells with thapsigargin in comparison to buffer control (Figure 5.2.3a).

In the continued presence of both thapsigargin and muscarinic receptor agonist, cells were subsequently stimulated with 10\mu M noradrenaline. In these cells, responses to noradrenaline were significantly inhibited in the presence of thapsigargin, due to the prior drainage of the SERCA pump-filled intracellular stores (Figure 5.2.3b). FLIPR traces of noradrenaline-mediated elevation of [Ca\textsuperscript{2+}]\textsubscript{i} in cells pre-treated with thapsigargin or buffer in the presence of 1mM muscarinic receptor agonists are represented in Figure 5.2.3b (Panel A). These results indicate that noradrenaline-mediated Ca\textsuperscript{2+} release is from thapsigargin-sensitive intracellular stores.
Fig. 5.2.3a. Effects of thapsigargin on Ca^{2+} signalling by 1mM muscarinic receptor agonists. Using the FLIPR, cells were initially challenged with 1mM muscarinic receptor agonists in the presence or absence of 2μM thapsigargin. Change in maximal fluorescence on muscarinic receptor agonist stimulation was quantified and taken as an index of change in the [Ca^{2+}]. Data are mean ± S.E.M. (n=3). **, p<0.01; ***, p<0.001, by unpaired Student's t-tests.
**Fig. 5.2.3b. Effects of thapsigargin on Ca²⁺ signalling mediated by noradrenaline in the continuous presence of 1mM muscarinic receptor agonists.** Using the FLIPR, cells were initially challenged with 1mM muscarinic receptor agonists in the presence or absence of 2μM thapsigargin. These cells were subsequently challenged with 10μM noradrenaline (Panel A). Data are representative of three experiments. Averaged data for responses to noradrenaline in the presence or absence of 2μM thapsigargin are represented in Panel B. Data are mean ± S.E.M. (n=3). ***, p<0.001, by unpaired Student’s t-tests.
5.2.4 Quantification of G-protein activation by $[^{35}\text{S}]$-GTP\textsubscript{yS} assay

GTP binding to an activated G-protein is critical in the initiation of signal transduction. Binding of the radiolabelled GTP-analogue $[^{35}\text{S}]$-GTP\textsubscript{yS} to activated G-proteins in membrane preparations allows quantification of G-protein activation by agonists (Sim \textit{et al.}, 1997). To assess whether the noradrenaline-mediated Ca\textsuperscript{2+} responses in the continuous presence of muscarinic receptor agonists were due to the enhanced activation of $\alpha_{q/11}$ or $\alpha_{i}$ G-proteins, HEK 293 membrane preparations were stimulated with methacholine, noradrenaline or both. The assay was carried out as described in Section 2.6.2. The radioactivity associated with agonist stimulation was expressed as the difference between radioactive counts per minute (CPM) bound in membrane preparations either under basal or stimulated conditions and the non-specific binding for each respective condition. To assess maximal agonist-mediated $\alpha_{q/11}$ protein activation, membrane preparations were stimulated with 1mM methacholine for 0-5min (Figure 5.2.4a). There was a significant difference in $[^{35}\text{S}]$-GTP\textsubscript{yS} bound in cell membrane preparations stimulated for 5min (Figure 5.2.4a; p<0.05). In all subsequent assays, membrane preparations were therefore stimulated with agonists for 5min.

Although, the radioactivity bound was less (as the decay of the isotope was not taken into consideration, at the time of the assay) the pattern of differences obtained were as expected (Figure 5.2.4b). In cell membrane preparations stimulated with noradrenaline, there were no significant differences in $[^{35}\text{S}]$-GTP\textsubscript{yS} bound to $\alpha_{q/11}$-protein or $\alpha_{i}$-protein, in comparison to basal (Figure 5.2.4b). Similarly, there were no significant differences in $[^{35}\text{S}]$-GTP\textsubscript{yS} bound to $\alpha_{q/11}$-protein or $\alpha_{i}$-protein in cell membrane preparations stimulated with methacholine or a combination of methacholine and noradrenaline (Figure 5.2.4b). While it is clear that further validation of this assay is required, in particular positive controls for activation of $\alpha_{i}$-coupling (e.g. by the use of membranes prepared from cells expressing a recombinant $\alpha_{i}$-coupled receptor), the data suggest an ability to determine activation of $\alpha_{q/11}$ by an endogenously expressed muscarinic receptor and further suggest that co-stimulation of muscarinic receptors and adrenoceptors does not enhance activation of $\alpha_{q/11}$ above the activation mediated by muscarinic receptors alone.
Fig. 5.2.4a. Effects of 1mM methacholine stimulation on Gaq/11-protein activation measured by [35S]-GTPγS assay. HEK 293 cell membrane preparations were stimulated with 1mM methacholine for 0-5 min. [35S]-GTPγS bound to Gaq/11-protein was quantified as the difference between radioactive counts per minute (CPM) bound in membrane preparations under either basal or stimulated conditions and the non-specific binding for each respective condition. Data are mean ± S.E.M. (n=4). *p<0.05, by unpaired Student’s t-tests.
**Fig. 5.2.4b.** Effects of stimulation of cell membrane preparations with methacholine, noradrenaline or both on G-protein activation measured by \[^{35}S\]GTP\(_\gamma\)S assay. HEK 293 cell membrane preparations were stimulated with either 1mM methacholine or 10\(\mu\)M noradrenaline or both for 5min. \[^{35}S\]GTP\(_\gamma\)S bound to Ga\(_{q/11}\)-protein (Panel A) or Ga\(_i\)-protein (Panel B) were quantified as the difference between radioactive counts per minute (CPM) bound in membrane preparations under either basal or stimulated conditions and the non-specific binding for each respective condition. Data are mean ± S.E.M. (n=4).
5.2.5 Sensitivity of noradrenaline-mediated Ca\textsuperscript{2+} signalling to pertussis and cholera toxins

Pertussis toxin (PTX) ADP-ribosylates and inactivates Ga\textsubscript{i}. To rule out any role for Ga\textsubscript{i} in mediating crosstalk, HEK 293 cells were treated with PTX (100ng/ml) for 18-20h prior to the FLIPR assay. PTX treatment had no effect on $E_{\text{max}}$ responses to muscarinic receptor agonists (Figure 5.2.5a; Panel A). There were no significant differences in the pEC\textsubscript{50} values of responses to methacholine in the presence or absence of PTX (6.30±0.21 Vs 6.27±0.27, (n=6); Figure 5.2.5b, Panel A). $E_{\text{max}}$ responses to subsequent addition of 10μM noradrenaline were also unaffected by PTX (Figure 5.2.5a; Panel B). The potency of responses to noradrenaline in the continued presence of muscarinic receptor agonists were unaffected by PTX (Figure 5.2.5b, Panel B; Table 5.2.5). The results indicate that Ga\textsubscript{i}-coupled receptors do not play a role in mediating Ca\textsuperscript{2+} responses to noradrenaline.

Cholera toxin (CTX), an activator of Ga\textsubscript{s}, on extended exposure abolishes Ga\textsubscript{s}-mediated signalling by down-regulation of Ga\textsubscript{s} (Seidel et al., 1999). To examine the ability of CTX to down-regulate Ga\textsubscript{s} in HEK 293 cells, cells were treated for 0-20h with 2μg/ml CTX. This concentration of CTX was previously shown to down-regulate Ga\textsubscript{s} signalling in these cells (Werry et al., 2002). Western blot analysis demonstrated the downregulation of Ga\textsubscript{s} by CTX in HEK 293 cells (Figure 5.2.5c). For subsequent FLIPR experiments, cells were treated with CTX for 18-20h prior to the assay. $E_{\text{max}}$ responses and the potency of responses to muscarinic receptor agonists were unaffected by incubation with CTX (Figure 5.2.5d; Panel A). In contrast, the $E_{\text{max}}$ responses to 10μM noradrenaline in the presence of 1mM muscarinic receptor agonists in CTX treated cells were significantly reduced in comparison to control (Figure 5.2.5d; Panel B). The pEC\textsubscript{50} value of concentration dependency of muscarinic receptor agonists to cause a facilitation of Ca\textsuperscript{2+} signalling to 10μM noradrenaline were methacholine: 5.48±0.19 (n=6) and oxotremorine: 6.14±0.03 (n=6; Figure 5.2.5e). However, in CTX treated cells a pEC\textsubscript{50} value could not be derived as the responses were abolished. These results confirm that noradrenaline-mediated Ca\textsuperscript{2+} responses are mediated via Ga\textsubscript{s}-coupled receptors.
Fig.5.2.5a. Effects of PTX on $\text{Ca}^{2+}$ signalling to 1mM muscarinic receptor agonists and 10µM noradrenaline. Cells incubated with 100ng/ml of PTX for 20h were stimulated with 1mM muscarinic receptor agonists and fluorescence recorded for 150s (Panel A). In its continuous presence, cells were challenged with 10µM noradrenaline and fluorescence recorded for further 150s (Panel B). Histogram represents the change in fluorescence on muscarinic receptor agonists or noradrenaline challenge in the presence or absence of PTX. Data are mean ± S.E.M. (n=6).
Fig. 5.2.5b. Effects of PTX on Ca$^{2+}$ signalling to muscarinic receptor agonists and noradrenaline. Cells incubated with 100ng/ml of PTX for 20h were stimulated with a range of concentrations of muscarinic receptor agonists and fluorescence recorded for 150s (Panel A; relate to Figure 5.2.5a, Panel A). In the continued presence of the muscarinic receptor agonists, cells were challenged with 10μM noradrenaline and fluorescence recorded for 150s (Panel B; relate to Figure 5.2.5a, Panel B). Change in fluorescence on stimulation with muscarinic receptor agonists and noradrenaline was quantified and taken as an index of change in the level of [Ca$^{2+}$]$_i$. Data are mean ± S.E.M. (n=6).
Table 5.2.5. The pEC₅₀ values determined from concentration-response curves to noradrenaline when cells were prestimulated with a range of concentrations of muscarinic receptor agonists, in the presence or absence of PTX, in a FLIPR assay (relate to Figure 5.2.5b, Panel B). Data are mean ± S.E.M. (n=3).

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<td>-PTX</td>
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<tr>
<td>Methacholine</td>
<td>5.54± 0.67</td>
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<td>Oxotremorine</td>
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Fig.5.2.5c. Down-regulation of Ga₅ by CTX. HEK 293 cells were treated with CTX for 0, 30min, 1h, 2h, 4h and 20h prior to cell lysis and subsequent analysis by Western blot. Panel A represents the Western blot demonstrating the effect of CTX on expression of Ga₅. This blot was stripped and reprobed for γ-tubulin expression to ensure equivalent protein loading (Panel B). Data are representative of three different experiments.
Fig. 5.2.5d. Effects of CTX on Ca\(^{2+}\) signalling to 1mM muscarinic receptor agonists and the subsequent addition of 10\(\mu\)M noradrenaline. Cells incubated with 2\(\mu\)g/ml of CTX for 20h were stimulated with 1mM muscarinic receptor agonists (Panel A). In the continued presence of muscarinic receptor agonist, cells were challenged with 10\(\mu\)M noradrenaline and fluorescence recorded (Panel B). Histogram represents the change in fluorescence on muscarinic receptor agonists or noradrenaline challenge in the presence or absence of CTX. Data are mean ± S.E.M. (n=6). *** p<0.001, by unpaired Student's t-tests.
Fig. 5.2.5e. Effects of CTX on intracellular Ca^{2+} signalling to muscarinic receptor agonists and noradrenaline. Cells incubated with 2μg/ml of CTX for 20h were stimulated with a range of concentrations of muscarinic receptor agonists and fluorescence recorded for 150s (Panel A; relate to Figure 5.2.5d, Panel A). In the continued presence of muscarinic receptor agonists, cells were challenged with 10μM noradrenaline and fluorescence recorded for a further 150s (Panel B; relate to Figure 5.2.5d, Panel B). Change in fluorescence on stimulation with muscarinic receptor agonists and noradrenaline was quantified and taken as an index of change in the [Ca^{2+}]. Data are mean ±S.E.M. (n=6).
5.2.6 Role for enhanced PLC-activity in mediating crosstalk

To address whether noradrenaline-mediated Ca\(^{2+}\) responses result from enhanced PLC activity, total inositol phosphate accumulation assays were carried out as described in Section 2.4.1. All data points obtained are expressed as fold increase in total inositol phosphates ([\(^{3}\)H]-InsP\(_x\)) relative to basal. Cells challenged with noradrenaline or buffer did not elevate [\(^{3}\)H]-InsP\(_x\). A time-dependent accumulation of [\(^{3}\)H]-InsP\(_x\) was obtained in cells stimulated with 100µM methacholine that reached a plateau after 20min (Figure 5.2.6a). For subsequent experiments, cells were stimulated for 12min with either muscarinic receptor agonists alone (full and partial agonists) or in combination with 10µM noradrenaline. A concentration-dependent accumulation of [\(^{3}\)H]-InsP\(_x\) was obtained on stimulation with methacholine (Figure 5.2.5b). However, neither the maximal responses nor the potency of the responses were affected by noradrenaline (Figure 5.2.6b). Arecoline, pilocarpine and oxotremorine were partial agonists in mediating [\(^{3}\)H]-InsP\(_x\) responses in comparison to methacholine (Figure 5.2.6c). However, a significant potentiation of [\(^{3}\)H]-InsP\(_x\) responses was not observed in cells co-stimulated with noradrenaline (Figure 5.2.6c). The experiments were also repeated with stimulation of cells for 20min. Under these conditions, noradrenaline did not potentiate [\(^{3}\)H]-InsP\(_x\) responses to full or partial muscarinic receptor agonists indicating that enhanced PLC activity was not crucial for mediating crosstalk (Figure 5.2.6d).

It should be emphasised that the [\(^{3}\)H]-InsP\(_x\) accumulation assay may not be sensitive enough to detect any transient increase in Ins(1,4,5)P\(_3\) and therefore this assay may not reveal whether a transient increase in Ins(1,4,5)P\(_3\) is responsible for crosstalk. To address this, HEK cells recombinantly expressing muscarinic M\(_3\) receptors (~1.7 pmol/mg protein; HEK-M\(_3\); Tovey et al., 2004) were transiently transfected with the Ins(1,4,5)P\(_3\) biosensor, eGFP-PH\(_{PLC\delta 1}\). This cell-line was used, as the level of expression of endogenous muscarinic M\(_3\) receptors in HEK 293 cells was insufficient to observe muscarinic receptor-mediated translocation of the eGFP-PH\(_{PLC\delta 1}\) construct from the plasma membrane to the cytosol (data not shown). In HEK-M\(_3\) cells, pilocarpine and arecoline behaved as full agonists, giving similar E\(_{\text{max}}\) responses to methacholine (Figure 5.2.6e). All of these agonists were more potent than in HEK 293 cells (compare Figure 5.2.6e with Figure 4.2.2a). At the lowest concentration of agonist used (1 nM), each of the muscarinic agonists enabled a Ca\(^{2+}\) response to a subsequent addition of
noradrenaline in the HEK-M₃ cells. However, as the concentration of muscarinic agonist was increased, the response to noradrenaline was diminished (Figure 5.2.6e). This is consistent with the muscarinic agonist causing a substantial depletion of the Ca²⁺ store in HEK-M₃ cells, such that the subsequent addition of noradrenaline was unable to further release Ca²⁺. PLC activity at the single cell level was assessed as previously described (Nash et al., 2001). In HEK-M₃ cells stimulated with 1 µM methacholine, the eGFP-Pₜ₈ construct translocated from the plasma membrane to the cytosol. This change in cytosolic fluorescence was quantified as a fold increase-over-basal fluorescence in regions of interest. Data quantification indicated a small increase in cytosolic fluorescence on noradrenaline addition, however, buffer challenge induced a similar increase in cytosolic fluorescence (Figure 5.2.6f).
Fig. 5.2.6a. Time-course for accumulation of [3H]-InsP₆ in cells stimulated with methacholine. HEK 293 cells were stimulated with 100µM methacholine for time-periods as indicated. Data are represented as fold increase of [3H]-InsP₆ accumulation in unstimulated cells. Basal values at t=0 were 2152±325 DPM/well. Data are mean ± S.E.M. (n=3).

Fig. 5.2.6b. Concentration-dependent accumulation of [3H]-InsP₆ in cells stimulated with either methacholine or methacholine in combination with noradrenaline. HEK 293 cells were stimulated with agonists for 20min. Data are represented as fold increase of [3H]-InsP₆ accumulation in unstimulated cells. Basal values at t=0 were 2582±655 DPM/well. Data are mean±S.E.M. (n=3).
Fig. 5.2.6c. Accumulation of $[^3]H$-InsP$_x$ in cells stimulated with partial or full muscarinic receptor agonists alone or in combination with noradrenaline for 12min. HEK 293 cells were stimulated with maximal (1mM; Panel A) or submaximal concentrations (0.1mM; Panel B) of muscarinic receptor agonists alone or in combination with 10μM noradrenaline, for 12min. Data are represented as fold increase of $[^3]H$-InsP$_x$ accumulation in unstimulated cells. Basal values at t=0 were 3214±321 DPM/well. Data are mean ± S.E.M. (n=3).
FIG. 5.2.6d. Accumulation of \[^3H\]-InsP\(_x\) in cells stimulated with partial or full muscarinic receptor agonists alone or in combination with noradrenaline for 20 min. HEK 293 cells were stimulated with either 1 mM concentration of muscarinic receptor agonists alone or in combination with 10 \(\mu\)M noradrenaline, for 20 min. Data are represented as fold increase of \[^3H\]-InsP\(_x\) accumulation in unstimulated cells. Basal values at \(t=0\) were 3015±547 DPM/well. Data are mean ± S.E.M. (n=3).
**Fig. 5.2.6e. Responses to muscarinic receptor agonists and to 10 μM noradrenaline in HEK-M3 cells.** Cells were stimulated with a range of concentrations of muscarinic receptor agonists. These cells were subsequently stimulated with 10 μM noradrenaline. Panel A represents Ca²⁺ responses to muscarinic receptor agonists in HEK-M3 cells and Panel B represents responses to 10 μM noradrenaline in the continued presence of muscarinic receptor agonists. Change in fluorescence on agonist stimulation were quantified and taken as an index of changes in the level of [Ca²⁺]ᵢ. Data are mean ± S.E.M. (n=3).
Fig. 5.2.6f. Quantification of agonist-mediated translocation of eGFP-PH $PLC\beta$ from plasma membrane to cytosol in HEK-M$_3$ cells. Representative traces of HEK-M$_3$ cells challenged with 1µM methacholine or buffer and subsequently stimulated with 10µM noradrenaline (Panel A). Change in cytosolic fluorescence in regions of interest induced by the translocation of the eGFP-PH $PLC\beta$ from the plasma membrane to the cytosol were quantified and represented as fold increase in fluorescence over baseline. Data are representative of four different experiments. Panel B represents quantification of responses to noradrenaline or buffer in cells challenged with buffer or methacholine. Data are mean± S.E.M. (n=4).
5.2.7 Role for PKC in mediating crosstalk

The muscarinic M₃ receptor couples to Goₐq¹₁ G-proteins that stimulates PLC-β. Hydrolysis of PtdIns(4,5)P₂ by PLC results in the generation of Ins(1,4,5)P₃ and DAG which in turn activates release of Ca²⁺ from intracellular stores and various PKC isoforms respectively. To address if PKC plays a role in regulating crosstalk, cells were pre-treated for 30min with 100μM myristoylated protein kinase C 20-28 cell-permeable inhibitor, prior to stimulation with muscarinic receptor agonists and noradrenaline (PKC inhibitor; Ward & O'Brian, 1993; Eichholtz et al., 1993). This synthetic substrate corresponds to the pseudosubstrate domain of PKC modified by attachment of a fatty acid to make the peptide cell-permeable. It has previously been demonstrated that the myristoylated peptide derived from PKA was unable to affect phosphorylation of myristoylated alanine-rich C kinase substrate (MARCKS) protein in comparison to the myristoylated protein kinase C peptide inhibitor (Eichholtz et al., 1993).

