The Activation of Guanine Nucleotide Binding Proteins by Muscarinic Acetylcholine Receptor Subtypes.

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By

Elizabeth Claire Akam, B.Sc.(Hons).

Department of Cell Physiology and Pharmacology
University of Leicester.

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The Activation of Guanine Nucleotide Binding Proteins by Muscarinic Acetylcholine Receptor Subtypes.

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Agonist-stimulation of human recombinant M₁, M₂, M₃ and M₄ receptors, expressed in Chinese hamster ovary cells, was investigated at the level of G protein activation. Functional responses were determined by a number of methods including [³⁵S]-GTPγS binding in membranes using both filtration-based and immunoprecipitation-based procedures; Ins(1,4,5)P₃ accumulation and ⁴⁵Ca²⁺ release from permeabilised cell suspensions; and cAMP accumulation in cell suspensions.

M₂ and M₄ receptors, with equivalent expression levels in this recombinant system, were found only to couple to pertussis toxin-sensitive G proteins with near equal kinetics. Methacholine appeared equipotent when activating the total G protein complement through the M₂ and M₄ receptors, however, it appeared more potent when activating G₁₃₂,α through the M₂ compared to the M₄ muscarinic receptor.

Using equivalent expression levels of M₁ and M₃ receptors both the subtypes were found to couple to both pertussis toxin-sensitive and -insensitive G proteins. CHO-M₁ and -M₃ mediated Ins(1,4,5)P₃ generation after pertussis toxin pre-treatment suggested the functional significance of coupling to multiple G protein classes may be in the stimulation of PLCβ by βγ-subunits derived from G₂-like G proteins. The activation of G₉₁ through the M₁ receptor subtype, after methacholine-stimulation, is faster, greater and more potent than that mediated by the M₃ receptor subtype, suggesting that the intrinsic activity of the M₁ subtype is greater than that of the M₃ subtype.

The ‘partial’ agonist pilocarpine also displayed very different G protein activation profiles after stimulation of M₁, M₂, M₃ and M₄ receptor subtypes, suggesting that agonists acting at different receptor subtypes may be capable of inducing relatively selective coupling of the occupied receptor to available G proteins.

This study therefore concludes that muscarinic receptor subtypes display divergent G protein activation profiles after either ‘full’ or ‘partial’ agonist-stimulation.
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Dedicated to Henry John David Akam.
Publications List

Akam E. C., Challiss R. A. J and Nahorski S. R.
Inverse agonist activity of atropine at human M_2 and M_4 muscarinic acetylcholine receptors revealed by [35S]-GTP\gamma S binding.

Use of [35S]-GTP\gamma S binding to assess type 1α metabotropic glutamate receptor activity in baby hamster kidney hamster cell membranes.

Pharmacological characterisation of type 1α metabotropic glutamate receptor-stimulated [35S]-GTP\gamma S binding.

Akam E. C., Challiss R. A. J and Nahorski S. R.
Methacholine-stimulated [35S]-GTP\gamma S binding and Ca^{2+} modulation in CHO-cells expressing M_1 and M_3 cholinoreceptors.

Akam E. C., Challiss R. A. J and Nahorski S. R.
Analysis of M_1, M_2, M_3 and M_4 muscarinic cholinoreceptor-G protein coupling using [35S]-GTP\gamma S binding and Gα-specific immunoprecipitation.

Akam E. C., Challiss R. A. J and Nahorski S. R.
Differential coupling of recombinant M_1, M_2, M_3 and M_4 muscarinic cholinoreceptors to subsets of guanine nucleotide binding proteins in Chinese hamster ovary cells.
In Preparation.
Chapter One.

Introduction.
1.0. Introduction.

The work contained in this thesis involves the characterisation of guanine nucleotide binding protein coupling to acetylcholine muscarinic receptor subtypes. Therefore, this introduction serves to outline G protein-coupled receptors and intracellular signalling and hopefully places this body of work in a wider context.