Pre-incubation with the PKC inhibitor had no effect either on the maximal response or plateau phase of the Ca²⁺ response to methacholine (Figure 5.2.7a). The pEC₅₀ value of the peak response to methacholine was unaffected by pre-treatment with the inhibitor (pEC₅₀ value 6.14±0.03 vs 6.18±0.04, n=3; Figure 5.2.7b, Panel A). The potency of oxotremorine responses could not be derived from the data obtained as the responses obtained were very small. Data was also analysed to include difference between fluorescence units 49s after agonist addition and baseline (plateau response). The potency of methacholine response obtained by this analysis was also unaffected by treatment with the PKC inhibitor (pEC₅₀ value 5.75±0.12 vs 5.64±0.15, n=3; Figure 5.2.7b, Panel B). In the continued presence of muscarinic receptor agonists, cells were subsequently stimulated with noradrenaline. The responses to noradrenaline were unaffected by the PKC inhibitor (Figure 5.2.7c). The pEC₅₀ values of peak responses to the concentration dependency of muscarinic receptor agonists to facilitate a subsequent Ca²⁺ response to 10μM noradrenaline were unaffected by pre-treatment with the inhibitor (Figure 5.2.7d; Table 5.2.7). The data obtained confirm that PKC does not play a role in mediating crosstalk.
Fig. 5.2.7a. Effects of the PKC inhibitor on muscarinic receptor agonists-mediated Ca$^{2+}$ signalling. Representative traces of cells stimulated with 1mM methacholine subsequent to incubation with 100μM PKC inhibitor for 30min. Data are representative of three or more experiments. Mean data are represented in Figure 5.2.5b.
Fig. 5.2.7b. Effects of the PKC inhibitor on muscarinic receptor agonists-mediated Ca\(^{2+}\) signalling. Cells were incubated with 100\(\mu\)M concentration of the PKC inhibitor for 30 min prior to stimulation with either methacholine or oxotremorine. Responses to agonists were represented either as a difference between peak and baseline fluorescence units (peak response; Panel A) or as a difference between fluorescence units 49s after agonist addition and baseline (plateau responses; Panel B). Data are mean ± S.E.M. (n=3).
Fig. 5.2.7c. Effects of the PKC inhibitor on noradrenaline-mediated $\text{Ca}^{2+}$ signalling in the presence of 1mM methacholine (Panel A) or oxotremorine (Panel B). Cells were incubated with 100μM concentration of the PKC inhibitor for 30min prior to stimulation with methacholine or oxotremorine and fluorescence recorded for 150s. These cells were subsequently stimulated with 10μM noradrenaline and fluorescence recorded for further 150s. Data are representative of three or more experiments. Mean data are represented in Figure 5.2.5d.
Fig. 5.2.7d. Effects of the PKC inhibitor on noradrenaline-mediated Ca\(^{2+}\) signalling in the presence of muscarinic receptor agonists. HEK 293 cells were incubated with 100\(\mu\)M concentration of the PKC inhibitor for 30 min prior to stimulation with muscarinic receptor agonists and fluorescence recorded for 150 s. These cells were subsequently challenged with 10\(\mu\)M noradrenaline and fluorescence recorded for a further 150 s (relate to Figure 5.2.7c). Change in maximal fluorescence on noradrenaline stimulation was quantified and taken as an index of change in the \([Ca^{2+}]_i\). Data are mean ± S.E.M. (n=3).

Table 5.2.7. The pEC\(_{50}\) values of muscarinic receptor agonists in their ability to reveal Ca\(^{2+}\) signalling by noradrenaline in a FLIPR assay (relate to Figure 5.2.7d). Data are mean ± S.E.M. (n=3).

<table>
<thead>
<tr>
<th>Prestimulating muscarinic receptor agonist</th>
<th>pEC(_{50}) of muscarinic receptor agonists ability to mediate a Ca(^{2+}) responses to 10(\mu)M noradrenaline</th>
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<tbody>
<tr>
<td></td>
<td>- PKC inhibitor</td>
</tr>
<tr>
<td>Methacholine</td>
<td>4.89± 0.11</td>
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<tr>
<td>Oxotremorine</td>
<td>6.00± 0.05</td>
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5.2.8 A role for PKA in mediating crosstalk?

PKA plays a key role in phosphorylation of $\Gamma_{\alpha}$-coupled receptor-mediated signalling (Section 1.3.2). In addition to its ability to down-regulate GPCR-mediated signalling, PKA has also been implicated in mediating crosstalk by phosphorylation of Ins(1,4,5)P$_3$ receptors, sensitising these receptors and leading to subsequent Ca$^{2+}$ release in response to cAMP elevating agents (Bruce et al., 2002; Brown et al., 2004). The $\beta_2$-adrenoceptor is a $\Gamma_{\alpha}$-coupled receptor. In order to address a role for PKA in mediating crosstalk between the muscarinic M$_3$ receptors and $\beta_2$-adrenoceptors, a range of commercially available PKA inhibitors including H-89, 14-22 myristoylated amide PKA inhibitor (14-22 peptide inhibitor), KT5720 and 4-cyano-3-methylisoquinoline (CMIQ) were used (Tovey et al., 2003; Kawakami et al., 2004; Chin et al., 2005).

Cells were incubated with H-89 (10µM), peptide inhibitor (25µM), KT5720 (10µM) and CMIQ (10µM) for 30min prior to stimulation with muscarinic receptor agonists and these cells were subsequently stimulated with noradrenaline. Pre-incubation with these inhibitors did not affect maximal responses to methacholine in comparison to buffer control (Figure 5.2.8a; Panel A). Maximal responses to 10µM noradrenaline in the presence of 1mM methacholine was also unaffected by pre-incubation with these inhibitors in comparison to buffer control (Figure 5.2.8a; Panel B). Having demonstrated a lack of effect of PKA inhibitors on maximal responses to muscarinic receptor agonists and subsequent responses to noradrenaline, the effect of a PKA inhibitor (KT5720) on the potency of muscarinic receptor agonists and noradrenaline-mediated responses were examined. Pre-incubation with KT5720 did not affect either the maximal responses or the pEC$_{50}$ values of methacholine to facilitate a subsequent Ca$^{2+}$ response to 10µM noradrenaline (6.26±0.07 vs 6.18±0.08, n=3; Figure 5.2.8b, Panel A). Similarly the pEC$_{50}$ values of oxotremorine to facilitate a subsequent Ca$^{2+}$ response to 10µM noradrenaline were also unaffected by KT5720 (5.93±0.11 vs 5.82±0.10, n=3; Figure 5.2.8b, Panel A). The lack of effect of these inhibitors on noradrenaline-mediated Ca$^{2+}$ responses suggests a mechanism for crosstalk independent of PKA.
Fig. 5.2.8a. Effects of PKA inhibitors on 1mM methacholine and 10μM noradrenaline-mediated Ca^{2+} responses. HEK 293 cells were pre-incubated with PKA inhibitors for 30min prior to stimulation with methacholine (Panel A) and subsequently challenged with 10μM noradrenaline (Panel B). Data are represented as a percentage of response in buffer control (tests for statistical significance was carried out on raw data). Data are mean ± S.E.M. (n=3).
Fig. 5.2.8b. Effects of KT5720 on methacholine and noradrenaline-mediated Ca\(^{2+}\) responses. HEK 293 cells were pre-incubated with 10μM KT5720 for 30 min prior to stimulation with muscarinic receptor agonists and subsequently challenged with 10μM noradrenaline. Panel A represents responses to methacholine or oxotremorine in cells in the presence or absence of KT5720. Panel B represents responses to noradrenaline in cells pre-stimulated with methacholine and oxotremorine in the presence or absence of KT5720. Change in fluorescence on agonist stimulation was quantified and taken as an index of change in the [Ca\(^{2+}\)]. Data are mean ± S.E.M. (n=3).
5.3 Discussion

5.3.1 Summary of data

The aim of this chapter was to characterise specific aspects of crosstalk and use a range of pharmacological agents to identify some of the critical components that may underlie the mechanism by which muscarinic receptor agonists reveal Ca\(^{2+}\) signalling in response to noradrenaline. The data demonstrate that noradrenaline-mediated Ca\(^{2+}\) responses are revealed only in the presence of an activated muscarinic receptor and are independent of the presence of extracellular Ca\(^{2+}\), but dependent on a thapsigargin-sensitive intracellular store. This crosstalk was shown to be independent of G\(\alpha_l\) but dependent on G\(\alpha_s\). Furthermore there was no evidence for the enhanced activation of PLC or involvement of either PKA or PKC in the regulation of crosstalk.

5.3.2 Potential mechanisms of crosstalk

5.3.2.1 Role for extracellular and intracellular Ca\(^{2+}\)

An important observation in this study was that noradrenaline was able to elevate [Ca\(^{2+}\)]\(_i\) in HEK 293 cells, in the presence or absence of Ca\(^{2+}\)\(_e\). There are a multitude of mechanisms by which GPCRs could interact to enhance [Ca\(^{2+}\)]\(_i\), however, some of these are highly unlikely, given the data contained in this chapter. For instance, any interaction that positively influences the flow of Ca\(^{2+}\)\(_e\) through plasma membrane Ca\(^{2+}\)\(_e\) channels is irrelevant, given that this phenomenon is independent of Ca\(^{2+}\)\(_e\).

Although methacholine-mediated peak Ca\(^{2+}\) responses were independent of Ca\(^{2+}\)\(_e\) the responses to oxotremorine were abolished. This indicates that either oxotremorine accesses intracellular stores close to the plasma membrane that are depleted in Ca\(^{2+}\) free media or that Ca\(^{2+}\) flux from the extracellular media plays a crucial role in mediating responses to partial agonists (Wheldon et al., 2001). Methacholine, on the other hand, could access additional intracellular stores as well as the ones close to the plasma membrane, hence the impact of a little loss from these stores are less obvious. However, it is important to consider that an accurate interpretation of the data is difficult when agonist-mediated Ca\(^{2+}\) responses are small, such as the oxotremorine-mediated Ca\(^{2+}\) responses. An interesting observation in HEK 293 cells was that in the presence of Ca\(^{2+}\)\(_e\), noradrenaline-mediated Ca\(^{2+}\) responses in the presence of 1mM methacholine
was significantly smaller in comparison to the absence of $\text{Ca}^{2+}$. One possible explanation is that in the absence of $\text{Ca}^{2+}$, the $\text{Ca}^{2+}$-ATPase pump does not eliminate cytosolic $\text{Ca}^{2+}$ to the same extent as when there is $\text{Ca}^{2+}$ efflux through the plasma membrane. This argument is further strengthened by the observations that the partial agonists responses to noradrenaline is unaffected by the presence or absence of $\text{Ca}^{2+}$. This may be due to the differences in the increase in concentration of cytosolic $\text{Ca}^{2+}$ in response to the initial muscarinic receptor stimulation and the extent to which the $\text{Ca}^{2+}$-ATPase pump is activated by various partial agonists. There are ample demonstrations of crosstalk between differentially coupled GPCRs that works independent of $\text{Ca}^{2+}$ (Werry et al., 2002; Tovey et al., 2003; Dyer et al., 2005). In rat parotid cells, isoproterenol-mediated potentiation of $\alpha$-adrenergic and muscarinic receptor-mediated $\text{Ca}^{2+}$ responses were similarly shown to be independent of $\text{Ca}^{2+}$ (Tanimura et al., 1999).

Similarly, the mobilisation of intracellular $\text{Ca}^{2+}$ in SHY-5Y cells by neuropeptide Y2 and somatostatin sst2 receptors in the continued presence of carbachol, is independent of $\text{Ca}^{2+}$ (Connor et al., 1997). The ability of noradrenaline to mediate $\text{Ca}^{2+}$ responses with no contribution from $\text{Ca}^{2+}$ in HEK 293 cells agrees with the literature.

The lack of dependence on $\text{Ca}^{2+}$ of the noradrenaline-mediated $\text{Ca}^{2+}$ responses in HEK 293 cells indicates that these responses are mediated through mobilisation from intracellular $\text{Ca}^{2+}$ stores. This store is likely to be the endoplasmic reticulum, although the $\text{Ca}^{2+}$ could be derived from elsewhere and released from the endoplasmic reticulum following a GPCR-mediated transfer from an alternate source (Short & Taylor, 2000). In HEK 293 cells treated with thapsigargin, responses to noradrenaline were also significantly inhibited, due to the prior drainage of the SERCA pump-filled intracellular stores.

In addition to Ins(1,4,5)P3, nicotinic acid adenine dinucleotide phosphate (NAADP) and cyclic ADP-ribose (cADPr) are novel $\text{Ca}^{2+}$ mobilising agents, initially described in sea urchin (Lee, 2001). Although these nucleotides have different structures and precursors, the same class of enzymes synthesise them. However, the $\text{Ca}^{2+}$ stores that they mobilise differ, with NAADP mobilising $\text{Ca}^{2+}$ from secretory lysosomes that are thapsigargin-insensitive. This is involved in plasma membrane repair following mechanical wounding (Galione & Petersen, 2005). It is unlikely that the $\text{Ca}^{2+}$ releasing agent
NAADP is responsible for noradrenaline-mediated elevation of \([\text{Ca}^{2+}]_i\), given that these responses are thapsigargin-sensitive.

cADPr mobilises Ca\(^{2+}\) from the endoplasmic reticulum stores through ryanodine receptors (Lee, 2001; Empson & Galione, 1997). However, there is conflicting evidence for the presence of ryanodine receptors in HEK 293 cells (Querfurth et al., 1998; Tong et al., 1999). Using quantitative PCR, SDS-PAGE analysis and \(^3\text{H}\)-ryanodine binding studies, Querfurth et al., (1998) demonstrated the existence of type 1 and type 2 ryanodine receptors in untransfected HEK 293 cells. However, Tong et al., (1999) argued that in HEK 293 cells, caffeine mediated mobilisation of \([\text{Ca}^{2+}]_i\), occurred only in cells transfected with cDNA coding for ryanodine receptors. The presence of functional ryanodine receptors and any role for cADPr in mobilising Ca\(^{2+}\) in HEK 293 cells will be addressed in Chapter 6.

### 5.3.2.2 Role for activated muscarinic receptor

In this study, noradrenaline mediates a Ca\(^{2+}\) response only in the continued presence of muscarinic receptor agonists. It has been suggested that Ca\(^{2+}\) release in response to G\(_{\alpha q/11}\)-coupled receptors may activate the proteolytic enzyme calpain. In human platelets, this enzyme has been shown to induce proteolytic cleavage of PLC-\(\beta 3\) following agonist-induced activation (Banno et al., 1995). The truncated fragment of PLC-\(\beta 3\) is hypersensitive to stimulation by G\(_{b\gamma}\) subunits (Banno et al., 1994). However, such change would be expected to persist for some time after removal of the primary agonist and would be reflected in \(^3\text{H}\)-InsP\(_x\) or Ins(1,4,5)P\(_3\) responses. Given the requirement for continued muscarinic receptor activation and lack of potentiation of \(^3\text{H}\)-InsP\(_x\) responses, it seems unlikely that these receptors cause irreversible intracellular changes such as protein cleavage, thereby mediating crosstalk.

Other aspects of receptor function that have the potential to mediate crosstalk include the ability of noradrenaline to influence receptor desensitisation or to enhance resensitisation of the muscarinic receptor enabling a second Ca\(^{2+}\) release from the intracellular stores. Following agonist stimulation, the receptor is desensitised rapidly by phosphorylation by GRKs or alternate kinases (Ferguson, 2001). Controlling desensitisation or resensitisation could enhance the ability of G\(_{\alpha s}\) or G\(_{\alpha i}\)-coupled
receptors to mediate signalling via the G_{i,0} subunits or allow the receptor to couple more efficiently to G_{q/11}. A range of factors, including Ca^{2+} sensor proteins and calmodulin, often regulates GRKs providing a route for attenuation of receptor desensitisation during elevation of [Ca^{2+}]. For example, dopamine D_{2} receptor desensitisation and internalisation are regulated by interactions of the neuronal calcium sensor (NCS-1) with GRK2 (Kabbani et al., 2002). In HEK 293 cells stably expressing the dopamine D_{2} receptors, transfection of NCS-1 does not effect agonist-independent receptor desensitisation. However, on agonist stimulation, desensitisation and internalisation of the receptor are inhibited by 50%. Furthermore, NCS-1 was shown to form a complex with GRK2 expressed endogenously in HEK 293 cells. The ability to coimmunoprecipitate NCS-1 with GRK2 was enhanced by forskolin and inhibited by the PKA inhibitor, H-89. Such interactions have implications for receptor crosstalk (Kabbani et al., 2002). In HEK 293 cells, if noradrenaline caused a reduction in desensitisation of the muscarinic receptors, we would expect the plateau phase of the Ca^{2+} response to muscarinic receptor agonists to be more enhanced (in comparison to a peak and plateau response kinetics observed on noradrenaline stimulation). Therefore, it is unlikely that noradrenaline causes a reduction in desensitisation of the muscarinic receptors given the nature of noradrenaline-mediated Ca^{2+} responses.

An alternate explanation for the requirement of continuous muscarinic receptor activation for noradrenaline-mediated Ca^{2+} release is enhanced receptor resensitisation. Such an increase in resensitisation may account for the ability of angiotensin converting enzyme (ACE) inhibitors to evoke Ca^{2+} signalling in the presence of activated bradykinin B_{2} receptors. Thus in CHO-K1 cells recombinantly expressing the ACE and or bradykinin B_{2} receptor, the ACE inhibitor enalaprilat evoked a Ca^{2+} signal in the presence of bradykinin only in cells expressing both ACE and the bradykinin B_{2} receptor (Minshall et al., 1997). Moreover, the Ca^{2+} signal evoked by enalaprilat was blocked by HOE 140, a bradykinin receptor antagonist (Erdos et al., 1999). These data would suggest that Ca^{2+} signalling by enalaprilat is a consequence of dephosphorylation and resensitisation of Bradykinin B_{2} receptors, allowing an additional round of Ca^{2+} signalling to be mediated by bradykinin (Erdos et al., 1999). However another study in porcine arteries has suggested that crosstalk is a metabolic process that occurs as result of the co-localisation of ACE-bradykinin B_{2} receptors on the endothelial cell membrane allowing enhanced concentrations of bradykinin in the immediate vicinity of the
receptor (Tom et al., 2002). Activation of $\mathrm{G}_{\alpha_5}$-coupled $\beta_2$-adrenoceptors by noradrenaline could accelerate the rate of resensitisation of the muscarinic receptor, increasing the number of functional receptors that in turn could allow another round of $\mathrm{Ca}^{2+}$ signalling. This could be analysed further using phosphatase inhibitors such as okadaic acid or cyclosporine A, prior to stimulation with noradrenaline.

5.3.2.3 Role for $\mathrm{G}_{\alpha_5}$, $\mathrm{G}_{\alpha_5}$ and enhanced PLC activity

The requirement for persistent muscarinic receptor activation for crosstalk highlights a possible synergy between components activated by the $\mathrm{G}_{\alpha_{q/11}}$-coupled muscarinic receptor and the $\mathrm{G}_{\alpha_5}$-coupled $\beta_2$-adrenoceptor. The use of CTX and PTX has helped to narrow down the potential mechanisms mediating this crosstalk. The use of PTX, which ADP-ribosylates $\mathrm{G}_{\alpha_i}$ and prevents receptor-activated $\mathrm{G}_{\alpha_i}$-dependent signalling, indicated that $\mathrm{G}_{\alpha_i}$ is not involved whether it is via class switching of the $\beta_2$-adrenoceptor or through the ability of other endogenous $\mathrm{G}_{\alpha_i}$-coupled GPCRs to mediate mobilisation of $\mathrm{Ca}^{2+}$ through $\mathrm{G}_{\beta\gamma}$ subunits. However, the ability of PTX to inhibit $\mathrm{G}_{\alpha_i}$-mediated signalling in HEK 293 cells was not confirmed in this study. Treatment of HEK 293 cells with PTX using a protocol essentially identical to that used in the present study has been shown previously to abolish $\mathrm{G}_{\alpha_i}$-mediated signalling (Werry et al., 2002; Friedman et al., 2002; Rumenapp et al., 2001). Ideally the efficacy of PTX in the present study could have been demonstrated by examining its ability to block agonist-mediated inhibition of forskolin-stimulated cAMP accumulation in HEK 293 cells recombinantly expressing $\mathrm{G}_{\alpha_i}$-coupled receptors.

Immunoblotting of HEK 293 cell lysates with $\mathrm{G}_{\alpha_5}$ antibodies demonstrated that prolonged exposure of cells to CTX substantially down-regulates $\mathrm{G}_{\alpha_5}$. Given the abolition of crosstalk by CTX, these data strongly suggest that crosstalk involves activation of $\mathrm{G}_{\alpha_5}$. Studies examining the functional inhibition of $\mathrm{G}_{\alpha_5}$ by CTX treatment (e.g. determination of agonist-mediated cAMP production) would further support the involvement of $\mathrm{G}_{\alpha_5}$-cAMP signalling. Another potential mechanism by which crosstalk could be mediated is through enhanced PLC activity. For example, in HEK 293 cells, the crosstalk between recombinantly expressed $\mathrm{G}_{\alpha_i}$-coupled CXCR2 and endogenous $\mathrm{G}_{\alpha_{q/11}}$-coupled purinergic receptors results not only in enhanced $\mathrm{Ca}^{2+}$ mobilisation but also enhanced PLC activity. This phosphoinositide accumulation was significantly
greater than the additive effect of activation of the purinergic receptors and CXCR2 separately (Werry et al., 2002). In addition, other studies have demonstrated that crosstalk between differentially coupled receptors involves enhanced phosphoinositide hydrolysis (Selbie et al., 1995; Yang et al., 2001; Werry et al., 2003). The mechanism for crosstalk resulting in enhanced PLC activity has been described mainly for Ga\textsubscript{q/11} and Ga\textsubscript{q}-coupled receptors. However, for crosstalk occurring between Ga\textsubscript{s} and Ga\textsubscript{q/11}-coupled receptors, other mechanisms have been implicated. For example, in rat parotid cells, isoproterenol potentiated Ga\textsubscript{q/11}-coupled α-adrenoceptor and muscarinic receptor-mediated Ca\textsuperscript{2+} responses. The authors suggested that potentiation of Ca\textsuperscript{2+} responses was mediated via the sensitisation of Ins(1,4,5)P\textsubscript{3} receptors by cAMP but not via enhanced PLC activity (Tanimura et al., 1999). In another example of crosstalk between Ga\textsubscript{s} and Ga\textsubscript{q/11}-coupled receptors in rat cerebellar astrocytes, the βγ subunit released on activation of the adenosine A\textsubscript{2B} receptors were implicated in potentiating Ca\textsuperscript{2+} responses to purinergic receptors (Jimenez et al., 1999). In this study, a role for enhanced PLC activity in HEK 293 cells has been similarly ruled out. Furthermore, single cell studies using eGFP-PH\textsubscript{PLC\textsubscript{B1}} construct has indicated that transient increases in Ins(1,4,5)P\textsubscript{3} are not responsible for mediating crosstalk. Taken together these data suggest a mechanism of crosstalk independent of enhanced PLC activity.

5.3.2.4 Role for PKC and PKA

One aspect of receptor desensitisation involves the phosphorylation of the receptor by the second messenger kinases, PKA and PKC. Inhibition of these kinases may result in the ability of the receptors to mediate signalling without attenuation. In HEK 293 cells expressing the Ga\textsubscript{s}-coupled dopamine D\textsubscript{2L} receptors, activation of PKC robustly enhanced dopamine-receptor mediated adenylyl cyclase sensitisation resulting in potentiation of the cAMP responses (Beazely & Watts, 2005). Similarly in rat pancreatic islets, PKC release mediated by stimulation of muscarinic receptors or cholecystokinin was demonstrated to play a key role in regulation of adenylyl cyclase isoforms (Tian & Laychock, 2001). In a study in CHO-K1 cells recombinantly expressing the muscarinic M\textsubscript{3} receptor and β2-adrenoceptors, the authors demonstrated that elevation of intracellular Ca\textsuperscript{2+} and decrease in PtdIns(4,5)P\textsubscript{2} mediated by the activation of muscarinic receptors did not effect the ability of endogenous GRKs to mediate β2-adrenoceptor phosphorylation (Budd et al., 1999). However, a role for PKC
in mediating heterologous phosphorylation of β2-adrenoceptors on stimulation of muscarinic M3 receptors was demonstrated (Budd et al., 1999). These examples highlight the diverse role PKC plays in mediating crosstalk. Although functional inhibition of PKC with the peptide inhibitor was not directly demonstrated in this study (e.g. by examining the inhibition of PKC-mediated phosphorylation of MARCKS protein), this concentration of inhibitor has been shown previously to be effective in various cell-types including arterial and vascular smooth muscle cells (Barman & Marrero, 2005; Hayabuchi et al., 2006). Thus, the data presented in the present study suggest that PKC is not involved in the observed crosstalk between muscarinic receptors and adrenoceptors.

A recent emerging regulatory role of PKA is via phosphorylation of Ins(1,4,5)P3 receptors. In mouse parotid acinar cells, PKA mediated phosphorylation of Ins(1,4,5)P3 receptors is the predominant mechanism for potentiation of Ca2+ signalling in response to carbachol by forskolin or other cAMP elevating agents (Bruce et al., 2002). Recently, an extension of the study in human parotid acinar cells similarly demonstrated a role for PKA mediated phosphorylation of Ins(1,4,5)P3 in potentiation of ATP-induced Ca2+ responses via purinergic receptors (Brown et al., 2004). In addition, PKA has also been implicated in the ability to potentiate Ca2+ signalling through simultaneous activation of Gαq/11-coupled receptors and Gαs-coupled-receptors in parotid cells, hepatocytes and pancreatic β-cells (Tanimura et al., 1999; Burgess et al., 1991; Liu et al., 1996). A few studies have also implicated a PKA-independent regulation of crosstalk. For example, in rat osteoblasts parathyroid hormone potentiates purinergic receptor-mediated mobilisation of intracellular Ca2+ was mediated via PKA independent pathway (Buckley et al., 2001). Similarly, other studies have implicated a PKA-independent pathway mediating crosstalk in different cell types (Short & Taylor, 2000; Tovey et al., 2003; Kang et al., 2001; Holz et al., 1999). In this study, the role for PKA in mediating crosstalk between muscarinic receptors and β2-adrenoceptors was investigated using pharmacological inhibitors. This second messenger kinase would be expected to mediate its action by negatively regulating muscarinic receptor-mediated Ca2+ release, with the β2-adrenoceptors negating this regulation. Alternatively, PKA could positively influence crosstalk, resulting in the inhibition of noradrenaline-mediated Ca2+ release with the use of inhibitors. However in this study, inhibition of PKA had no effect on the
magnitude of noradrenaline-mediated Ca\textsuperscript{2+} release. Although PKA inhibition by the inhibitors was not directly assessed in this study (e.g. by assessing their ability to inhibit a PKA-dependent event such as phosphorylation of cAMP response element binding protein (CREB)), the inhibitors used here have been shown previously to be effective in these and other cell types (Tovey et al., 2003; Kawakami et al., 2004; Chin et al., 2005) and the data therefore suggest that crosstalk does not involve PKA by, for example, sensitisation of Ins(1,4,5)P\textsubscript{3} receptors. The data obtained suggest a Ga\textsubscript{s}-dependent and PKA-independent mechanism mediates crosstalk. This aspect of crosstalk will be examined in more detail in Chapter 6.

Based on these findings there are a number of mechanistic indications for crosstalk. It was therefore considered necessary to assess the relative effects of a variety of intracellular factors to determine some of the components that characterise this crosstalk and will be described in detail in Chapter 6.
Chapter 6: Potential mechanism(s) of crosstalk between the muscarinic M₃ receptor and the β₂-adrenoceptor

6.1 Introduction

In the previous chapter, it was demonstrated that Ca²⁺ signalling in response to noradrenaline was dependent on Gαs and thapsigargin-sensitive intracellular Ca²⁺ stores. The data obtained suggested that crosstalk was independent of either enhanced PLC activity, Gαt or extracellular Ca²⁺. Using pharmacological inhibitors, a role for PKA and PKC in crosstalk was ruled out. Based on these observations, this chapter aims to examine some of the possible mechanisms by which Ca²⁺ signalling occurs in response to noradrenaline, in the continued presence of muscarinic receptors activation.

The stimulation of Gα₅-coupled β₂-adrenoceptors activates adenylyl cyclases, which results in the hydrolysis of ATP to cAMP (Kobilka et al., 1987). Crosstalk between muscarinic M₃ receptors and β₂-adrenoceptors resulting in enhanced mobilisation of intracellular Ca²⁺ may be due to regulation of additional signal transduction pathways, like cAMP. Ca²⁺ can regulate components of the cAMP signalling machinery by either activating or inhibiting different adenylyl cyclases and phosphodiesterases (Section 1.6.1.1). For example, a recent study in MIN6 pancreatic β-cells utilised simultaneous measurements of Ca²⁺ and cAMP using fura-2 and a biosynthetic FRET-based cAMP sensor, Epac1-camps to demonstrate that these signalling pathways interact as a coordinated network, capable of producing diverse temporal signalling patterns in these cells (Landa et al., 2005). Conversely, cAMP and PKA can modulate intracellular Ca²⁺ channels or the plasma membrane Ca²⁺-ATPase, thereby regulating Ca²⁺ signalling.

The enhancing effects of cAMP on Ca²⁺ signalling have been demonstrated in several tissues including rat hepatocytes, where this occurs through sensitisation of the Ins(1,4,5)P₃ receptors (Bird et al., 1993). In these cells sulphydryl reagents cause Ca²⁺ spikes only if the threshold level of Ins(1,4,5)P₃ was lowered by pre-stimulation with low concentrations of Ca²⁺ mobilising agents or by microinjection of the Ins(1,4,5)P₃ analogue Ins(2,4,5)P₃. In these cells, isoprenaline or dibutyryl cAMP only caused an elevation of intracellular Ca²⁺ if the intracellular Ins(1,4,5)P₃ levels was lowered to subthreshold levels (Bird et al., 1993). The effects of cAMP on Ca²⁺ signalling have
also been demonstrated in mouse pancreatic β-cells. These cells induce different types of Ca\(^{2+}\) oscillations in response to glucose, particularly large amplitude Ca\(^{2+}\) oscillations. Subsequent addition of cAMP-elevating agents such as forskolin and glucagon triggers pronounced transient oscillations superimposed upon the large amplitude oscillations (Liu et al., 1996). In cells treated with thapsigargin or carbachol, the transient oscillations in response to glucose or forskolin were inhibited but the large amplitude oscillations in response to glucose were unaffected. Methoxyverapamil abolished large amplitude oscillations but had no effect on smaller transients indicating lack of dependence of these responses on voltage-operated Ca\(^{2+}\) channels (Liu et al., 1996). More recently, other studies have also highlighted the ability of these distinct pathways to co-ordinate signalling at multiple levels (Gorbunova & Spitzer, 2002; Rich et al., 2001). This chapter aims to determine if this interaction between cAMP and Ins(1,4,5)P\(_3\) receptors underlie the ability of noradrenaline to mediate Ca\(^{2+}\) signalling.

In addition, there is evidence suggesting a role for ryanodine receptors in mediating cAMP-dependent Ca\(^{2+}\) signalling. For example, in cardiomyocytes, β\(_2\)-adrenoceptor-mediated elevation of intracellular Ca\(^{2+}\) is mediated via cAMP/PKA signalling, that in turn influencing cADPr acting through ryanodine sensitive Ca\(^{2+}\) channels (Xie et al., 2005). β\(_2\)-Adrenoceptor-mediated elevation of intracellular Ca\(^{2+}\) was inhibited by a cADPr antagonist and high concentrations of ryanodine (Xie et al., 2005). This implicates a co-ordinating role for cAMP, cADPr and ryanodine receptors. Evidence is also emerging for a role of cADPr in muscarinic receptor-mediated Ca\(^{2+}\) signalling in pancreatic acinar cells. The majority of cADPr has been ascribed to the activity of ADP-ribosyl cyclase of CD38 (Fukushi et al., 2001). In pancreatic acinar cells from CD-38 knockout mice, stimulation with low concentrations of acetylcholine did not result in Ca\(^{2+}\) oscillations in comparison to control wild type cells (Fukushi et al., 2001). In control cells, oscillations were blocked by ryanodine. This indicates a CD38-dependent Ca\(^{2+}\) release mechanism that plays a crucial role in generation of Ca\(^{2+}\) oscillations (Fukushi et al., 2001). The precise role for ryanodine receptors in mediating muscarinic receptor-mediated Ca\(^{2+}\) signalling and crosstalk need to be addressed, as there are conflicting evidences for presence of ryanodine receptors in HEK 293 cells (Querfurth et al., 1998; Tong et al., 1999).
This Chapter details exploration of some of the potential mechanism(s) by which noradrenaline-mediates Ca$^{2+}$ signalling in the continued presence of activated muscarinic receptors. The role for ryanodine and Ins(1,4,5)P$_3$ receptor channels was investigated using pharmacological inhibitors and their contribution to Ca$^{2+}$ signalling mediated by muscarinic receptor agonists and noradrenaline examined. Furthermore, a range of factors that may influence the mechanism by which noradrenaline can mediate Ca$^{2+}$ signalling was examined.
6.2 Results

6.2.1 Effect of the adenylyl cyclase inhibitor SQ22,536 on noradrenaline-mediated Ca^{2+} release

To determine the role of adenylyl cyclases and cAMP in Ca^{2+} signalling by noradrenaline in HEK 293 cells, cells were pre-treated with 1 mM SQ22,536 (9-(tetrahydro-2-furyl) adenine). SQ22,536 inhibits adenylyl cyclases and hence cAMP production, in various tissues including tracheal smooth muscle cells and HEK 293 cells (Turcato & Clapp, 1999; Tovey et al., 2003; Koike et al., 2004; Yang et al., 1996). Cells pre-treated with SQ22,536 for 30 min were stimulated with muscarinic receptor agonists and subsequently with 10 μM noradrenaline. In cells pre-incubated with the SQ22,536 the maximal responses and potency of responses to methacholine and oxotremorine were not significantly different to buffer control (Figure 6.2.1; Table 6.2.1) confirming the lack of effect of SQ22,536 on muscarinic receptor-mediated Ca^{2+} signalling. These cells were subsequently stimulated with 10 μM noradrenaline.

Although the responses to 10 μM noradrenaline in the presence of higher concentrations of muscarinic receptor agonists were not inhibited by SQ22,536, the responses at lower concentrations of methacholine were reduced (Figure 6.2.1b). A possible explanation for this is that all adenylyl cyclases in HEK 293 cells are not completely inhibited by SQ22,536 pretreatment and full noradrenaline-mediated adenylyl cyclase activity is not required for mediating a maximum response. Future experiments measuring accumulation of cAMP in the presence or absence of SQ22,536 could give a direct indication of the extent of the adenylyl cyclase inhibition mediated by this compound. A concentration-dependent curve could not be derived for the ability of methacholine to mediate Ca^{2+} signalling by 10 μM noradrenaline in the presence of SQ22,536. Although the potency of oxotremorine-mediated facilitation of Ca^{2+} signalling by noradrenaline was attenuated by SQ22,536, this difference did not attain statistical significance (Table 6.2.1). It is unclear why SQ22,536 should influence responses to noradrenaline in the presence of methacholine more than in the presence of oxotremorine. This may be due to distinct mechanisms of crosstalk mediated by full agonists versus partial agonists and requires further validation.
Fig. 6.2.1a. Muscarinic receptor-mediated Ca$^{2+}$ signalling in the presence or absence of the adenylyl cyclase inhibitor, SQ22,536. Cells incubated with 1mM SQ22,536 for 30min were stimulated with muscarinic receptor agonists and fluorescence recorded for 150s. Histogram represents maximal change in fluorescence on stimulation with 1mM agonist in the presence or absence of SQ22,536. Panel B represents concentration response curves to agonists in the presence or absence of SQ22,536. Change in fluorescence on stimulation with muscarinic receptor agonists was quantified and taken as an index of changes in the [$Ca^{2+}]_i$. Data are mean +/- S.E.M. (n=3).
Fig. 6.2.1b. Effects of SQ22,536 on noradrenaline-mediated Ca$$^{2+}$$ signalling in the continued presence of muscarinic receptor agonists. Cells incubated with 1mM SQ22,536 for 30min were stimulated with muscarinic receptor agonists and fluorescence recorded for 150s. These cells were subsequently stimulated with 10μM noradrenaline and fluorescence recorded for further 150s. Graph represents the normalised responses in fluorescence units when stimulated with 10μM noradrenaline. Data are mean ± S.E.M. (n=3).
Table 6.2.1. The pEC₅₀ values of muscarinic receptor agonist-mediated Ca²⁺ responses and their ability to reveal Ca²⁺ signalling by 10μM noradrenaline, in a FLIPR assay in the absence or presence of 1mM SQ22,536 (relate to Figure 6.2.1a, 6.2.1b). Data are mean ± S.E.M. (n=3).

<table>
<thead>
<tr>
<th>Muscarinic receptor agonists</th>
<th>pEC₅₀ of muscarinic receptor agonist-mediated Ca²⁺ responses</th>
<th>pEC₅₀ of muscarinic receptor agonists ability to mediate a Ca²⁺ responses to 10μM noradrenaline</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methacholine</td>
<td>6.02± 0.22</td>
<td>-</td>
</tr>
<tr>
<td>Methacholine+SQ22,536</td>
<td>5.91± 0.21</td>
<td>5.64± 0.22</td>
</tr>
<tr>
<td>Oxotremorine</td>
<td>5.81± 0.27</td>
<td>5.64± 0.27</td>
</tr>
<tr>
<td>Oxotremorine+SQ22,536</td>
<td>6.08± 0.27</td>
<td>5.11± 0.22</td>
</tr>
</tbody>
</table>

6.2.2 Forskolin can reveal Ca²⁺ signalling in the continued presence of muscarinic receptor agonists

To examine if direct activation of adenylyl cyclases with forskolin would mediate Ca²⁺ signalling in the continued presence of muscarinic receptor activation, cells were stimulated with 100μM forskolin subsequent to stimulation with 1mM methacholine. The change in fluorescence evoked by forskolin in the presence of methacholine was comparable to the responses evoked by 10μM noradrenaline in the presence of 1mM methacholine (Figure 6.2.2a). Moreover, forskolin evoked a significantly greater Ca²⁺ response in the presence of 1mM methacholine than in its absence (Figure 6.2.2b). These results indicate that forskolin can reveal Ca²⁺ signalling in the continued presence of muscarinic receptor agonists.

To determine if Ca²⁺ responses mediated by forskolin depend on the concentration of the pre-stimulating muscarinic receptor agonist, cells were stimulated with 100μM forskolin (or 10μM noradrenaline) subsequent to stimulation with varying concentrations of muscarinic receptor agonists (Figure 6.2.2c). The potency of oxotremorine to mediate Ca²⁺ signalling by 100μM forskolin was similar to the potency of oxotremorine to mediate Ca²⁺ signalling by 10μM noradrenaline (pEC₅₀ value of
5.27±0.35 Vs. 5.68±0.41, n=3, respectively). Although forskolin mediated a robust elevation of [Ca\textsuperscript{2+}], when cells were prestimulated with 1mM methacholine, at lower concentrations of methacholine the responses to forskolin were much smaller (Figure 6.2.2c). As such the potency of methacholine to mediate Ca\textsuperscript{2+} signalling by forskolin could not be derived. To overcome any potential difficulties in intracellular Ca\textsuperscript{2+} store access by forskolin, concentration response curves to muscarinic receptor agonists were constructed subsequent to stimulation with either forskolin or buffer.

Methacholine evoked a Ca\textsuperscript{2+} response consisting of a rapid transient peak followed by a lower sustained plateau response. In cells pre-stimulated with forskolin, both 1mM and an EC\textsubscript{50} concentration of methacholine caused a Ca\textsuperscript{2+} response that resulted in an enhanced plateau phase (Figure 6.2.2d; Panel A). While only the latter evoked greater maximal responses in the presence of forskolin (Figure 6.2.2d; Panel A and B). In cells prestimulated with forskolin, oxotremorine-mediated peak and plateau responses were enhanced (Figure 6.2.2e). The differences in maximal responses observed however, did not reach statistical significance (Figure 6.2.2f).

The plateau phase of the muscarinic receptor agonist-mediated Ca\textsuperscript{2+} responses was significantly different at higher concentrations of agonists (Figure 6.2.2g and 6.2.2h). As an index of this, changes in fluorescence 49s after agonist addition was calculated (Section 4.2.6). Although there were significant differences in the plateau responses to muscarinic receptor agonists, the difference in potency of the muscarinic receptor agonist-mediated Ca\textsuperscript{2+} responses, obtained by the two analyses did not reach statistical significance. These values are summarised in Table 6.2.2.
Fig. 6.2.2a. Demonstration of the ability of forskolin to reveal Ca^{2+} signalling in the continued presence of methacholine. Representative FLIPR traces of cells challenged with 10\mu M noradrenaline (Panel A) or 100\mu M forskolin (Panel B) subsequent to stimulation with 1mM methacholine. Buffer addition following methacholine, failed to elevate [Ca^{2+}]_i. Cells challenged with buffer also failed to reveal an elevation of [Ca^{2+}]_i to noradrenaline or forskolin. However, in the continued presence of methacholine, noradrenaline and forskolin caused elevations in [Ca^{2+}]_i. Data are representative of 3 or more experiments. Mean data are represented in Figure 6.2.2b.
Fig. 6.2.2b. Average responses to 10μM noradrenaline or 100μM forskolin in HEK 293 cells prestimulated with either 1mM methacholine or buffer. Using the FLIPR cells were initially challenged with buffer or 1mM methacholine and subsequently stimulated with either 10μM noradrenaline, 100μM forskolin or buffer (relate to Figure 6.2.2a). Change in fluorescence on agonist stimulation were quantified and taken as an index of changes in the [Ca^{2+}]_i. Data are mean ± S.E.M. (n=3). ***, p<0.001; **, p<0.01, by one-way ANOVA with Bonferroni’s post tests.
Fig 6.2.2c. Responses to 10 μM noradrenaline or 100 μM forskolin in the continued presence of varying concentrations of muscarinic receptor agonists in HEK 293 cells. Cells were stimulated in a FLIPR with a range of concentrations of muscarinic receptor agonists (t=10s) and fluorescence recorded for 150s. Either 10 μM noradrenaline or 100 μM forskolin was subsequently added and the cells fluorescence recorded for a further 150s. Change in fluorescence on forskolin or noradrenaline stimulation was quantified and taken as an index of change in the [Ca^{2+}]_{i}. Data are mean ± S.E.M. (n=3).
Fig. 6.2.2d. Responses to methacholine in cells pre-stimulated with 100μM forskolin or buffer. Representative FLIPR traces of cells challenged with 1mM (Panel A) or 1μM (Panel B) methacholine in the continued presence or absence of 100μM forskolin. Data are representative of 3 experiments. Mean data are represented in Figure 6.2.2f.
**Fig. 6.2.2e.** Responses to oxotremorine in cells pre-stimulated with 100μM forskolin or buffer. Representative FLIPR traces of cells challenged with 1mM (Panel A) or 1μM (Panel B) oxotremorine in the continued presence or absence of 100μM forskolin. Data are representative of 3 experiments. Mean data are represented in Figure 6.2.2f.
Fig. 6.2.2f. Responses to muscarinic receptor agonists in cells pre-stimulated with 100μM forskolin. Cells were challenged with muscarinic receptor agonists subsequent to challenge with either 100μM forskolin or buffer (relate to Figure 6.2.2d and 6.2.2e). Histogram represents maximal changes in fluorescence on agonist stimulation taken as an index of the change in the [Ca^{2+}]. Data are mean ± S.E.M. (n=3).
Fig. 6.2.2g. Responses to methacholine in cells pre-stimulated with 100 μM forskolin. Cells were stimulated in a FLIPR with a range of concentrations of methacholine following challenge with 100 μM forskolin or buffer. Panel A represents the responses to methacholine calculated as a difference between peak and baseline fluorescence units (peak responses). Panel B represents methacholine responses calculated as a difference between fluorescence units 49s after agonist addition and baseline (plateau responses; ***, p<0.001, by two-way ANOVA). Data are mean±S.E.M. (n=3).
Fig. 6.2.2h. Responses to oxotremorine in cells pre-stimulated with 100μM forskolin. Cells were stimulated in a FLIPR with a range of concentrations of oxotremorine following challenge with 100μM forskolin or buffer. Panel A represents the responses to oxotremorine calculated as a difference between peak and baseline fluorescence units (peak responses). Panel B represents oxotremorine responses calculated as a difference between fluorescence units 49s after agonist addition and baseline (plateau responses; *** , p<0.001, by two-way ANOVA). Data are mean±S.E.M. (n=3).
Table 6.2.2. The pEC₅₀ values determined from concentration-response curves to muscarinic receptor agonists prestimulated with either 100μM forskolin or buffer, in a FLIPR assay (relate to Figure 6.2.2g, 6.2.2h). Data are mean ± S.E.M. (n=3).