1.1. Cell surface receptors.

Cell surface receptors have been characterised according to the mechanism that converts ligand binding events into intracellular signals. Upon interaction with agonists ligand-gated ion channels, such as nicotinic acetylcholine, glutamate and GABA \(_A\) (\(\gamma\)-aminobutyric acid) receptors, alter their conformation to form a pore through which ions may rapidly flow across the plasma membrane. Other receptors possess intrinsic tyrosine kinase activity including those for insulin, platelet derived growth factor (PDGF) and other growth factors. These receptors form oligomeric structures to allow both auto-phosphorylation and substrate-phosphorylation, and elicit relatively slow responses (White and Kahn, 1994). A further class of receptors are coupled to effector molecules via guanine-nucleotide binding proteins (G-proteins). Signal transducing G-proteins occur in two forms, “small G-proteins” which are single polypeptides of approx. 200 amino acids e.g. Ras and Rho (Exton, 1998; Vojtek and Der, 1998; Mackay and Hall, 1998) and heterotrimeric G proteins which play a fundamental role by being coupled functionally to various secondary messenger systems and ion channels (Gilman, 1987). Heterotrimeric G proteins are involved in a plethora of transmembrane signalling pathways, and are found associated with cell surface receptors which share a characteristic topological structure consisting of seven transmembrane domains. Receptors coupled to heterotrimeric G proteins now form the largest family of receptor genes, currently believed to consist of around 1000 members.
1.2. G proteins.

G protein-coupled receptors (GPCRs) exert their various biological actions by interacting with guanine nucleotide binding proteins (G proteins) (Ji et al., 1998). G proteins are heterotrimeric in structure, being composed of α, β and γ subunits (refer Gilman, 1987). The α subunit is responsible for the binding and hydrolysis of guanine nucleotides, whereas the β and γ subunits function as a single heterodimer.

G proteins provide a solution to the compromise that cells must make between responding rapidly and being able to respond to very low concentrations of extracellular stimulus (Taylor, 1990). G proteins exist in two main states, guanosine 5'-diphosphate (GDP) bound (inactive), and guanosine 5'-triphosphate (GTP) bound (active) conformations. The transition between these conformations is catalysed by agonist-occupation of the receptor to which the G-protein is coupled (Figure 1.1). This causes the exchange of GTP for GDP on the α subunit, which then dissociates from the βγ complex and modulates the function of target effector systems. The intrinsic GTPase activity of the α subunit then cleaves the terminal phosphate group and α-GDP and βγ recombine to complete the cycle (Figure 1.1). The activity of the intrinsic GTPase of Gα subunit, and hence the duration of signalling, has been shown to be regulated by GTPase activating proteins (GAPs) such as PLCβ, cGMP-PDE-γ subunit and a new group of signalling proteins termed regulators of G protein signalling (RGS proteins) (For further discussion of the Gα GTPase regulation see below).

The activated receptor has a high affinity for the G protein conformation in which its α and βγ subunits are associated, and the single guanine nucleotide binding site of the α subunit is empty. Thus, activated receptors promote the release of GDP from the α subunit and serve to hold open the guanine nucleotide binding site. They may even increase the α subunit affinity for GTP relative to GDP, but due to the differing physiological concentration of GTP compared to GDP, GTP will bind preferentially even if they have similar affinities for the guanine nucleotide binding site of the α subunit (Taylor, 1990). Although it is the α subunit that undergoes exchange of GTP
The regulatory cycle of heterotrimeric G proteins.

When an activated receptor interacts with the G protein the $\text{G}_\alpha$ subunit affinity for GDP decreases so that GDP comes away from the active site. The higher concentration of GTP in the cell means that GTP replaces the GDP. Once GTP is bound, the $\alpha$ subunit assumes its activated conformation and dissociates from both the $\beta\gamma$ subunits and the receptor. The activated GTP-$\text{G}_\alpha$ lasts until the GTP is hydrolysed by the intrinsic GTPase activity of the $\text{G}_\alpha$. 