<table>
<thead>
<tr>
<th>pEC₅₀ values of muscarinic receptor agonists measured as</th>
<th>difference between peak fluorescence and baseline (peak responses)</th>
<th>difference between fluorescence 49 sec after peak response and baseline (plateau responses)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer+ Methacholine</td>
<td>5.84±0.16</td>
<td>5.56±0.06</td>
</tr>
<tr>
<td>Forskolin+ Methacholine</td>
<td>6.12±0.17</td>
<td>5.82±0.15</td>
</tr>
<tr>
<td>Buffer+ Oxotremorine</td>
<td>6.03±0.06</td>
<td>-</td>
</tr>
<tr>
<td>Forskolin+ Oxotremorine</td>
<td>6.50±0.40</td>
<td>5.63±0.37</td>
</tr>
</tbody>
</table>

6.2.3 Effect of dibutyryl cAMP on methacholine-mediated Ca²⁺ signalling

To examine whether the cell permeable cAMP analogue dibutyryl cAMP (dbcAMP) could affect methacholine-mediated Ca²⁺ signalling, cells were incubated with 1mM dbcAMP or buffer control, for 5min prior to stimulation with methacholine. In cells stimulated with methacholine, a Ca²⁺ response that consists of a transient peak and plateau response was obtained. In cells pre-stimulated with dbcAMP, methacholine-mediated Ca²⁺ responses resulted in an enhanced peak and plateau phase (Figure 6.2.3a, 6.2.3b, Panel A). Although there were significant differences in the maximal responses, the potency of the peak methacholine responses was not different (pEC₅₀ value 6.57±0.11 and 6.44±0.02, n=3 in the presence or absence of dbcAMP respectively). As an index of the enhanced plateau phase response, changes in fluorescence 49 sec after agonist addition was calculated. The differences in maximal responses to methacholine in cells pre-stimulated with dbcAMP versus buffer were significant (Figure 6.2.3b, Panel B). However, the potencies of the plateau phase methacholine responses were not different (pEC₅₀ value 5.98±0.18 vs 6.14±0.07, n=3 in the presence and absence of dbcAMP, respectively).
Fig. 6.2.3a. Effect of pre-stimulating HEK 293 cells with 1mM dbcAMP or buffer on methacholine-mediated Ca\textsuperscript{2+} responses. Cells incubated with 1mM dbcAMP for 5 min were subsequently stimulated with methacholine. Representative FLIPR traces of cells challenged with 1mM (Panel A) or 1μM (Panel B) methacholine (t=10 sec). Data are representative of 3 experiments. Mean data are represented in Figure 6.2.3b.
Fig. 6.2.3b. Effect of pre-stimulating HEK 293 cells with dbcAMP or buffer on methacholine-mediated Ca²⁺ responses. Cells incubated with 1mM dbcAMP for 5min were subsequently stimulated with methacholine. Cells were challenged with methacholine (t=10s) and fluorescence recorded for 135 sec. Panel A represents changes in fluorescence on methacholine stimulation represented as a difference between peak and baseline fluorescence units (peak responses; **, p<0.01, by two-way ANOVA). Panel B represents the concentration responses to methacholine represented as a difference between fluorescence units 49 sec after agonist addition and baseline (plateau responses; ***, p<0.001, by two-way ANOVA). Data are mean ± S.E.M. (n=3).
6.2.4 Functional ryanodine receptors in HEK 293 cells

To examine if HEK 293 cells express functional ryanodine receptors, cells were challenged with a range of concentrations of caffeine, a known activator of ryanodine receptors. Cells were stimulated with 1, 5 or 15 mM caffeine, concentrations which have been previously shown to mediate Ca\(^{2+}\) release via ryanodine receptors in HEK 293 cells (Luo et al., 2003). In this study, caffeine mediated a concentration-dependent release of Ca\(^{2+}\) (Figure 6.2.4a). At a 1 mM concentration of caffeine, the response mediated was comparable to the response evoked by buffer (Figure 6.2.4a, Panel B). However at 5 mM and 15 mM concentrations of caffeine, a significant increase in fluorescence was observed (6-fold and 8-fold respectively; Figure 6.2.4a, Panel B). These results indicate the presence of functional ryanodine receptors in HEK 293 cells, consistent with previous observations (Luo et al., 2003; Querfurth et al., 1998).

In the continuous presence of 1, 5 or 15 mM caffeine, cells were stimulated with methacholine. Methacholine still evoked a peak and plateau response in the presence of caffeine (Figure 6.2.4b). In cells pre-treated with buffer or 1 mM caffeine, methacholine evoked comparable responses over the concentration range. However, the methacholine response was inhibited in cells pre-stimulated with 5 mM and 15 mM caffeine with greater inhibition observed at 15 mM (Figure 6.2.4b, Panel B). This may be due to caffeine partially depleting Ca\(^{2+}\) stores possessing Ins(1,4,5)P\(_3\) receptors, or the block of Ins(1,4,5)P\(_3\) receptors, as methylxanthines can inhibit Ins(1,4,5)P\(_3\) receptors in a variety of cells (Karaki et al., 1997; Ehrlich et al., 1994). The effect of caffeine on Ins(1,4,5)P\(_3\) receptors could be examined by determining the efflux of \(^{45}\)Ca\(^{2+}\) in permeabilised cells upon stimulation with exogenous Ins(1,4,5)P\(_3\) in the presence or absence of varying concentrations of caffeine. The potency of methacholine responses in the presence of caffeine did not vary significantly (Figure 6.2.4c; Table 6.2.4).

When HEK 293 cells were treated with a concentration of ryanodine known to inhibit ryanodine receptors (30 \(\mu\)M), the peak phase of the methacholine response was unaffected (Figure 6.2.4d). The potency of the methacholine-mediated Ca\(^{2+}\) response was also unaffected by ryanodine. However, when the plateau phase of the methacholine response was examined, the response was significantly inhibited by ryanodine (Figure 6.2.4e). The potency of the methacholine-mediated plateau Ca\(^{2+}\)
response was significantly reduced (pEC50 value 4.93±0.13 vs 5.87±0.10, n=3, in the presence or absence of ryanodine respectively). These results suggest that ryanodine receptors contribute to the sustained, plateau phase of the Ca2+ response to methacholine, in addition to influx of Ca2+ from the extracellular media demonstrated in Chapter 5 (Section 5.2.2). However, in cells treated with ryanodine oxotremorine-mediated an enhanced elevation of intracellular Ca2+, in comparison to buffer treated cells (Figure 6.2.4f, Panel A). Ryanodine, however, significantly inhibited the potency of the oxotremorine-mediated Ca2+ responses (Figure 6.2.4f; pEC50 value of 4.68±0.25 vs 6.16±0.01, n=3 in the presence or absence of ryanodine respectively). These results are consistent with previous demonstrations of functional ryanodine receptors and indicate a possible involvement of ryanodine receptor-dependent Ca2+ release in HEK 293 cells (Querfurth et al., 1998; Luo et al., 2003).
Fig. 6.2.4a. Effect of challenging HEK 293 cells with caffeine. Cells were challenged with varying concentrations of caffeine (t=15s) and fluorescence recorded for 80s. Panel A represents FLIPR trace of cells challenged with either caffeine or buffer. Data are representative of 3 or more experiments. Panel B represents the average responses to caffeine or buffer represented as the maximal difference between peak and baseline fluorescence units. Data are mean ± S.E.M. (n=3). * p<0.05, by one-way ANOVA with Bonferroni's post tests.
Fig. 6.2.4b. Effect of stimulating HEK 293 cells with methacholine following challenge with caffeine. Cells stimulated with caffeine (Fig. 6.2.4a) were subsequently stimulated with 1mM methacholine. Panel A is representative FLIPR trace of cells challenged 1mM methacholine (t=10s) and fluorescence recorded for 80s, in the continued presence of various concentrations of caffeine. Data are representative of 3 or more experiments. Panel B represents the average responses to 1mM methacholine in the presence of either caffeine or buffer. Data are mean ± S.E.M. (n=3). **, p<0.01, by one-way ANOVA with Bonferroni's post tests.
Fig. 6.2.4c. \( \text{Ca}^{2+} \) responses to methacholine in the continued presence of varying concentrations of caffeine. Cells were challenged with varying concentrations of methacholine, in the continued presence of caffeine. Data represent the responses to methacholine plotted as a difference between peak and baseline fluorescence units (peak responses). Data are mean ± S.E.M. (n=3).

Table 6.2.4. The \( pE_{C50} \) values determined from concentration-response curves to methacholine in cells prestimulated with either varying concentrations of caffeine or buffer, in a FLIPR assay (relate to Figure 6.2.4c). Data are mean ± S.E.M. (n=3).

<table>
<thead>
<tr>
<th>Pre-addition</th>
<th>( pE_{C50} ) values of responses to methacholine</th>
</tr>
</thead>
<tbody>
<tr>
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<tr>
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</tr>
<tr>
<td>5 mM Caffeine</td>
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</tr>
<tr>
<td>15 mM Caffeine</td>
<td>4.97±0.12</td>
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</table>
Fig. 6.2.4d. Effect of stimulating HEK 293 cells with methacholine following pre-incubation with ryanodine. Cells were pre-incubated with 30μM ryanodine for 10 min prior to stimulation with methacholine. Panel A represents FLIPR trace of cells challenged 1mM methacholine (t=10s) and fluorescence recorded for 125 s, in the continued presence of ryanodine. Data are representative of 3 or more experiments. Panel B represents the responses to methacholine as the difference between peak and baseline fluorescence units (peak responses; *, p<0.05, by two-way ANOVA). Data are mean ± S.E.M. (n=3).
**Fig. 6.2.4e.** Response to methacholine in HEK 293 cells pre-incubated with 30\(\mu\)M ryanodine or buffer prior to stimulation with muscarinic agonist stimulation. Cells were pre-incubated with 30\(\mu\)M ryanodine for 10min prior to stimulation with methacholine. Graph represents the responses to methacholine as a difference between fluorescence units 49s after agonist addition and baseline (plateau responses; ***, \(p<0.001\), by two-way ANOVA). Data are mean ± S.E.M. (n=3).
Fig. 6.2.4f. Response to oxotremorine in HEK 293 cells pre-incubated with 30μM ryanodine or buffer prior to stimulation with muscarinic agonist stimulation. Cells were pre-incubated with 30μM ryanodine for 10min prior to stimulation with oxotremorine. Panel A represents FLIPR trace of cells challenged 1μM oxotremorine (t=10s) and fluorescence recorded for 125s, in the continued presence of ryanodine. Data are representative of 3 or more experiments. Panel B represents the concentration-dependent responses to oxotremorine in the presence or absence of ryanodine (***, p<0.001, by two-way ANOVA). Change in fluorescence on agonist stimulation was quantified and taken as an index of changes in the [Ca^{2+}]i. Data are mean ± S.E.M. (n=3).
6.2.5 Effect of inhibiting Ins(1,4,5)P$_3$ and ryanodine receptors on noradrenaline-mediated Ca$^{2+}$ release

To assess the role for Ins(1,4,5)P$_3$ receptors and ryanodine receptors in noradrenaline-mediated Ca$^{2+}$ release, cells were treated with inhibitors of these channels (2-aminoethoxydiphenylborane (2-APB) and high concentrations of ryanodine respectively), either prior to or subsequent to stimulation with muscarinic receptor agonists. These cells were then challenged with noradrenaline. In cells pre-incubated with 100µM 2-APB for 30min, the maximal responses to muscarinic receptor agonists were significantly inhibited (Figure 6.2.5a). This concentration of 2-APB has been previously shown to be effective in inhibiting Ins(1,4,5)P$_3$ receptor function (Soulsby & Wojcikiewicz, 2002). Methacholine evoked a significantly less potent response in cells pre-incubated with 2-APB (Figure 6.2.5a; pEC$_{50}$ value of 5.61±0.04 and 6.13±0.11, n=3 in the presence or absence of 2-APB respectively). The potency of oxotremorine-mediated Ca$^{2+}$ responses could not be derived in the presence of 2-APB, as the responses were abolished (Figure 6.2.5a).

To examine the effect of these intracellular Ca$^{2+}$ channel inhibitors on noradrenaline-mediated Ca$^{2+}$ responses, cells were treated with the compounds separately or in combination, subsequent to stimulation with muscarinic receptor agonists. In cells pre-stimulated with methacholine the maximal responses to noradrenaline were inhibited in cells treated with the combination of 2-APB and ryanodine (Figure 6.2.5b, Panel A). However, this difference in the maximal responses to noradrenaline in the combined presence of 2-APB and ryanodine did not reach statistical significance. Responses to noradrenaline in the continued presence of oxotremorine were significantly inhibited by 2-APB and 2-APB in combination with ryanodine (Figure 6.2.5b, Panel B). The potency of muscarinic receptor agonist to reveal Ca$^{2+}$ signalling to noradrenaline in the combined presence of 2-APB and ryanodine were attenuated in comparison to the effect of either compounds individually (Figure 6.2.5c). Table 6.2.5 summaries these values. This result suggests coordination between Ins(1,4,5)P$_3$ receptors and ryanodine receptors in mediating Ca$^{2+}$ responses to noradrenaline.
**Fig. 6.2.5a. Effect of pre-incubating HEK 293 cells with 100µM 2-APB or buffer prior to muscarinic agonist stimulation.** Cells were incubated in the presence or absence of 100µM 2-APB for 30min prior to challenge with muscarinic receptor agonists (***, p<0.001 and **, p<0.01, for methacholine and oxotremorine response by two-way ANOVA in the presence or absence of 2-APB respectively). Change in fluorescence on agonist stimulation was quantified and taken as an index of changes in the [Ca^{2+}]. Data are mean ±S.E.M. (n=3).
Fig. 6.2.5b. Maximal responses to noradrenaline in the presence of muscarinic receptor agonists and intracellular Ca^{2+} channel blockers. Cells were incubated with either 100μM 2-APB, 30μM ryanodine or a combination of both for 30min prior to challenge with 1mM muscarinic receptor agonists. These cells were subsequently challenged with 10μM noradrenaline. Histogram represents maximal change in fluorescence on noradrenaline stimulation quantified and taken as an index of changes in the [Ca^{2+}]_i. Data are mean ± S.E.M. (n=3). *** p<0.001, * p<0.05, by one-way ANOVA with Bonferroni’s post test.
Fig. 6.2.5c. Responses to noradrenaline in the presence of muscarinic receptor agonists and intracellular Ca\(^{2+}\) channel blockers. Cells were incubated with either 100\(\mu\)M 2-APB, 30\(\mu\)M ryanodine or a combination of both for 30min prior to challenge with muscarinic receptor agonists. These cells were subsequently challenged with 10\(\mu\)M noradrenaline. Change in fluorescence on noradrenaline stimulation was quantified and taken as an index of changes in the \([Ca^{2+}]_i\). Data are mean\(\pm\) S.E.M. (n=3).
Table 6.2.5. The pEC_{50} values of muscarinic receptor agonists’ ability to mediate Ca^{2+} signalling by 10μM noradrenaline in the presence or absence of 2-APB, ryanodine or a combination of both, in a FLIPR assay (relate to Figure 6.2.5c). Data are mean ± S.E.M. (n=3). **, p<0.01, *, p<0.05, by one-way ANOVA with Bonferroni’s post tests.

<table>
<thead>
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<th>Ryanodine</th>
<th>2-APB+ Ryanodine</th>
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<td>Methacholine</td>
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<td>Oxotremorine</td>
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</table>

6.2.6 A role for cyclic ADP-ribose (cADPr) in mediating crosstalk

cADPr is a naturally occurring cyclic nucleotide that mediates release of intracellular Ca^{2+} via ryanodine receptors, independent of Ins(1,4,5)P₃ receptor-mediated intracellular Ca^{2+} release (Bai et al., 2005). To investigate a potential role of cADPr in mediating crosstalk, cells were pre-treated with 100 μM 8-bromo cyclic ADP ribose (8-BrcADPr), 100 μM 2-APB or both. 8-BrcADPr is a cell-permeable cADPr antagonist that selectively inhibits the Ca^{2+} releasing effect of cADPr in various tissues, including porcine airway smooth muscle cells and cardiomyocytes (White et al., 2003; Xie et al., 2005). Pre-incubation with 8-BrcADPr alone had no effect on either the maximal responses or the potency of the methacholine-mediated Ca^{2+} responses (Figure 6.2.6a). The plateau phase of the methacholine response was also unaffected in the presence of 8-BrcADPr (Figure 6.2.6a). The degree of inhibition of the maximal responses to methacholine in the presence of 2-APB was identical to the inhibition mediated by the combination of 8-BrcADPr and 2-APB (Figure 6.2.6a). Similarly there were no significant differences in the potency of methacholine response in the presence of 2-APB versus the combination of 8-BrcADPr and 2-APB. These results show that there is no direct involvement of cADPr in mediating Ca^{2+} responses to methacholine.
HEK 293 cells challenged with methacholine in the presence or absence of 8-BrcADPr were subsequently challenged with 10 μM noradrenaline. Under these circumstances, the maximal responses to noradrenaline were not significantly attenuated (Figure 6.2.6b). The potency of methacholine to mediate Ca\(^{2+}\) signalling by noradrenaline varied depending on the inhibitor used (Figure 6.2.6b; Table 6.2.6). Noradrenaline-mediated Ca\(^{2+}\) responses were inhibited by 8-BrcADPr alone and 2-APB in combination with 8-BrcADPr (Table 6.2.6). However, there was no significant difference in the potency of the ability of methacholine to reveal Ca\(^{2+}\) signalling by noradrenaline between cells treated with just 8-BrcADPr in comparison to a combination of 8-BrcADPr and 2-APB. Although these results indicate a possible role for cADPr in Ca\(^{2+}\) release by noradrenaline, the combined role for Ins(1,4,5)P\(_3\) receptors and cADPr in mediating crosstalk remains inconclusive as a synergistic effect of 8-BrcADPr and 2-APB on noradrenaline-mediated Ca\(^{2+}\) responses were not observed.
Fig. 6.2.6a. Effect of pre-incubating HEK 293 cells with the cADPr antagonist 8-BrcADPr, 2-APB or a combination of both prior to stimulation with methacholine. Cells were incubated with 100μM 8-BrcADPr, 100μM 2-APB or a combination of both for 30min prior to challenge with methacholine. Panel A represents the time-course of the methacholine response in the presence or absence of inhibitors. Data are representative of 3 or more experiments. Panel B represents the concentration-response curves to methacholine in the presence or absence of inhibitors. Change in fluorescence on agonist stimulation was quantified and taken as an index of changes in the [Ca²⁺]. Data are mean ± S.E.M. (n=3).
Fig. 6.2.6b. Responses to noradrenaline in cells pre-incubated with the cADPr antagonist 8-BrcADPr, 2-APB or a combination of both prior to stimulation with methacholine. Cells were incubated with 100μM 8-BrcADPr, 100μM 2-APB or a combination of both for 30min prior to challenge with methacholine. These cells were subsequently challenged with 10μM noradrenaline. Change in fluorescence on noradrenaline stimulation was quantified and taken as an index of changes in the [Ca^{2+}]_{i}. Data are mean ± S.E.M. (n=3).

Table 6.2.6. The pEC_{50} values of muscarinic receptor agonist’s ability to reveal Ca^{2+} signalling by 10μM noradrenaline in cells pre-incubated with either 2-APB, 8-BrcADPr or both, in a FLIPR assay (relate to Figure 6.2.6b). Data are mean ± S.E.M. (n=3). *** p<0.001, * p<0.05, by one-way ANOVA with Bonferroni’s post tests.

<table>
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<tr>
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<th>Buffer</th>
<th>2-APB</th>
<th>8-BrcADPr</th>
<th>2-APB+ 8-BrcADPr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methacholine</td>
<td>6.18±0.10</td>
<td>5.49±0.09</td>
<td>4.90±0.02</td>
<td>5.00±0.08</td>
</tr>
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</table>

pEC_{50} value of methacholine to mediate Ca^{2+} responses to 10 µM noradrenaline in the presence of
6.2.7 **Ins(1,4,5)P₃-mediated release of $^{45}\text{Ca}^{2+}$ from intracellular stores of permeabilised cells**

One potential mechanism by which cAMP is likely to mediate Ca$^{2+}$ release is through the sensitisation of the intracellular Ca$^{2+}$ channels. To determine if the sensitisation of Ins(1,4,5)P₃-receptors by cAMP is instrumental in mediating crosstalk, Ins(1,4,5)P₃-mediated Ca$^{2+}$ release from intracellular stores were determined in the presence or absence of cAMP. Cells were permeabilised with saponin, loaded with 2.3mCi/ml of $^{45}\text{CaCl}_2$ and allowed to incubate for 15-20 min. This time-period has been demonstrated to be sufficient to allow sufficient loading of the rapidly releasable intracellular Ca$^{2+}$ stores (Willars et al., 2001). The concentration-dependent release of $^{45}\text{Ca}^{2+}$ by Ins(1,4,5)P₃ from the intracellular stores was initially assessed (Figure 6.2.7a). In permeabilised HEK 293 cells, the potency of Ins(1,4,5)P₃ to mediate $^{45}\text{Ca}^{2+}$ release from a rapidly releasable pool was 0.25 μM (−log₁₀ (M) Kₐ, 6.59±0.08; n=3).

To determine the effect of cAMP on the release of $^{45}\text{Ca}^{2+}$ from the intracellular stores, permeabilised cells were incubated with 1mM cAMP for 10min in the presence or absence of the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX) and subsequently stimulated with 30μM Ins(1,4,5)P₃ (Figure 6.2.7b). cAMP and IBMX did not mediate release of $^{45}\text{Ca}^{2+}$. In cells prestimulated with cAMP there was no enhanced release of $^{45}\text{Ca}^{2+}$ in comparison to control. Ionomycin on the other hand (positive control) mediated release of 84% of the total $^{45}\text{Ca}^{2+}$ loaded. To determine if prestimulation with varying concentrations of cAMP would potentiate Ins(1,4,5)P₃-mediated $^{45}\text{Ca}^{2+}$ release, cells preincubated with cAMP were stimulated for 3min with 30μM Ins(1,4,5)P₃. In cells prestimulated with cAMP, no enhanced release of $^{45}\text{Ca}^{2+}$ was observed (Figure 6.2.7c). These experiments indicate that the sensitisation of Ins(1,4,5)P₃ receptors by cAMP does not play a role in mediating crosstalk. (These experiments were carried out by Neil Johnston, Research Technician, Department of Cell Physiology and Pharmacology, University of Leicester).
**Fig. 6.2.7a.** Ins(1,4,5)P₃-mediated ⁴⁵Ca²⁺ release in permeabilised HEK 293 cells. Saponin-permeabilised HEK 293 cells loaded with ⁴⁵Ca²⁺ were incubated with varying concentrations of Ins(1,4,5)P₃ for 1 min prior to determination of ⁴⁵Ca²⁺ release. ⁴⁵Ca²⁺ retained in the intracellular stores is represented as a percentage of total loaded. Data are mean ± S.E.M. (n=3).

**Fig. 6.2.7b.** ⁴⁵Ca²⁺ release in permeabilised HEK 293 cells. Saponin-permeabilised HEK 293 cells loaded with ⁴⁵Ca²⁺ were preincubated with 1mM cAMP, IBMX or buffer for 10 min. were subsequently stimulated with 30μM Ins(1,4,5)P₃ or ionomycin prior to determination of ⁴⁵Ca²⁺ release. ⁴⁵Ca²⁺ retained in the intracellular stores was represented as a percentage of total loaded. Data are mean ± S.E.M. (n=3). **, p<0.01; ***, p<0.001 by one-way ANOVA with Bonferroni’s post tests (Only relevant comparisons denoted for clarity).
Fig. 6.2.7c. **45Ca**²⁺ release in permeabilised HEK 293 cells. Saponin-permeabilised HEK 293 cells loaded with **45Ca**²⁺ were preincubated with varying concentrations of cAMP or buffer for 10 min. These cells were subsequently stimulated with 30 μM Ins(1,4,5)P₃ prior to determination of **45Ca**²⁺ release. **45Ca**²⁺ retained in the intracellular stores was represented as a percentage of total loaded. Data are mean ± S.E.M. (n=3).
6.2.8 A Role for Epac in mediating crosstalk

The effect of the Epac activator, 8-(4-chloro-phenylthio)-2'-O-methyladenosine-3':5'-cyclic monophosphate (8-pCPT-2'-O-Me-cAMP) on crosstalk was assessed. This compound has been previously demonstrated to selectively activate Epac, with no effect on PKA (Kang et al., 2003; Holz, 2004). Addition of Epac in the presence of muscarinic agonist failed to elevate Ca\(^{2+}\) (data not shown). Previous experiments demonstrated that preincubation with noradrenaline can increase both the E\(_{\text{max}}\) and potency of muscarinic receptor-mediated Ca\(^{2+}\) responses (Chapter 4). To determine if the Epac activator can similarly influence muscarinic receptor agonist-mediated Ca\(^{2+}\) responses, HEK 293 cells were preincubated with 100 μM Epac activator or 10 μM noradrenaline for 5 min prior to stimulation with methacholine.

In cells pre-stimulated with the Epac activator and noradrenaline methacholine-mediated Ca\(^{2+}\) responses resulted in a bigger peak response. However, these differences did not attain statistical significance (Figure 6.2.8a; Panel A). The potency of the methacholine response was not significantly different in the three cases (pEC\(_{50}\) value of 5.98±0.06, 6.12±0.04 and 5.92±0.09, n=3, in the presence of either the Epac activator or noradrenaline and buffer control respectively). As an index of the difference in the plateau phase of the Ca\(^{2+}\) response, the differences between fluorescence intensity 49s after agonist addition and baseline was calculated. There were significant differences in responses to methacholine in cells pre-stimulated with Epac activator versus buffer (Figure 6.2.8a, Panel B, p=0.03, by two-way ANOVA). The potency of the methacholine response was however not significantly different in the three cases (pEC\(_{50}\) value of 5.70±0.04, 5.56±0.09 and 5.48±0.08, n=3, in the presence of either the Epac activator or noradrenaline and buffer control respectively). These experiments suggest a potential role for Epac, however, the comparatively poor effect of noradrenaline-pre-incubation on methacholine-mediated Ca\(^{2+}\) responses complicate data interpretation. Additional experiments, for example utilising the Epac dominant-negative mutant (Kang et al., 2005; Rangarajan et al., 2003), could be used to confirm a role for Epac in mediating crosstalk.
Fig. 6.2.8a. Effect of pre-incubating HEK 293 cells with the Epac activator or noradrenaline prior to stimulation with methacholine. HEK 293 cells preincubated with 100μM Epac activator, 10μM noradrenaline or buffer for 5min were subsequently stimulated with methacholine in a FLIPR. Panel A is the responses to methacholine represented as the difference between peak and baseline fluorescence units (peak responses). Panel B are the responses to methacholine represented as the difference between fluorescence units 49s after agonist addition and baseline (plateau responses). Data are mean ± S.E.M. (n=3). **, p<0.01; *, p<0.05, by two-way ANOVA with Bonferroni's post tests.
6.3 Discussion

6.3.1 Summary of data

A range of pharmacological inhibitors was used to rule out critical components regulating the mechanism(s) of crosstalk between muscarinic M3 receptors and β2-adrenoceptors. Findings presented here suggest that Ins(1,4,5)P3 and ryanodine Ca2+ release channels mediate the Ca2+ mobilising actions of noradrenaline. The adenylyl cyclase inhibitor SQ22,536 attenuated noradrenaline-mediated Ca2+ responses. In addition, forskolin and dbcAMP were able to mimic crosstalk, suggesting a crucial role for cAMP. Experiments using an Epac specific activator have implicated a potential role for cAMP-Epac signalling complex in mediating crosstalk.

6.3.2 A role for Ins(1,4,5)P3 receptors, ryanodine receptors and cADPr?

Various pharmacological tools were utilised to determine the potential mechanisms by which crosstalk occurs. Although pharmacological inhibitors of the intracellular Ca2+ channels are not very specific, they still form a very integral part of identifying specific roles for them in various cell types (Taylor & Broad, 1998). The significant loss in potency of the muscarinic receptor agonists to mediate Ca2+ signalling by noradrenaline in cells pre-treated with ryanodine and 2-APB, implicate a potential synergy between these intracellular Ca2+ channels. However, the specificity of these inhibitors can be questioned. For example, in DT40 chicken B-lymphocytes, 2-APB (1-10μM) is a potent activator of store-operated Ca2+ channels (SOC) while higher concentrations (75-100μM) induces release of Ca2+ from intracellular stores and inhibits SOCs (Ma et al., 2002; Bootman et al., 2002). However, a Ca2+ elevation was not observed in HEK 293 cells upon administration of 2-APB, suggesting that 2-APB did not mobilise intracellular Ca2+ on its own (data not shown). Moreover, given that the noradrenaline-mediated Ca2+ response is independent of extracellular Ca2+, 2-APB’s inhibitory effect on SOCs should not affect the noradrenaline response. Furthermore, a previous study demonstrated that 2-APB inhibits Ins(1,4,5)P3 receptor function, ubiquitination and downregulation, but with variable characteristics in different cell types (Soulsby & Wojcikiewicz, 2002). These authors demonstrated that in HEK 293 cells, 100μM 2-APB inhibits Ins(1,4,5)P3-induced Ca2+ release but does not mobilise intracellular Ca2+ on its own. These findings are in agreement with the present study. Based on these
observations, it was concluded in the current study that 2-APB acted primarily by blocking Ins(1,4,5)P$_3$ receptors.

Numerous studies have provided evidence for the synergistic roles between the two-intracellular Ca$^{2+}$ release channels in several cell-types, including rat vascular myocytes, rat portal vein, rat gastric myocytes, rat cerebral artery, mouse luteinized-granulosa cells and rabbit portal vein (Boittin et al., 1999; Boittin et al., 1998; White & McGeown, 2000; Jaggar & Nelson, 2000; Morales-Tlalpan et al., 2005; Gordienko & Bolton, 2002). For example, in mouse luteinized-granulosa cells, ATP induces an elevation of [Ca$^{2+}$], by stimulating PLC through purinergic receptors, leading to the production of Ins(1,4,5)P$_3$ and subsequent release of Ca$^{2+}$ (Morales-Tlalpan et al., 2005). ATP-mediated Ca$^{2+}$ release was significantly inhibited in the presence of dantrolene and ryanodine, which block the ryanodine receptors. When these cells were pre-stimulated with caffeine or 2μM ryanodine, ATP-mediated responses were also attenuated. However, ryanodine receptor modulators did not affect Ins(1,4,5)P$_3$ production by ATP, indicating that the actions of these modulators are independent of purinergic receptor-associated PLC activity. These data indicate a synergy between the Ins(1,4,5)P$_3$ and ryanodine receptors, both contributing to patterns of agonist-mediated Ca$^{2+}$ release. Another example for interactions between the two channels was provided in rabbit portal vein myocytes. In these cells, localised spontaneous Ca$^{2+}$-release events visualised by line scanning imaging revealed two distinct spatiotemporal patterns consisting of small amplitude fast events and larger but slower events (Gordienko & Bolton, 2002). Both of these events were unaffected by nicardipine, but were abolished by treatment with ryanodine and thapsigargin. However, the small-amplitude events (similar to Ca$^{2+}$ sparks in cardiomyocytes) were unaffected by 2-APB, but the coupling between neighbouring Ca$^{2+}$ release domains that results in spontaneous Ca$^{2+}$ waves, was abolished. From these observations, the authors propose that activation of Ins(1,4,5)P$_3$ receptors recruits neighbouring domains of ryanodine receptors leading to larger Ca$^{2+}$ release and saltatory propagation of Ca$^{2+}$ waves in portal vein myocytes (Gordienko & Bolton, 2002). cADPr is a naturally occurring cyclic nucleotide that mediates release of intracellular Ca$^{2+}$ via ryanodine receptors. Evidence also suggests a role of cADPr in Ca$^{2+}$ signalling in response to muscarinic receptor activation in bovine tracheal smooth muscles, porcine airway smooth muscles and rat duodenal myocytes (White et al., 2003; Fritz et al., 2005; Franco et al., 2001). However, in the present study the cADPr-
selective antagonist 8-BrcADPr did not affect the potency of the muscarinic-receptor mediated Ca\(^{2+}\) response, indicating that in HEK 293 cells, cADPr do not contribute to Ca\(^{2+}\) signalling mediated by muscarinic receptors. Similarly, \(\beta\)-adrenoceptor-mediated increase of ADP-ribosyl cyclase activity has been reported in crude membrane preparations of rat cardiomyocytes (Higashida et al., 1999). However, the \(\beta\)-adrenoceptor subtype mediating this response was not characterised. More recently, a direct coupling of cADPr to cAMP signalling was demonstrated in rat cardiomyocytes (Xie et al., 2005). In these cells, isoproterenol-mediated elevation of intracellular Ca\(^{2+}\) was inhibited by 8-BrcADPr, H-89 and high concentrations of ryanodine. Moreover 8-BrcADPr and H-89 blocked the Ca\(^{2+}\) responses induced by a cAMP-analogue. Based on these observations, the authors speculate that \(\beta\)-adrenoceptor-mediated regulation of Ca\(^{2+}\) responses in rat cardiomyocytes are primed by activation of cyclic ADPr-cyclases via a cAMP/PKA-signalling pathway (Xie et al., 2005). This mechanism is not applicable for crosstalk in HEK 293 cells in this study, as a role for PKA could not be demonstrated.

In addition to the combined role for ryanodine receptors and Ins(1,4,5)P\(_3\) receptors in agonist-mediated Ca\(^{2+}\) responses, a role for cADPr in contributing to these Ca\(^{2+}\) responses was also described (Churchill & Galione, 2001; Fellener & Arendshorst, 2005; Burdakov et al., 2001). For example, in sea urchin eggs, it was demonstrated that NAADP-induced Ca\(^{2+}\) oscillations were partially blocked by the Ins(1,4,5)P\(_3\) receptor antagonist heparin or 8-amino-cADPr individually, but eliminated by the presence of both, indicating a requirement for both Ins(1,4,5)P\(_3\) and cADPr-dependent Ca\(^{2+}\) release (Churchill & Galione, 2001). Furthermore, in rat afferent arterioles it was demonstrated that cADPr acting via ryanodine receptors and the subsequent sensitisation of the ryanodine receptors to CICR are major components of the total increase of intracellular Ca\(^{2+}\) on stimulation with Angiotensin II (Fellener & Arendshorst, 2005). Based on their observations, it was proposed that stimulation of afferent arterioles with Angiotensin II results in the initial release of Ca\(^{2+}\) via the Ins(1,4,5)P\(_3\) receptors subsequently activating CICR at ryanodine receptors. However, the exact mechanisms by which activation of ADPR cyclases are linked to increases in intracellular Ca\(^{2+}\) remains unclear. Based on the literature and data presented, it is apparent that there is a functional coordination between the ryanodine receptors and Ins(1,4,5)P\(_3\) receptors in mediating Ca\(^{2+}\) signalling.
to noradrenaline. Although it is unclear how cADPr contributes to this Ca$^{2+}$ signal in HEK 293 cells, it is possible that Ins(1,4,5)P$_3$ may be used to initiate Ca$^{2+}$ signalling, while cADPr and ryanodine receptors contributes to the prolonged Ca$^{2+}$ signals such as Ca$^{2+}$ oscillations demonstrated in Chapter 4 (Churchill & Galione, 2002; Galione et al., 1993; Lee et al., 1993).

6.3.3 A role for cAMP?

An important observation in this study was that in the presence of adenylyl cyclase inhibitors, the ability of noradrenaline in the presence of muscarinic receptor activation to elevate [Ca$^{2+}$], was attenuated. However, there was a marked difference in the ability of SQ22,536 to inhibit Ca$^{2+}$ responses to noradrenaline in the continued presence of methacholine versus oxotremorine. This is also consistent with the previous observations that partial agonists of the muscarinic receptor are more efficacious in mediating a subsequent response to noradrenaline than full agonists. These differences could be due to distinct mechanisms mediating crosstalk by full muscarinic receptor agonists versus partial agonists. In addition, forskolin and dbcAMP were able to mimic crosstalk. These results cumulatively suggest a role for adenylyl cyclase and cAMP in crosstalk. This observation is contradictory to a number of studies (Short & Taylor, 2002; Tovey et al., 2003; Lezcano & Bergson, 2002), which demonstrated that despite the ability of Go$_{q/11}$-coupled receptors to potentiate Ca$^{2+}$ responses to subsequent stimulation of Ga-coupled receptors, agents such as 8-BrcAMP, forskolin and dbcAMP could not mimic crosstalk. For example, the ability of Ga$_{q}$-coupled parathyroid hormone to increase the Ca$^{2+}$ mobilisation in response to carbachol was independent of extracellular Ca$^{2+}$ and PKA, in agreement with this study, adenylyl cyclase inhibitors, however, did not influence crosstalk (Tovey et al., 2003). Moreover the inability of 8-BrcAMP to mimic the ability of parathyroid hormone to increase the Ca$^{2+}$ mobilisation in response to carbachol was demonstrated in an earlier study (Short & Taylor, 2000). These studies propose that parathyroid hormone potentiate Ca$^{2+}$ release through sensitisation of Ins(1,4,5)P$_3$ receptors by a cAMP-independent mechanism (Tovey et al., 2003).

In this study, a crucial role for cAMP in mediating crosstalk was demonstrated. This is similar to the ability of glucagon-like peptide-1 (GLP-1) ability to mobilise intracellular
Ca\(^{2+}\) in pancreatic β-cells through generation of cAMP. The GLP-1 receptor is a G\(\alpha_s\)-coupled receptor that on activation induces insulin secretion that is crucial for maintaining glucose homeostasis. The insulin secretory effect of GLP-1 is attenuated by treatments that inhibit elevation of \([\text{Ca}^{2+}]_i\) (Holz et al., 1999). Furthermore, cAMP analogues and forskolin can mimic elevation of \([\text{Ca}^{2+}]_i\) in a manner similar to GLP-1. However, in these cells (unlike in HEK 293 cells), there was functional coupling between the influx of Ca\(^{2+}\) through the voltage gated calcium channels and mobilisation of Ca\(^{2+}\) through ryanodine receptors, as methoxyverapamil or nimodipine attenuated the \([\text{Ca}^{2+}]_i\) responses (Holz et al., 1999; Liu et al., 1996). Based on these observations, it was proposed that PKA-mediated phosphorylation promotes the opening of L-type voltage-gated Ca\(^{2+}\) channels and influx of Ca\(^{2+}\). PKA also sensitises the ryanodine receptors to the stimulatory effects of cytosolic Ca\(^{2+}\) inducing CICR (Holz et al., 1999).

Further studies in pancreatic β-cells were able to demonstrate a signal transduction pathway that also allowed a cAMP-dependent CICR that was independent of PKA (Kang et al., 2001). In these cells, transient transfection of a dominant negative mutant isoform of cAMP-regulated guanine nucleotide exchange factor (cAMP-GEFII or exchange protein directly activated by cAMP 2, Epac2) blocked the Ca\(^{2+}\) responses to forskolin. This result confirmed a role for GLP-1 receptor mediated sensitisation of intracellular Ca\(^{2+}\) release independent of PKA, but dependent on Epac (Kang et al., 2001). Recent studies by the same group used an Epac selective cAMP analogue 8-pCPT-2'-O-Me-cAMP to demonstrate that it acted as a stimulus for CICR and exocytosis in pancreatic β-cells (Kang et al., 2003).

There are however, conflicting reports on the role for ryanodine receptors in mediating CICR in response to cAMP elevating agents. Dyachok & Gylfe, (2004) argued that CICR in pancreatic β-cells is mediated exclusively by Ins(1,4,5)P\(_3\) receptors, as ryanodine pre-treatment failed to attenuate CICR, whereas in cells treated with 2-APB CICR was inhibited. Using a PKA-specific activator, Sp-5,6-DCI-cBIMPS, a role for PKA in triggering exocytosis in pancreatic β-cells was demonstrated (Dyachok & Gylfe, 2004). Although opinions differed on the role of the Ca\(^{2+}\) channel involved in triggering Ca\(^{2+}\) release and a role for PKA, both groups were unanimous on the role for cAMP in mediating CICR by Ca\(^{2+}\) channel sensitisation. The authors argue that the
controversies regarding the type of receptors mediating CICR in pancreatic β-cells may be due to the involvement of different mechanisms in clonal versus primary β-cells (Dyachok & Gylfe, 2004). Recently, an extension of this study examined the effect of exendin-4, a peptide related in structure to GLP-1, in mouse-pancreatic β-cells. Exendin-4 could mobilise intracellular Ca^{2+} in a manner similar to GLP-1. However, H-89 exerted a partial inhibitory effect on exedin-4-mediated CICR, implicating operation of a PKA-dependent pathway as well as a PKA-independent, Epac-dependent pathway in these cells (Kang et al., 2005). Furthermore, the combined use of heparin and ryanodine abolished mobilisation of [Ca^{2+}], demonstrating the coordinating roles of these intracellular Ca^{2+} channels. Based on these observations, it was proposed that the Ca^{2+} mobilising actions of GLP-1 is mediated through both ryanodine and Ins(1,4,5)P_{3} receptors. Both these channels act as cAMP and Ca^{2+} coincidence detectors as their opening is regulated by the simultaneous increase in cAMP and Ca^{2+} (Kang et al., 2005).

In the present study, although Ins(1,4,5)P_{3}-mediated 45 Ca^{2+} release experiments indicate that sensitisation of Ins(1,4,5)P_{3} receptors by cAMP does not play a role in mediating crosstalk, this however cannot be ruled out completely. Permeabilisation of cells often result in impaired G-protein and receptor functioning. Moreover, there is evidence that cell permeabilisation may cause the loss of key cytosolic factors that may have important regulatory control over SERCA activity (Kang et al., 2001; Bruce et al., 2002). In HEK 293 cells, there is sufficient evidence for the involvement of CICR via the ryanodine receptors in mediating crosstalk. It is possible that cell permeabilisation abolishes CICR therefore any potentiation of 45 Ca^{2+} release mediated by cAMP is abolished.

In HEK 293 cells, given that noradrenaline-mediated Ca^{2+} signalling in the presence of muscarinic receptor activation is independent of PKA and dependent on cAMP, it is possible that Epac could play a role in crosstalk. Two isoforms of Epac (1 and 2) has been described, differing only in the number of cAMP-binding domains in the N-terminal region of the protein. Epac proteins are distributed differentially in the mammalian brain and organs. They bind cAMP and selectively activate Rap, a Ras-like G-protein (Kawasaki et al., 1998; de Rooji et al., 1998). A number of different stimuli induce activation of the Rap signalling pathway, including cAMP and Ca^{2+} (Franke et al., 1997; Altschuler et al., 1995). For example, stimulation of platelets with α-thrombin
results in the dramatic increase of activated Rap1. Furthermore, this increase correlates with an increase in intracellular Ca^{2+} suggesting a crucial role for Ca^{2+}-mediated signalling in platelet aggregation (Franke et al., 1997; Franke et al., 2000).

In a human immature megakaryocyte cell line MEG-01, cAMP elevating agents revealed Ca^{2+} signalling in a manner identical to this study (den Dekker et al., 2002). The authors demonstrated a direct correlation between the activation of Rap1 and increase of Ca^{2+} mediated by cAMP elevating agents such as forskolin and a prostacyclin receptor agonist, iloprost. In addition to demonstrating an independence from extracellular Ca^{2+} and independence of PKA, a role for Ins(1,4,5)P_3 receptors was also proposed. However a complete inhibition of the Ca^{2+} responses could not be achieved with pre-incubation with 2-APB, leading the authors to propose a role for 2-APB-insensitive Ins(1,4,5)P_3 receptor channels. Based on their observations, they proposed Rap1 to be a molecular switch that regulates SERCA activity, with the cAMP-induced Rap1 activation leading to the inhibition of SERCA and a rise in [Ca^{2+}], (den Dekker et al., 2002). However, another study has reported a PLC and Ca^{2+}-signalling pathway that is triggered by cAMP-mediated by Epac (Schmidt et al., 2001). In their study, in HEK 293 cells recombinantly expressing the β_2-adrenoceptor, stimulation with adrenaline resulted in the elevation of [Ca^{2+}], that was independent of PKA. Transient transfection of PLC-ε enhanced the ability of the Gα_ε-coupled receptors to stimulate PLC. The authors propose on the basis of their observations, a Ca^{2+} signalling pathway that is triggered by the activation of Gα_ε and adenylyl cyclase. This is dependent on cAMP production, with Epac serving as a cAMP effector activating Rap2B, which in turn leads to specific activation of PLC-ε (Schmidt et al., 2001). However, this model is unlikely to account for mediating crosstalk in this study, since the β_2-adrenoceptors cannot elevate [Ca^{2+}] without an activated muscarinic receptor and there is no evidence for enhanced PLC activity mediating crosstalk.

There is evidence for functional roles of cAMP-mediated signalling through Epac. For example, in ovarian carcinoma cells (Ovcar3) endogenously expressing β_2-adrenoceptors, stimulation of the receptor with isoproterenol induced integrin-mediated cell adhesion to fibronectin. This was mediated by an Epac-Rap1 signalling pathway, as the Epac activator 8-pCPT-2'-O-Me-cAMP enhanced cell adhesion while introduction
of Rap1GAPII, an inhibitor of Rap1 attenuated cell adhesion (Rangarajan et al., 2003). More recently, Ca\(^{2+}\)-induced acrosomal exocytosis was demonstrated to act via cAMP through a PKA-independent, Epac-dependent pathway (Branham et al., 2006). These examples highlight a range of PKA-independent, Epac-dependent physiologically relevant interactions between cAMP and Ca\(^{2+}\).

There may be different mechanisms mediating crosstalk, one potential mechanism may be through a cAMP-dependent sensitisation of intracellular Ca\(^{2+}\) release channels mediated by Epac-cAMP signalling complex. In pancreatic β-cells, it is proposed that the ability of GLP-1 to mediate mobilisation of [Ca\(^{2+}\)]\(_i\), is mediated by this mechanism (Kang et al., 2001; Kang et al., 2003; Kang et al., 2005). Based on the data obtained in HEK 293 cells, a possible mechanism by which noradrenaline can mediate Ca\(^{2+}\) signalling in the presence of activated muscarinic receptors is by binding of cAMP to Epac that in turn activates Ca\(^{2+}\) release through the ryanodine receptors. It is possible that L-type voltage-gated Ca\(^{2+}\) channels are not activated as in pancreatic β-cells (Kang et al., 2001; Kang et al., 2003; Kang et al., 2005), as the threshold of intracellular Ca\(^{2+}\) release is lowered by the ongoing muscarinic receptor activation. The extent of the noradrenaline-mediated Ca\(^{2+}\) responses in the presence of various muscarinic receptor agonists may be governed by a series of factors including the regulation of plasma membrane Ca\(^{2+}\)-ATPases, Ca\(^{2+}\) uptake into the ER by SERCA or uptake into the mitochondria (Bruce et al., 2002). However, it is unclear if Rap1 mediates any role in crosstalk. In platelets, SERCA isoform 3b (SERCA 3b) was demonstrated to be the sole target for Rap1 (Lacabaratz-Porret et al., 1998). Moreover, platelet inhibition was demonstrated to occur upon an increase in cAMP concentration, resulting in Rap1 phosphorylation and its subsequent dissociation from SERCA 3b, which in turn regulates the Ins(1,4,5)P\(_3\)-sensitive Ca\(^{2+}\) pool (Lacabaratz-Porret et al., 1998). Similarly Rap1 may play a role in regulating crosstalk in HEK 293 cells. The role for Epac could be explored further using the Epac dominant negative isoform while any role for Rap in modulating SERCA activity could be examined using the Rap1 inhibitor (Kang et al., 2005; Rangarajan et al., 2003). The role for the regulation of plasma membrane Ca\(^{2+}\)-ATPases can also be examined by ImM La\(^{3+}\), a known inhibitor (Furukawa et al., 1988; Bruce et al., 2002).
However, it is established that the affinity of cAMP for Epac is lower than for PKA (Holz, 2004; Kraemer et al., 2001; de Rooij et al., 2000). Such observations suggest that Epac could be activated in regions of the cell where cAMP formation is strongly stimulated or in subcellular compartments (Holz, 2004). The advent of fluorescent indicators of cAMP and Epac has revealed differential dynamics of cAMP signalling within discrete subcellular compartments (DiPilato et al., 2004; Zaccolo et al., 2000; Ponsioen et al., 2004). For example, using a FRET-based reporter assay with full-length Epac1, differential dynamics of cAMP signalling in response to activation of endogenously expressed β-adrenoceptor and prostanoid receptors was demonstrated in HEK 293 cells (DiPilato et al., 2004). It was demonstrated that cAMP production at the plasma membrane immediately produces a pool of cAMP in the nucleus. This pool is not sufficient to generate responses through PKA, in the given time-scale. This is however sufficient to induce conformational changes in Epac1 targeted to the nucleus (DiPilato et al., 2004). Furthermore a previous study has demonstrated the co-localisation of Epac1 to mitotic spindles and the nuclear membrane in a cell-cycle dependent manner (Qiao et al., 2002). In another study using cAMP ligand gated ion channels as biosensors for cAMP, it was demonstrated that in HEK 293 cells, stimulation with prostaglandin E1 caused a transient increase in cAMP near the plasma membrane, while total cellular cAMP content rose to a steady level (Rich et al., 2001). Such evidence highlights the possibility of highly localised cAMP events influencing signalling within a cell. Similarly, in HEK 293 cells, crosstalk may be influenced by such localised changes in cAMP. In summary, the mechanism governing crosstalk between the muscarinic M3 receptors and β-adrenoceptors are influenced a range of factors including cAMP, Epac and could possibly involves compartmentalisation of cAMP signalling.

To assess if crosstalk between the muscarinic receptors and the β-adrenoceptors are mediated in physiologically relevant cells, this phenomenon was examined in rat tracheal smooth muscle cells and human airway epithelial NCI H292 cell line. Chapter 7 addresses if the crosstalk occurs and attempts to address certain basic pharmacological parameters governing the crosstalk.
Chapter 7: Demonstration of crosstalk in tracheal smooth muscle cells and lung epithelial cells

7.1 Introduction

This study has been focused on characterising crosstalk between the muscarinic M₃ receptors and the β₂-adrenoceptors endogenously expressed in HEK 293 cells and trying to address the mechanism(s) by which this occurs. This has provided fresh insight into the way GPCRs interact with one another and influence signal transduction. The phenomenon of crosstalk has been demonstrated in several recombinant systems. However, demonstrations in physiologically relevant cell systems are fewer. The aim of the work described in this chapter was to extend the studies from the recombinant system into a series of primary cell types, expressing both receptor subtypes, to assess if this phenomenon occurs in these cells.

Muscarinic M₃ receptors and β₂-adrenoceptors are co-expressed in numerous cell types including smooth muscle cells. However, the significance of crosstalk at the level of intracellular Ca²⁺ or beyond, between these receptor subtypes is unclear. Potentiation of Ca²⁺ responses may enhance cellular responses such as secretion or contraction. However, considering that these receptor subtypes are expected to mediate contrasting physiological effects, the relevance of the findings in this study, is difficult to interpret. There are suggestions in the literature that β₂-adrenoceptors contribute to the contractile effect mediated by the muscarinic M₃ receptors. For example, in β-adrenoceptor knockout mice, contradictory to expectations, the ability of Ga₅/₁₁-coupled receptor agonists mediated contractile responses was attenuated (McGraw et al., 2003). Moreover, in mice overexpressing β₂-adrenoceptors, the contractile effect mediated by Ga₅/₁₁-coupled receptor agonists was enhanced. This was demonstrated both in-vivo and in-vitro preparations (McGraw et al., 2003; McGraw et al., 2000). In the β₁/₂-adrenoceptor-knockout airway smooth muscle cells, there was no change in the levels of expression of either Ga₅ or Ga₅/₁₁, but instead the PLC-β₁ content was reduced by 60%. While in cells overexpressing the β₂-adrenoceptors, there was a 2-fold increase in PLC-β₁ expression. Based on their observations, it was proposed that enhanced PLC-β₁ expression is the mechanism by which β-adrenoceptor activation leads to an increase in
airway hyperresponsiveness over time with chronic use of β-adrenoceptor agonists
(McGraw et al., 2003). More recently, in an in-vitro model of bronchoprotection,
salbutamol-induced hyperresponsiveness to acetylcholine was investigated (Girodet et al., 2005). In cultured human airway smooth muscle cells, a 10min pre-treatment with salbutamol enhanced contractions mediated by high concentrations of acetylcholine but inhibited contractions mediated by low concentrations of acetylcholine. Following an 18h pre-treatment with salbutamol there was no protective effect and responses to acetylcholine were significantly increased. Pre-treatment with salbutamol also enhanced the intracellular Ca\(^{2+}\) response to acetylcholine. The Ca\(^{2+}\) channel blocker nicardipine abolished salbutamol-mediated enhanced contractile responses to acetylcholine. Based on these observations, it was proposed that potentiation of salbutamol-induced acetylcholine response is mediated by Ca\(^{2+}\) channel activation (Girodet et al., 2005). In a similar study, the role for Ca\(^{2+}\) and Cl\(^{-}\) ion channels in 5-hydroxytryptamine (5-HT)-mediated contractile responses in tracheae from ovalbumin challenged rats were examined in-vitro (Moura et al., 2005). In rat trachea challenged with an antigen, 5-HT induced a stronger contraction in comparison to control. Pre-treatment with nifedipine or the Ca\(^{2+}\)-activated Cl\(^{-}\) channel blocker, niflumic acid, abolished enhanced contractile responses to 5-HT. Based on these observations, it was proposed that rat airway hyperresponsiveness is likely to be associated with increased Ca\(^{2+}\) and Cl\(^{-}\) ion channel activity (Moura et al., 2005). In addition other factors such as receptor desensitisation, upregulation of β-adrenoceptors and polymorphisms associated with the β-adrenoceptor have been implicated in this phenomenon (Hall, 2004; French & Hall, 2002).

Having characterised factors contributing to the potential mechanism for interaction between GPCRs in HEK 293 cells, this chapter attempts to establish if crosstalk occurs in rat tracheal smooth muscle cells and human lung epithelial cell line, NCI H292. Initial studies were aimed at replicating the existence of this phenomenon in primary cells. Furthermore, a range of factors that may influence the mechanism by which noradrenaline can mediate Ca\(^{2+}\) signalling in the continued presence of muscarinic receptor agonists was examined.
7.2 Results

7.2.1 α-Actin staining of tracheal smooth muscle cells

Rat tracheal smooth muscle cells were isolated as described in Section 2.7 (Figure 7.2.1a). Isolated cells were cultured for up to 7 days. These cells were initially stained with the smooth muscle specific α-actin and visualised using confocal microscopy on day 4 and day 7 of culture (Figure 7.2.1b). Immunofluorescent staining was positive in all cells with the smooth muscle α-actin filaments stained in strands within the cytoplasm. However, α-actin immunofluorescence was not observed in human lung epithelial NCI H292 cells indicating specific staining of actin filaments in the smooth muscle cells (Figure 7.2.1b).
Fig. 7.2.1a. Acutely dissociated rat tracheal smooth muscle cells. Male Wistar rats (<300gm) were culled by concussion followed by cervical dislocation in accordance with Schedule 1 of the Animals (Scientific Procedures) Act 1986. A mid-line ventral incision was made and the trachea isolated. Tissue was digested initially with papain in the presence of 1,4-dithiothreitol for 30min. This was followed by digestion with collagenase and hyaluronidase for 45-60min. Smooth muscle cells was dissociated by mechanical sheer by repeated triturations through a fire-polished Pasteur pipette. These cells were collected by centrifugation at 200g for 5min and resuspended in medium 231. Image represents acutely isolated cells resuspended in media. Further details in text.
Fig. 7.2.1b. α-Actin immunofluorescence in rat tracheal smooth muscle cells. Rat tracheal smooth muscle cells were isolated and cultured for up to 7 days. To analyse smooth muscle α-actin, cells were permeabilised and fixed using 100% methanol. These cells were subsequently incubated with α-actin antibody and FITC-conjugated secondary antibody and imaged using a confocal microscope (Section 2.7.2). Panel A and B represents tracheal smooth muscle cells at day 4 and day 7 of culture respectively. Panel C represents NCI H292 human lung epithelial cells, processed as described above. Data are representative of 3 or more experiments.
7.2.2 Demonstration of crosstalk in rat tracheal smooth muscle cells

Rat tracheal smooth muscle cells obtained were initially imaged during several stages in culture. Cells at day 1-2 of culture could not be used for Ca\textsuperscript{2+} imaging by confocal microscopy because they contracted on agonist stimulation (methacholine, UTP) making agonist-mediated fluorescence changes difficult to quantitate. Instead, cells at day 4 of culture were used in all subsequent experiments. In cells stimulated with noradrenaline, there was no change in fluorescence, indicating a lack of noradrenaline-mediated Ca\textsuperscript{2+} release via α or β-adrenoceptors (Figure 7.2.2a; Panel A). As for the responses to methacholine, these were attenuated in comparison to cells at day 1-2 of culture (Figure 7.2.2a; Panel B). This observation was consistent with literature evidence and was associated with the difficulty in the maintenance of muscarinic M\textsubscript{3} receptors in smooth muscle preparations (Yang et al., 1991; Panettieri et al., 1989). However these cells demonstrated robust responses to UTP and this was taken as a measure of cell viability and responsiveness (Figure 7.2.2a; Panel B).

A series of experiments were carried out to determine the best protocol using confocal microscopy to identify crosstalk. Cells stimulated with methacholine were subsequently stimulated with noradrenaline and changes in fluorescence recorded (bath additions; Protocol 1; Figure 7.2.2b; Panel A). Alternatively, cells were stimulated with methacholine in combination with noradrenaline (bath additions; Protocol 2; Figure 7.2.2b; Panel B). In rat tracheal smooth muscle cell preparations, noradrenaline-mediated a Ca\textsuperscript{2+} response in the continued presence of methacholine. However, the proportion of responding cells varied from one culture to another. Due to the inconsistency of responses obtained and to avoid further difficulty in data interpretation, Protocol 1 was used for subsequent experiments. The data analysis included either averaged responses from all smooth muscle cells in the field of view or peak responses from just the cells that responded to agonists (Figure 7.2.2c). An average of 70% of cells responded to noradrenaline in the presence of methacholine, while all cells responded to UTP. Stimulation with 10 μM noradrenaline alone failed to elevate [Ca\textsuperscript{2+}]\textsubscript{i} (Figure 7.2.2a; Panel A). The data obtained implicate crosstalk between the muscarinic receptors and the β-adrenoceptors occurs in rat tracheal smooth muscle cells.
Fig. 7.2.2a. Ca^{2+} responses to agonists in rat tracheal smooth muscle cells. Rat tracheal smooth muscle cells cultured for 4 days were imaged by confocal microscopy. Panel A represents cells challenged with 10μM noradrenaline (bath addition). Panel B represents lack of response to 1mM methacholine in these cells. UTP mediated a robust response in these cells indicating cell viability and responsiveness (bath addition). Data are representative of three or more experiments.
Fig. 7.2.2b. Protocols to examine crosstalk in rat tracheal smooth muscle cells. Rat tracheal smooth muscle cells cultured for 4 days were imaged by confocal microscopy. Cells were challenged with 10μM noradrenaline (bath addition) subsequent to challenge with 1mM methacholine (bath addition). Cell viability and responsiveness was assessed by stimulation with 2μM UTP (bath addition). Panel A represents responses to noradrenaline obtained using Protocol 1. Panel B represents response to noradrenaline in cells obtained using Protocol 2. Data are representative of three or more experiments.
Fig. 7.2.2c. \(Ca^{2+}\) responses to agonists in rat tracheal smooth muscle cells. Rat tracheal smooth muscle cells cultured for 4 days were challenged with agonists using protocol 1 and imaged by confocal microscopy. Histograms represent responses to 1mM methacholine, 10\(\mu\)M noradrenaline, 10\(\mu\)M noradrenaline in the continued presence of 1mM methacholine and 2\(\mu\)M UTP. Panel A represents averaged responses to agonists in all cells. Panel B represents peak responses from just the cells that responded to agonists (noradrenaline response in the continued presence of methacholine 53/76 cells). Data are mean \pm S.E.M. (n=3). **, p<0.01, by one-way ANOVA with Bonferroni's post tests.
7.2.3 Effect of CTX and PTX pre-treatment on crosstalk in rat tracheal smooth muscle cells

Rat tracheal smooth muscle cells obtained were treated with 100 ng/ml of PTX or 2 μg/ml of CTX for 20 h prior to imaging by confocal microscopy. In cells pre-treated with PTX, only 25% of cells responded to noradrenaline in the continued presence of methacholine compared to 67% of cells in untreated cells (Figure 7.2.3a, 7.2.3b; Panel A). UTP-mediated Ca²⁺ responses were unaffected by PTX treatment. These results indicate that in the rat tracheal smooth muscle cells, there is an involvement of Goᵢ or Gdi-coupled receptors in mediating Ca²⁺ responses to noradrenaline. However, if only the responding cells are analysed, the peak responses were unaffected by PTX treatment (Figure 7.2.3b; Panel B).

In cells pre-treated with CTX, the responses to noradrenaline were enhanced in comparison to untreated cells (Figure 7.2.3b). This indicates that in the absence of the Go₅-mediated component of signalling, Go₉/₁₁ or Go₁-coupled adrenoceptors mediate Ca²⁺ responses to noradrenaline. Since noradrenaline mediated a Ca²⁺ response in CTX treated cells, this complicated subsequent data interpretation. Moreover, there was inter-experimental variability in the percentage of cells responding to noradrenaline in the continued presence of methacholine (control responses). This may arise due to a heterogeneous cell population in primary cultures and/or differences in the levels of receptor expression over the period of cell culture. In the continued presence of methacholine, the responses evoked by noradrenaline were also enhanced in CTX-treated cells (Figure 7.2.3c; Panel B). However, a smaller percentage of cells responded to noradrenaline in the continued presence of methacholine in CTX-treated cells in comparison to control (17% vs 26% respectively; Figure 7.2.3a). From these data, it was inconclusive which adrenoceptor subtype is responsible for noradrenaline-mediated Ca²⁺ responses. However due to time constraints the adrenoceptor subtype mediating this effect was not dissected out using selective antagonists.
Fig. 7.2.3a. Percentage of tracheal smooth muscle cells responding to 10 μM noradrenaline in the continued presence of methacholine in cells treated with either PTX (Panel A) or CTX (Panel B). Rat tracheal smooth muscle cells were pre-treated with 100 ng/ml of PTX or 2 μg/ml of CTX for 20 h prior to challenge with 1 mM methacholine and subsequently with 10 μM noradrenaline. Data are mean ± S.E.M. (n=3).
average response to 10µM noradrenaline in the presence of methacholine ± PTX

peak response to 10µM noradrenaline in the presence of methacholine ± PTX

Fig.7.2.3b. Ca^{2+} responses to noradrenaline in the continued presence of methacholine in rat tracheal smooth muscle cells treated with PTX. Rat tracheal smooth muscle cells were pre-treated with 100ng/ml of PTX for 20h prior to challenge with 1mM methacholine and subsequently with 10µM noradrenaline. Cells were imaged by confocal microscopy and maximal responses quantified. Histograms represent responses to noradrenaline in the continued presence of methacholine. Panel A represents averaged responses to agonists in all 98 cells. Panel B represents responses evoked to agonists taking into account only those cells that responded to noradrenaline. Data are mean±S.E.M. (n=3).
Fig. 7.2.3c. Ca^{2+} responses to noradrenaline in the continued presence of methacholine in rat tracheal smooth muscle cells treated with CTX. Rat tracheal smooth muscle cells were pre-treated with 2μg/ml of CTX for 20h prior to challenge with 1mM methacholine and subsequently with 10μM noradrenaline. Cells were imaged by confocal microscopy and maximal responses quantified. Histograms represent responses to noradrenaline in the presence of methacholine. Panel A represents averaged responses to noradrenaline in the presence or absence of CTX. Panel B compares responses evoked by noradrenaline taking into account just the responding cells and averaged responses. Data are mean ± S.E.M. (n=3).
7.2.4 Demonstration of crosstalk in NCI H292 cells

To assess whether crosstalk occur in human lung epithelial NCI H292 cells, using a FLIPR, cells were stimulated with methacholine and in its continued presence stimulated with the β₂-adrenoceptor selective agonist formoterol (1 μM) or noradrenaline (10 μM). Noradrenaline and formoterol did not mediate a Ca²⁺ response in these cells. However, in the continued presence of methacholine, formoterol and noradrenaline evoked a Ca²⁺ response that was dependent on the concentration of prestimulating methacholine (Figure 7.2.4a). The ability of methacholine to facilitate the Ca²⁺ response to subsequent stimulation with 1 μM formoterol (pEC₅₀ value for methacholine of 5.17±0.17, n=3) was not significantly different to when cells were stimulated with 10 μM noradrenaline (pEC₅₀ value for methacholine of 4.95±0.11, n=3).

To confirm that the adrenoceptor mediating these responses was β₂-adrenoceptor, cells were incubated with β₂-adrenoceptor selective antagonist ICI 118,551 (1 μM) for 10 min prior to stimulation with methacholine (Hoffmann et al., 2004; Baker, 2005). The maximal response to methacholine was unaffected by the presence of ICI 118,551. However, the responses to methacholine were slightly more potent in the presence of the β-adrenoceptor antagonist. Although, this difference was not statistically significant (pEC₅₀ value of 5.24±0.05 vs 4.89±0.19, n=3 in the presence or absence of ICI 118,551; Figure 7.2.4b; Panel A). 1 μM ICI 118,551 abolished responses to noradrenaline in the presence of methacholine. This indicates that β₂-adrenoceptors play a major role in mediating crosstalk in NCI H292 (Figure 7.2.4b; Panel B).
Fig. 7.2.4a. Ca\textsuperscript{2+} responses to agonists in human lung epithelial NCI H292 cells. Cells were stimulated with a range of concentrations of methacholine in NCI H292 cells and fluorescence recorded for 150s (Panel A). These cells were subsequently challenged with 10\,\mu\text{M} noradrenaline or 1\,\mu\text{M} formoterol and fluorescence recorded for a further 150s (Panel B). Change in fluorescence on noradrenaline or formoterol stimulation was quantified and taken as an index of change in the [Ca\textsuperscript{2+}]\textsubscript{i}. Data are mean ± S.E.M. (n=3).
Fig. 7.2.4b. $\text{Ca}^{2+}$ responses to agonists in human lung epithelial NCI H292 cells. Following a 10 min pre-incubation with 1 $\mu$M ICI 118,551, cells were stimulated with a range of concentrations of methacholine and fluorescence recorded for 150s using a FLIPR (Panel A). These cells were subsequently challenged with 10 $\mu$M noradrenaline and fluorescence recorded for a further 150s (Panel B). Change in fluorescence on agonist stimulation was quantified and taken as an index of change in the [Ca$^{2+}$]. Data are mean ± S.E.M. (n=3).
7.2.5 Effect of CTX on formoterol-mediated Ca\textsuperscript{2+} responses in NCI H292 cells

NCI H292 cells were treated with 2 μg/ml of CTX for 20 h prior to stimulation with a range of concentrations of methacholine and subsequently with 1 μM formoterol. The maximal responses to methacholine were unaffected by incubation with CTX (Figure 7.2.5a; Panel A). In contrast, in CTX treated cells, maximal responses to 1 μM formoterol in the continued presence of 1 mM methacholine were attenuated (Figure 7.2.5a; Panel B). CTX had no effect on the potency of responses to muscarinic receptor agonists (Figure 7.2.5b; pEC\textsubscript{50} values of 5.04±0.15 and 5.09±0.11, n=3 in the presence or absence of CTX respectively). The potency of responses to formoterol in the continued presence of methacholine was 4.72±0.09, n=3 (Figure 7.2.5b; Panel B). However, a pEC\textsubscript{50} value of the response to formoterol in the presence of methacholine in CTX-treated cells could not be derived as the responses were abolished. These results confirm that formoterol-mediated Ca\textsuperscript{2+} responses in NCI H292 cells are mediated via G\alpha\textsubscript{q}-coupled receptors.
Fig. 7.2.5a. Effects of CTX on Ca\(^{2+}\) signalling to 1mM methacholine and to the subsequent addition of 1\(\mu\)M formoterol. NCI H292 cells incubated with 2\(\mu\)g/ml of CTX for 20h were stimulated with 1mM methacholine and fluorescence recorded for 150s (Panel A). In the continued presence of methacholine, cells were challenged with 1\(\mu\)M formoterol and fluorescence recorded for further 150s (Panel B). Histograms represent the change in fluorescence on methacholine or formoterol challenge in the presence or absence of CTX. Data are mean + S.E.M. (n=3). ***, p<0.001, by unpaired Student's t-tests.
Fig. 7.2.5b. Effects of CTX on $Ca^{2+}$ signalling to methacholine and the subsequent addition of 1μM formoterol. NCI H292 cells incubated with 2μg/ml of CTX for 20h were stimulated with 1mM methacholine and fluorescence recorded for 150s (Panel A). In the continued presence of methacholine, cells were challenged with 1μM formoterol and fluorescence recorded for further 150s (Panel B). Change in fluorescence on agonist stimulation was quantified and taken as an index of change in the $[Ca^{2+}]_i$. Data are mean ± S.E.M. (n=3).
7.2.6 Effect of PKA inhibition on formoterol-mediated Ca$^{2+}$ responses in NCI H292 cells

To investigate the potential role of PKA in mediating crosstalk, NCI H292 cells were preincubated with 10 μM 4-cyano-3-methylisoquinoline (CMIQ; Tovey et al., 2003) for 30 min prior to stimulation with methacholine. In the continued presence of methacholine, cells were challenged with 1 μM formoterol. Maximal responses and potency of methacholine-mediated Ca$^{2+}$ response was unaffected by CMIQ (Figure 7.2.6a; pEC$_{50}$ values of responses 5.02±0.08 and 4.99±0.09 (n=3) in the presence or absence of CMIQ respectively). The response evoked by formoterol in the continued presence of methacholine was similarly unaffected by CMIQ (Figure 7.2.6a; pEC$_{50}$ values of responses 5.10±0.09 vs 5.15±0.12 (n=3) in the presence or absence of CMIQ respectively). These results indicate that formoterol-mediated Ca$^{2+}$ responses are independent of any regulation by PKA in NCI H292 cells. This is in agreement with the study in HEK 293 cells. However, it should be emphasized that the lack of data demonstrating a positive inhibitory effect of CMIQ on PKA in these cells, means that the data should be interpreted with some caution.
**Fig. 7.2.6a.** Ca$^{2+}$ responses to agonists in NCI H292 cells. NCI H292 cells were pre-treated with 10μM CMIQ for 30 min prior to stimulation with a range of concentrations of methacholine in a FLIPR and fluorescence recorded for 150 s (Panel A). These cells were subsequently stimulated with 1μM formoterol and fluorescence recorded for a further 150 s (Panel B). Change in fluorescence on agonist stimulation was quantified and taken as an index of change in the [Ca$^{2+}$]. Data are mean ± S.E.M. (n=3).
To determine whether direct activation of adenylyl cyclases with forskolin would potentiate methacholine-mediated Ca^{2+} responses, NCI H292 cells were pre-incubated with 100 μM forskolin for 5 min prior to stimulation with methacholine. Similarly, cells were pre-incubated with 1 μM formoterol for 5 min prior to stimulation with methacholine. In cells pre-treated with formoterol or forskolin the maximal responses to methacholine were enhanced in comparison to control (p<0.05; Figure 7.2.7a, 7.2.7b). There were no significant changes in the plateau phase response of methacholine. In the presence of forskolin, methacholine-mediated a Ca^{2+} response with a pEC_{50} value of 5.36±0.09 (n=3) while in cells pre-treated with formoterol, methacholine-mediated a Ca^{2+} response with a pEC_{50} value of 5.32±0.11 (n=3), that was not statistically different to control (5.37±0.15, n=3). These results indicate that forskolin can mimic crosstalk in a manner identical to formoterol in NCI H292 cells.
Fig. 7.2.7a. \( \text{Ca}^{2+} \) responses to 1mM methacholine in NCI H292 cells pre-incubated with either forskolin or formoterol. NCI H292 cells were pre-treated with 1\( \mu \)M formoterol (Panel A) or 100\( \mu \)M forskolin (Panel B) for 5min prior to stimulation with 1mM methacholine in a FLIPR. Data are representative of 3 experiments. Averaged responses are represented in Figure 7.2.7b; Panel A.
**Fig. 7.2.7b. Ca²⁺ responses to methacholine in NCI H292 cells prestimulated with either forskolin or formoterol.** NCI H292 cells were pre-treated with 1µM formoterol or 100µM forskolin for 5min prior to stimulation with a range of concentrations of methacholine in a FLIPR. Panel A represents averaged responses to 1mM methacholine in the presence of forskolin and formoterol (relate to Figure 7.2.7a). Panel B represents the concentration-dependent response to methacholine in the presence or absence of either formoterol or forskolin. Data are mean ± S.E.M. (n=3). *, p<0.05, by one-way ANOVA with Bonferroni’s post tests.
7.2.8 Effect of the putative Ins(1,4,5)P₃ receptor antagonist, 2-APB on formoterol-mediated Ca²⁺ mobilisation in NCI H292 cells

To assess the role of Ins(1,4,5)P₃ receptors in formoterol-mediated Ca²⁺ mobilisation, cells were treated with an inhibitor of this channel (2-APB), prior to stimulation with methacholine. These cells were then subsequently challenged with formoterol. In cells pre-incubated with 100 pM 2-APB for 30 min, methacholine-evoked Ca²⁺ responses were abolished (Figure 7.2.8a; Panel A). This result indicates that in these cells, Ca²⁺ responses evoked by methacholine are mediated by Ins(1,4,5)P₃ receptors with little or no contribution from other Ca²⁺ release channels. Similarly, responses to 1 μM formoterol in the continued presence of methacholine were abolished in cells pre-treated with 2-APB. These results indicate that formoterol-mediated Ca²⁺ responses are mediated by Ins(1,4,5)P₃ receptors with little or no contribution from other Ca²⁺ release channels. A role for ryanodine receptors in these cells was not investigated in these cells, as caffeine did not induce a Ca²⁺ response (data not shown).
Fig. 7.2.8a. Ca^{2+} responses to agonists in NCI H292 cells in the presence or absence of 2-APB. NCI H292 cells were pre-treated with 100μM 2-APB for 30min prior to stimulation with a range of concentrations of methacholine and fluorescence recorded for 150s (Panel A). These cells were subsequently stimulated with 1μM formoterol and fluorescence recorded for a further 150s. Change in fluorescence on agonist stimulation was quantified and taken as an index of change in the [Ca^{2+}]_i. Data are mean ± S.E.M. (n=3).
7.2.9 Effect of methacholine on formoterol-mediated cAMP responses in NCI H292 cells

NCI H292 cells mediate a robust cAMP response following stimulation with formoterol (Mandy Lawson, AstraZeneca R&D, Charnwood, personal communication). In this study, cAMP accumulation was quantified in these cells using the Alphascreen kit (Section 2.5). Formoterol-mediated a maximal concentration-dependent cAMP accumulation in cells stimulated for 10 min with the agonist (Figure 7.2.9a; Panel A). The pEC$_{50}$ value of formoterol-mediated cAMP accumulation was 8.35±0.16 (n=3).

To assess whether activation of muscarinic receptors would influence cAMP responses mediated by β$_2$-adrenoceptors, cells were stimulated with 1 μM formoterol in the presence or absence of 1 mM methacholine for 1, 3 or 10 min (Figure 7.2.9a; Panel B). The cAMP accumulation in response to formoterol was not enhanced by 1 mM methacholine at any time-point. To determine if prestimulation with lower concentrations of methacholine would potentiate formoterol-mediated cAMP responses, cells were stimulated with 1 mM, 1 μM or 0.01 μM methacholine and subsequently stimulated with a range of concentrations of formoterol (Figure 7.2.9b). There were no significant differences either in the maximal responses (Figure 7.2.9b; Panel A) or potency of formoterol-mediated cAMP responses in the presence or absence of methacholine (Figure 7.2.9b; Panel B). These results indicate that activation of muscarinic receptors does not influence β$_2$-adrenoceptor-mediated cAMP responses.
Fig. 7.2.9a. cAMP responses to formoterol in NCI H292 cells. NCI H292 cells were stimulated with a range of concentrations of formoterol for 1, 3 or 10 min (Panel A). Histogram (Panel B) represents cAMP accumulation in cells stimulated with 1 μM formoterol in the presence of 1 mM methacholine for 1, 3 or 10 min. cAMP accumulation was quantified by the Alphascreen kit as described in Section 2.5. Data are mean ± S.E.M. (n=3).
maximal response to formoterol in the presence of

- Buffer
- 1mM Methacholine
- 1µM Methacholine
- 0.01µM Methacholine

**Fig. 7.2.9b. cAMP responses to agonists in NCI H292 cells.** Histogram (Panel A) represents cAMP accumulation in NCI H292 cells stimulated with 1µM formoterol for 10min in the presence or absence of varying concentrations of methacholine. Panel B represents the concentration-dependent response of cells stimulated with formoterol in the presence of 1mM, 1µM or 0.01µM methacholine for 10min. cAMP accumulation was quantified by the Alphascreen kit as described in Section 2.5. Data are mean +/− S.E.M. (n=3).
7.3 Discussion

7.3.1 Summary of data

The ability of noradrenaline to reveal Ca$^{2+}$ signalling in the continued presence of muscarinic receptor agonists were successfully demonstrated in both rat tracheal smooth muscle cells and human lung epithelial NCI H292 cells. The complete characterisation of crosstalk in rat tracheal smooth muscle cells has been difficult as expression of other adrenoceptor subtypes complicate data interpretation. In NCI H292 cells, a range of factors contributing to the mechanism of crosstalk was characterised. In these cells crosstalk is mediated in a cAMP-dependent and PKA-independent manner, similar to HEK 293 cells. Moreover Ins(1,4,5)P$_3$ receptor channels was demonstrated to be responsible for mediating Ca$^{2+}$ responses to the β$_2$-adrenoceptor agonists. The activation of muscarinic receptors did not affect the β$_2$-adrenoceptor-mediated cAMP responses, demonstrating the non-reciprocal nature of this crosstalk.

7.3.2 Crosstalk in physiologically relevant cells

The ability of noradrenaline to reveal Ca$^{2+}$ signalling in the continued presence of muscarinic receptor agonists was successfully demonstrated in both rat tracheal smooth cells. In most smooth muscle tissues, both muscarinic M$_2$ and M$_3$ receptors are co-expressed (Eglen et al., 1994; Hornigold et al., 2003). Muscarinic receptor knockouts mice have been used to study receptor subtype specific effects in the organs where they are co-expressed. In muscarinic M$_1$, M$_2$, M$_3$ receptor single and double knockout mice studies were carried out to determine which of the muscarinic receptor subtypes regulate the constriction of intrapulmonary airways (Struckmann et al., 2003). In wild type mice, muscarinic agonist-induced bronchoconstriction consists of three phases: a robust bronchoconstriction develops within minutes of agonist administration followed by a transient relaxation phase and finally a sustained bronchoconstriction.

In muscarinic M$_3$ receptor knockout animals, although lower concentrations of agonist were required to induce bronchoconstriction, increasing concentration of agonist significantly reduced bronchoconstriction. The transient relaxation phase observed was identical to wild types. In muscarinic M$_1$ receptor knockout mice, at lower concentrations of muscarinic agonists, there was a significantly higher bronchoconstriction in comparison to wild type mice. At higher concentrations of
agonists, the bronchoconstriction was identical to controls. Interestingly, the transient relaxation phase observed in wild type mice was completely abolished in knockout mice. In muscarinic M2 receptor knockout mice, the initial bronchoconstriction was identical to wild type mice, in comparison with the transient relaxation and sustained bronchoconstriction, which was significantly effected. Taken together these results indicate that the muscarinic M3 and M2 receptor subtypes affect peripheral airway tone by theirconcerted action, while muscarinic M1 receptors counteract this bronchoconstriction perhaps by mediating release of a relaxing agent (Struckmann et al., 2003). Another study has implicated that the tracheal and main bronchus constriction is largely mediated by the muscarinic M3 receptors (Stengel et al., 2002). Therefore it is likely that the crosstalk mediated in rat tracheal smooth muscle cells is mediated mainly by muscarinic M3 receptors. However an indirect role for muscarinic M2 receptors cannot be ruled out.

Evidence for the involvement of crosstalk between muscarinic M2 and M3 receptors in the regulation of second messenger and extracellular signal-regulated kinase (ERK) activity pathways have been presented in CHO-K1 cells stable expressing these receptor subtypes (Hornigold et al., 2003). In these cells, a contribution of muscarinic M2 receptors was demonstrated to the muscarinic M3 receptor-mediated Ins(1,4,5)P3 responses as well as ERK activation. The ERK response was approximately 50-fold more potent in cells co-expressing both muscarinic M2 and M3 receptors in comparison to either receptor subtypes on their own. Moreover, co-stimulation of the endogenous purinergic receptors also caused a 10-fold shift in methacholine-mediated ERK activation in CHO-K1 cells stably expressing the muscarinic M2 receptors suggesting a role for crosstalk between G Purdue11-coupled receptors and Ga coupled receptors in ERK activation (Hornigold et al., 2003). In the rat tracheal smooth muscle cells, it seems unlikely that crosstalk between the muscarinic receptor subtypes mediate crosstalk. However an interaction between Ga-coupled muscarinic receptors and G Purdue11-coupled α-adrenoceptor or Ga-coupled β-adrenoceptors could mediate crosstalk in rat tracheal smooth muscle cells.

There is overwhelming evidence for the involvement of β2-adrenoceptors and not other adrenoceptors in airway smooth muscle relaxation (Tanaka et al., 2004; Tanaka et al., 2005; Kompa et al., 1995). In this study, although CTX treatment reduced the
percentage of cells responding to noradrenaline in the presence of methacholine these results were not conclusive for an exclusive role for \( \beta_2 \)-adrenoceptors. In CTX-treated cells, noradrenaline alone mediated a \( \text{Ca}^{2+} \) response (implicating a role for either \( \text{G}_{\alpha_{q11}} \)-coupled or \( \text{G}_{\alpha_5} \)-coupled \( \alpha \)-adrenoceptors). This suggests the dominance of \( \beta \)-adrenoceptor-mediated relaxant effect over contractile \( \alpha \)-adrenoceptor activity. Furthermore, the percentage of cells responding to noradrenaline was reduced by PTX treatment implicating a potential role for \( \text{G}_{\alpha_1} \)-coupled \( \alpha \)-adrenoceptors in this crosstalk. There is evidence for both expression and functional roles for the \( \alpha_2 \)-adrenoceptors in tracheal smooth muscle cells (Goldie et al., 1990). The role for \( \alpha \)-adrenoceptors in mediating crosstalk could be dissected out in future experiments using commercially available selective adrenoceptor agonists and antagonists.

There is a wealth of literature investigating crosstalk between the \( \text{G}_{\alpha_{q11}} \) and \( \text{G}_{\alpha_5} \)-coupled receptors, resulting in enhanced phosphoinositide responses (Werry et al., 2003). If a role for \( \text{G}_{\alpha_1} \)-coupled and \( \text{G}_{\alpha_{q11}} \)-coupled receptors is identified in mediating crosstalk, one of the potential mechanism by which this crosstalk may occur is through pooling of liberated \( \text{G}_{\beta\gamma} \) subunits (Section 1.4.1.3). Activation of muscarinic M_2 receptors in cells overexpressing \( \text{G}_{\beta\gamma} \) subunits elicited agonist-mediated PLC responses that were pertussis toxin insensitive in comparison to cells expressing the complete \( \text{G}_{\alpha_{q11}} \text{G}_{\beta\gamma} \) subunits (Chan et al., 2000). This indicates the ability of the \( \text{G}_{\beta\gamma} \) subunits to stimulate PLC. Using constitutive active mutants of different \( \text{G}_{\alpha_{q11}} \) members, it was demonstrated that preactivation of PLC-\( \beta \) is required for \( \text{G}_{\alpha_1} \)-mediated potentiation of inositol phosphate responses mediated via the \( \text{G}_{\beta\gamma} \) subunits (Chan et al., 2000). A role for \( \text{G}_{\beta\gamma} \) subunits could be analysed further using \( \text{G}_{\beta\gamma} \) scavengers such as the C-terminal tail of GRK2 or the transducin \( \text{G}_{\alpha} \) subunit.

In the human lung epithelial NCI H292 cells using a \( \beta_2 \)-adrenoceptor selective agonist and antagonist, a role for this adrenoceptor in mediating crosstalk was confirmed. Although we have been unable to demonstrate all the factors influencing crosstalk in these cells (due to time-constraints), it is reasonably clear that crosstalk is mediated by a cAMP-dependent, PKA independent mechanism with a crucial role for \( \text{Ins}(1,4,5)\text{P}_3 \) sensitive \( \text{Ca}^{2+} \) channels. However, due to the lack of positive controls, such as the lack of data demonstrating a positive inhibitory effect of CMIQ on PKA, the data must be
interpreted with caution. This is in agreement with the data in HEK 293 cells. A number of other factors such as a role for thapsigargin-sensitive intracellular Ca\textsuperscript{2+} stores, extracellular Ca\textsuperscript{2+}, G\alpha, and Epac need to be confirmed before a mechanism by which crosstalk occurs is proposed.

Although the muscarinic receptor subtypes and β\textsubscript{2}-adrenoceptors are generally known to mediate opposing physiological actions, evidence is emerging that β\textsubscript{2}-adrenoceptors may contribute to the contractile effect mediated by the muscarinic M\textsubscript{3} receptors. However, while this may reflect crosstalk of the type described within this study, other mechanisms may exist. For example, in β-adrenoceptor knockout mice, the ability of G\alpha\textsubscript{q/11}-coupled receptor agonists to mediate a contractile response was attenuated and this was attributed to the ~60% loss of PLC-β1 expression (McGraw et al., 2003). However in human airway smooth muscle cells, although β\textsubscript{2}-adrenoceptor agonists augmented spasmogen responses, a role for enhanced PLC-β1 expression could not be demonstrated (Sayers et al., 2006). Other studies have demonstrated that upregulation of G\alpha\textsubscript{q/11}-coupled receptors on exposure to β\textsubscript{2}-adrenoceptor agonists could contribute to airway hyperresponsiveness (Mak et al., 2000; Katsunuma et al., 2000). On the other hand, downregulation of G\alpha\textsubscript{s}-coupled β\textsubscript{2}-adrenoceptors on stimulation with G\alpha\textsubscript{q/11}-agonists are implicated as a potential cause for hyperresponsiveness. In bovine tracheal smooth muscle cells, it was demonstrated that stimulation with carbachol or PKC activation significantly reduced the maximum binding capacity of [\textsuperscript{125}I]-iodocyanopindolol (Grandordy et al., 1993). Contrary to these findings, a study in mice model of either hyporesponsive or hyperresponsive to acetylcholine, demonstrated that there were no significant differences in the level of receptor expression of either G\alpha\textsubscript{q/11}-coupled muscarinic M\textsubscript{3} receptors or G\alpha\textsubscript{s}-coupled β\textsubscript{2}-adrenoceptors in comparison to wild type (Gavett & Wills-Karp, 1993). In addition, other potential causes for hyperresponsiveness include a role for cytokines, cADPR, β-adrenoceptor desensitisation, upregulation of β-adrenoceptors and polymorphisms associated with the β-adrenoceptors (Deshpande et al., 2004; Amrani et al., 2004; Shore & Moore, 2003; French & Hall, 2002; Hall, 2004).

This study in HEK 293 and NCI H292 cells suggest a cAMP-dependent, PKA independent mechanism that may provide a novel aspect that contributes to airway
hyperresponsiveness. Furthermore, a recent study has demonstrated that the anti-spasmogenic effect of isoprenaline on guinea-pig trachea is mediated by a PKA-independent mechanism (Spicuzza et al., 2001). Using cell-permeant phosphodiesterases-resistant cAMP analogues that selectively inhibit PKA, attenuation of acetylcholine-induced tension was demonstrated to occur in isolated guinea-pig tracheal rings. Moreover, a PKG inhibitor failed to antagonise the relaxatory effect of isoprenaline under conditions in which antispasmogenic effect of a cGMP analogue was abolished, suggesting a pathway independent of regulation by PKG. These findings demonstrate that PKA-independent pathways are operational in tracheal smooth muscle cells and are in agreement with this study.

In summary, the data obtained in this chapter are indicative of crosstalk occurring in physiologically relevant cell-systems. The exact mechanism by which this occurs remains to be fully characterised but initial findings point to a cAMP-dependent and PKA-independent regulation of crosstalk.
Chapter 8: General Discussion

8.1 Summary of data

This thesis was aimed at characterising the ability of partial agonists of the muscarinic receptor to drive cellular signalling and examining their ability to mediate crosstalk between two differentially coupled GPCRs. The concept of partial agonism was examined in cell systems recombinantly expressing either high or low levels of muscarinic M₃ receptors. Partial agonists are often considered therapeutically relevant as they may avoid adverse effects such as desensitisation, tolerance or dependence associated with receptor activation by full-agonists (Zhu, 2005). Despite evidence in the literature for a good correlation between agonist-coupling efficiency and desensitisation, phosphorylation and internalisation, this study has demonstrated that this concept is not applicable to all circumstances (Clark et al., 1999; January et al., 1997; January et al., 1998; Yu et al., 1997). Of particular interest is the agonist oxotremorine. Despite being able to phosphorylate the muscarinic M₃ receptors (in Buckley-M₃ cells with higher receptor expression) to a lesser extent than methacholine, it mediates internalisation of the receptor comparable to the full agonist. Pilocarpine and arecoline, similarly, mediate second messenger responses and receptor phosphorylation comparable to methacholine. However, these agonists were poor in mediating receptor internalisation. These results demonstrate a discrepancy between agonist-coupling efficiency and desensitisation, phosphorylation and internalisation, similar to the literature evidence (Szekeres et al., 1998; Edwardson & Szekeres, 1999; Keith et al., 1996). This may be due to factors such as the rate constant for receptor recycling and receptor endocytosis influencing internalisation, effectively allowing agonists with relatively lower intrinsic efficacy to cause significant internalisation (Edwardson & Szekeres, 1999).

This thesis explores crosstalk between the $G_{q/11}$-coupled muscarinic M₃ receptors and $G_{s}$-coupled $\beta_2$-adrenoceptors that result in intracellular $\mathrm{Ca}^{2+}$ signalling. It was demonstrated in this study that stimulation of $\beta_2$-adrenoceptors with noradrenaline does not elevate $[\mathrm{Ca}^{2+}]_i$ in HEK 293 cells unless they are pre-stimulated with agonists acting at the muscarinic receptors. This is in agreement with the literature evidence of the
ability of Gαo-coupled receptors to mediate a Ca²⁺ signal in the continued presence of activated Gαq₁₁-coupled receptors (Werry et al., 2002; Tovey et al., 2003). These responses were dependent on both the concentration of the pre-stimulating muscarinic receptor agonist and noradrenaline. Facilitation of noradrenaline-mediated Ca²⁺ responses occur only in the presence of activated muscarinic receptors, as treatment with atropine, subsequent to stimulation with methacholine, abolished responses to noradrenaline. Crosstalk was independent of the presence of extracellular Ca²⁺, but dependent on thapsigargin-sensitive intracellular stores and is consistent with literature evidence (Werry et al., 2002; Tovey et al., 2003; Dyer et al., 2005; Tanimura et al., 1999; Connor et al., 1997). This study provides the first demonstration of the ability of partial agonists of the muscarinic receptor to mediate Ca²⁺ signalling to noradrenaline. Despite the apparent inability of some of the partial agonists to mediate a Ca²⁺ response, these agonists mediated robust elevation of intracellular Ca²⁺ in response to noradrenaline with similar or greater magnitude to full agonists.

Experiments were performed to determine the mechanism underlying crosstalk between muscarinic M₃ receptors and β₂-adrenoceptors. Data obtained from [³⁵H]-InsPₓ accumulation studies provided evidence for a lack of enhanced PLC activity in mediating crosstalk. This suggests that potential mechanisms involving increases in Ins(1,4,5)P₃ production such as cooperativity between Gαq₁₁ and Gβγ subunits or gain of Gαq₁₁-coupling are not responsible for mediating crosstalk. However, while measurement of [³⁵H]-InsPₓ accumulation at extremely short time-periods may reflect the initial level of PLC activity, measurement for prolonged time-periods reflects PLC activity only during the plateau phase of Ins(1,4,5)P₃ generation (Willars & Nahorski, 1995). These experiments cannot rule out a role for enhanced PLC activity, but suggest this is unlikely given other studies examining crosstalk between different receptor combinations have been able to use such techniques to show an involvement for PLC activity (Werry et al., 2002; Selbie et al., 1995; Yang et al., 2001; Werry et al., 2003). To assess transient increases in Ins(1,4,5)P₃ generation in single cells, the eGFP-PH PLC₈₁ construct was used. Stimulation of cells with noradrenaline did not result in a detectable translocation of eGFP-PH PLC₈₁ construct either in methacholine or buffer challenged cells. An alternate mechanism for crosstalk was suggested based on the ability of adenylyl cyclase inhibitors to attenuate noradrenaline-mediated Ca²⁺ responses. In addition, forskolin and dbcAMP were able to mimic crosstalk, suggesting
a crucial role for cAMP. Crosstalk between $\text{G}_{\alpha_4}$-coupled receptors and $\text{G}_{\alpha_{q/11}}$-coupled receptors described previously has implicated a role for PKA (Tanimura et al., 1999; Burgess et al., 1991; Liu et al., 1996; Brown et al., 2004; Bruce et al., 2002). Interestingly, crosstalk described in this study was independent of PKA. The dependence of crosstalk on cAMP and independence from PKA suggested a possible role for Epac in crosstalk, as demonstrated in the literature (Kang et al., 2001; Kang et al., 2003; Dekker et al., 2002; Kang et al., 2005). Experiments using the Epac activator 8-pCPT-2'-'O-Me-cAMP indicated an involvement for this cAMP-GEF in crosstalk. This compound has been demonstrated to bind to and activate Epac with a higher affinity than cAMP (EC$_{50}$ value 2.2 $\mu$M versus 30 $\mu$M for cAMP; Kang et al., 2003).

The data presented suggest a role for cAMP, Epac and intracellular Ca$^{2+}$ channels in mediating crosstalk. The most likely hypothesis for the ability of noradrenaline to mediate Ca$^{2+}$ responses is through the sensitisation of intracellular Ca$^{2+}$ release channels by cAMP-Epac signalling complex, as proposed in the literature (Kang et al., 2001; Kang et al., 2003; Dekker et al., 2002; Kang et al., 2005). However, due to the lack of data confirming the positive effects of the pharmacological tools used in this study, these results should be interpreted with caution.

Examination of crosstalk at the single cell level revealed that noradrenaline influences Ca$^{2+}$ oscillations in the presence of submaximal concentrations of muscarinic receptor agonists. In particular, there was a significant increase in the number of cells oscillating and frequency of baseline and sinusoidal Ca$^{2+}$ oscillations. The relevance of such alterations in Ca$^{2+}$ oscillations as a result of crosstalk is unclear. However, given that Ca$^{2+}$ oscillations influence aspects such as gene transcription it is possible that such crosstalk could influence aspects of cell function yet to be explored (Dolmetsch et al., 1998; Hu et al., 1999; Shore & Moore, 2003).

The ability of noradrenaline to mediate Ca$^{2+}$ signalling that was dependent on the continued presence of muscarinic receptor agonists was successfully demonstrated in rat tracheal smooth muscle cells. To characterise the receptor subtype mediating the noradrenaline response, rat tracheal smooth muscle cells were treated with CTX and PTX. Treatment with CTX reduced the percentage of cells responding to noradrenaline but did not inhibit responses completely. While in PTX treated cells, the percentage of cells responding to noradrenaline was reduced, indicating a $\text{G}_{\alpha_4}$ involvement. These
results indicate that in addition to β2-adrenoceptors, Gαi-coupled α-adrenoceptors may be mediating Ca²⁺ responses to noradrenaline in rat tracheal smooth muscle cells. A human lung epithelial NCI H292 cell line that mediates a robust cAMP response following stimulation with the β2-adrenoceptor selective agonist formeterol was used to determine if crosstalk was reciprocal. In these cells, activation of muscarinic receptors did not potentiate cAMP responses by β2-adrenoceptors despite the ability of noradrenaline to mediate a Ca²⁺ response, in the continued presence of muscarinic receptor agonists. In summary, this study has contributed to our knowledge of crosstalk by demonstrating the ability of partial agonists to mediate crosstalk and their ability to influence Ca²⁺ oscillations. In addition, the occurrence of this phenomenon in physiologically relevant cell-type was demonstrated.

8.2 Physiological implications for crosstalk

One critical aspect uncovered in this study is the ability of noradrenaline to influence Ca²⁺ oscillations including aspects such as frequency, types of oscillation and the number of cells oscillating. The physiological relevance of Ca²⁺ oscillations has been demonstrated in T-lymphocytes using a unique Ca²⁺ clamp technique. In T-lymphocytes, oscillations reduce the effective Ca²⁺ threshold for activating pro-inflammatory transcription factors such as NF-AT, Oct/OAP and NF-κB (Dolmetsch et al., 1998). Oscillatory frequency regulates the specificity with which the transcription factors are activated. For example, rapid oscillations activate all three-transcription factors whereas infrequent oscillations activate only NF-κB (Dolmetsch et al., 1998). Another study in human aortic endothelial cells demonstrated that Ca²⁺ oscillations induced by histamine receptors activate NF-κB (Hu et al., 1999). The frequency of oscillations induced was inhibited by ~50% by a Ins(1,4,5)P₃ receptor blocker and this change in oscillatory frequency was paralleled by a reduction of NF-κB activation. This suggests that changes in oscillatory frequency may be a mode of regulation of nuclear transcription, providing cells with specific mechanisms to control gene expression on agonist stimulation (Hu et al., 1999).

Muscarinic M₃ receptors and β₂-adrenoceptors are co-expressed in numerous cell types including smooth muscle cells. Potentiation of Ca²⁺ responses may enhance cellular
responses such as secretion or contraction. However, considering that these receptor subtypes are expected to mediate contrasting physiological effects, the relevance of the findings in this study, is difficult to interpret. However, changes in oscillatory frequency described in this study may influence gene expression driven by either muscarinic receptors or β2-adrenoceptors. There is evidence in the literature that β2-adrenoceptor agonists influence changes in the transcription of several genes, for example, eotaxin and cyclin-D1 (Shore & Moore, 2003). In human airway smooth muscle cells, using a reporter gene driven by cAMP response element (CRE), the effect of a range of β2-adrenoceptor agonists on gene expression was characterised (Scott et al., 1999). Many target genes in these cells contain CRE and their expression is likely to be driven by the direct effect of cAMP or PKA stimulating binding of cAMP response element binding protein (CREB) to CRE in target genes (Scott et al., 1999). A time-dependent increase in reporter gene activity was obtained in cells stimulated with β2-adrenoceptor agonists, indicating that difference in cAMP levels can produce changes in gene expression of a reporter construct under the control of CRE. Another study utilised a reporter construct containing the cDNA for human placental secreted alkaline phosphatases (SPAP) under the transcriptional control of CRE, to compare effects of isoprenaline and salmeterol on gene expression in cell lines expressing the human β2-adrenoceptor at varying levels (McDonnell et al., 1998). Both agonists were able to stimulate gene transcription in cells via the activation of CRE controlling the production of SPAP, irrespective of receptor numbers. However, there was a marked contrast between the ability of agonists to mediate cAMP responses and gene transcription. The results obtained suggest that long duration action of salmeterol activates gene transcription to a greater extent than high efficacy, short-acting agonists like isoproterenol (McDonnell et al., 1998). This indicates agonist-specific upregulation of gene transcription. Similarly, crosstalk described in this study may also define the profile of gene transcription responses.

Activation of muscarinic receptors by agonists induces formation of immediate early gene expression (Trejo et al., 1991; Trejo et al., 1992). One prominent member of the immediate early gene family is the proto-oncogene c-fos, which encodes for nuclear proteins (Trejo et al., 1991). The induction of immediate early genes is a primary event that correlates with the initiation of DNA synthesis by growth factors (Trejo et al., 1991; Greenberg & Ziff, 1984). The promoter region of c-fos contains specific
regulatory elements that confer responsiveness to phorbol esters and Ca\(^{2+}\). Induction of c-fos is dependent on intracellular Ca\(^{2+}\) and PKC. More recently, stimulation of muscarinic M\(_3\) receptors endogenously expressed in human T and B-cells, were shown to induce Ca\(^{2+}\) oscillations, which in turn upregulated c-fos expression (Fuji & Kawashima, 2000; Fuji & Kawashima, 2000a). Upregulation of c-fos in these cells is perhaps indicative of the mechanism by which muscarinic receptors contribute to regulation of immune cell function. These examples highlight the ability of agonist stimulation of muscarinic receptors to influence gene transcription.

Crosstalk between differentially coupled receptor subtypes has been demonstrated to increase gene transcription. For example, crosstalk between Ga\(_s\)-coupled parathyroid hormone receptors and Ga\(_q/11\)-coupled purinergic receptors affects expression of c-fos in rat osteoblasts (Buckley et al., 2001). This proto-oncogene is implicated in the regulation of osteoblast functions, including proliferation and differentiation (Buckley et al., 2002; Lee et al., 1994). Similarly, the crosstalk between muscarinic M\(_3\) receptors and \(\beta_2\)-adrenoceptors demonstrated here may influence gene expression. This could be a potential route for investigation for any future study into the functional relevance of crosstalk.

8.3 Future directions

There are several approaches for the continuation of this study, including the dissection of the exact mechanism of crosstalk and examining its physiological relevance. The muscarinic M\(_3\)-\(\beta_2\)-adrenoceptor crosstalk mechanism has been proposed to involve contributions from Epac, cAMP and the intracellular Ca\(^{2+}\) channels. However, the mechanism by which these factors influence crosstalk has not been conclusively proven. The role for Epac in mediating crosstalk could be analysed by transiently transfecting the dominant negative isoform (Kang et al., 2001) in HEK 293 cells and assessing its effect on crosstalk. Using quantitative PCR, expression of Epac1 and Epac2 can be quantified and any differences in expression on noradrenaline stimulation determined. Furthermore, small interference RNA (siRNA) targeted against Epac could be used to knockdown Epac expression and its effect on crosstalk analysed.
A study in human immature megakaryocyte cell line MEG-01, demonstrated a direct correlation between the activation of Rap1 and increase of Ca\(^{2+}\) mediated by cAMP elevating agents (Dekker et al., 2002). The role for Rap1, if any, in this study is unclear. Rap1 may influence crosstalk by its ability to act as a molecular switch for SERCA as demonstrated previously (Lacabaratz-Porret et al., 1998). The difference in the regulation of Ca\(^{2+}\)-ATPases or SERCA by Rap may determine the magnitude of responses to noradrenaline in the presence of muscarinic receptor agonists. Activation of Rap1, following muscarinic receptor agonist stimulation and subsequent noradrenaline stimulation could be determined by Western blotting using antibodies described previously (Lacabaratz-Porret et al., 1998; Schmidt et al., 2001). Any difference in the extent of activation of Rap1 by partial and full muscarinic receptor agonists could be similarly quantified. In addition, the role for Rap in modulating SERCA activity could be examined using the Rap1 inhibitor, Rap1GAPII (Kang et al., 2005; Rangarajan et al., 2003).

The demonstration of crosstalk in primary cells highlights the importance of examining its physiological relevance. Given the literature evidence for upregulation of gene transcription by activation of muscarinic receptors and β-adrenoceptors (Section 8.2), it would be critical to investigate the effect of crosstalk on pro-inflammatory transcription factors and the proto-oncogene c-fos, in primary cells coexpressing these receptor subtypes. Furthermore, recent studies have demonstrated the influence of Epac on transcriptional regulation in Jurkat T-cells and primary T-cells, indicating influence of Epac on gene transcription (Fuld et al., 2005; Gerlo et al., 2006). The effect of crosstalk on gene transcription could be examined by reporter gene assays, as described previously (McDonnell et al., 1998; Scott et al., 1999). Additionally, cDNA microarray analysis could also be used to potentially identify the genes regulated either positively or negatively by crosstalk.

**8.4 Summary and conclusions**

This study set out to explore partial agonism in a variety of cell lines with varying receptor expression and to characterise signalling in these cells. Aspects such as second messenger accumulation, receptor phosphorylation and internalisation were examined.
A good correlation between agonist-coupling efficiency with desensitisation, phosphorylation and internalisation could not be demonstrated for oxotremorine, arecoline or pilocarpine in Buckley-M₃ cells with higher receptor expression. The ability of these agonists to mediate crosstalk was also examined. In HEK 293 cells, the partial agonist of the muscarinic receptor mediated Ca²⁺ responses to noradrenaline of a bigger magnitude than the full agonist methacholine. Data obtained demonstrate that noradrenaline-mediated Ca²⁺ responses are revealed only in the presence of an activated muscarinic receptor and are independent of the presence of extracellular Ca²⁺, but dependent on thapsigargin-sensitive intracellular stores. This crosstalk was shown to be independent of Gα₁ but dependent on Gα₅. Furthermore, there was no evidence for the enhanced activation of PLC or involvement of either PKA or PKC in the regulation of crosstalk. In addition, factors contributing to the mechanism of crosstalk include cAMP and Epac. It is likely that the ability of muscarinic receptor agonists to reveal Ca²⁺ signalling by noradrenaline is by Epac-cAMP signalling complex-mediated sensitisation of the intracellular Ca²⁺ channels. Furthermore, the ability of noradrenaline to reveal Ca²⁺ signalling in the continued presence of muscarinic receptor agonists has been demonstrated in rat tracheal smooth muscle cells. Further studies have the potential to explore aspects including the mechanism of sensitisation of intracellular Ca²⁺ channels by Epac-cAMP signalling complex, relevance of changes in Ca²⁺ oscillations, influence on gene transcription and differences between the ability of partial agonists and full agonists to drive gene transcription.
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