![Diagram of the regulatory cycle of heterotrimeric G proteins](image-url)
for GDP, the βγ complex may play an essential role in presenting the α subunit to the receptor. Without the βγ there is little effect of receptors on the α subunit (Florio and Sternweis, 1989). The activated GTP-bound α subunit interacts with particular effector molecules including certain ion channels, phospholipases and adenylyl cyclases to give rise to a change in intracellular concentrations of second messengers e.g. cAMP, inositol polyphosphates and Ca²⁺ (Birnbaumer and Birnbaumer 1995; Neer, 1995a) thus allowing the exchange of information between an extracellular and intracellular environment. The βγ subunits also play an important signalling role and it has now been illustrated, in a variety of studies, that Gβγ can stimulate PLCβ activity (Blank et al., 1992; Blank et al., 1993; Boyer et al., 1992; Camps et al., 1992a; Camps et al., 1992b; Katz et al., 1992; Park et al., 1993). Also AC activity can be regulated both positively and negatively by βγ (Katada et al., 1987; Tang and Gilman, 1991; Federman et al., 1992; Taussig et al., 1993; Taussig et al., 1994). Therefore the interaction between GPCR and heterotrimeric G proteins represents the initial biochemical step in the transmission of information from the extracellular environment to the intracellular response.


The first evidence of a receptor communicating with an effector molecule via a G protein came from Rodbell and his collaborators (Rodbell et al., 1971a,b). The identification of phototransducin (Wheeler and Bilensky, 1977) and the S49 cell mutant “cyc-“ (Bourne et al., 1975) (lacking Gs), which affected GTP-dependent adenylyl cyclase activity (Ross and Gilman, 1977), increased the research interest in this area. This led, by 1980, to the purification of transducin and Gs, which were both crucial in the subsequent cloning and sequencing of further members of the G protein family (Godchaux and Zimmerman, 1979; Kuhn 1980; Bitensky et al., 1981; Stryer et al, 1981). Cloning and sequencing techniques have identified and classified 20, 5 and 11 mammalian genes encoding α, β and γ subunits respectively (Simon et al, 1991; Conklin and Bourne, 1993; Neer, 1995a; Neubig, 1998).
1.2.2. Structure of G proteins.

The G proteins that transmit information from cell surface receptors to intracellular effectors are heterotrimeric. Each G-protein is composed of an α subunit (molecular mass = 39-52kDa) that binds guanine nucleotides, a β subunit (molecular mass = 37kDa) and a γ subunit (molecular mass = 8kDa) that are always tightly associated. Although the βγ complex can be shared by different α subunits to form the heterodimer, it is the identity of the α subunit that is currently used to define individual G proteins (Hepler and Gilman, 1992).

1.2.3. G protein ADP-ribosylating toxins.

The identification of G proteins lead to the subsequent identification of bacterial exotoxins which can covalently modify specific residues of the α subunit and alter the normal functioning of the protein. These toxins rapidly became invaluable tools for G protein identification. Cholera toxin, isolated from cultures of Vibrio cholerae, is able to catalyse the transfer of the ADP-ribose moiety of NAD to a specific arginine residue of Gα. The target arginine residue is located at the C-terminal of the Gα subunit, the exact residue (Arg 187/188 or 201/202) depends on which of the four splice variants of Gα is under investigation. This modification leads to the inhibition of the intrinsic GTPase activity, and the α subunit remains in a permanently active state (Cassel and Selinger, 1977). Pertussis toxin (PTx), isolated from cultures of Bordatella pertussis, catalyses the transfer of the ADP-ribose moiety to a specific cysteine residue four amino acids removed from the C-terminus of Gα/Gγ. In contrast to the ADP-ribosylation of Gα when Gγ-like G proteins are ADP-ribosylated receptor-mediated activation of the G protein is prevented (Katada and Ui, 1981, West et al., 1985).

1.2.4. Mg2+ requirements.

G protein activation cannot occur in the absence of magnesium ions (Mg2+) (Hulme et al., 1983). Mg2+ at concentrations of 5-100 mM stimulates the rate of binding of
guanosine 5-O-(3-thiophosphate) (GTPγS) to both \( G_s \) and \( G_i \) (Hanski et al., 1981; Sternweis et al., 1981; Northup et al., 1982; Bokoch et al., 1984), and this event directly correlated to the rate of dissociation stimulated by \( M_{g}^{2+} \) in \( G_i \) (Higashijima et al., 1987). Ligand activation of GPCRs serves to lower the \( M_{g}^{2+} \) requirement for G protein activation. Under physiological conditions (0.5 mM \( M_{g}^{2+} \)) receptor activation changes the affinity of a regulatory \( M_{g}^{2+} \)-binding site from millimolar to micromolar eliciting its saturation with cytosolic \( M_{g}^{2+} \). This system has been termed the 'Mg\(^{2+}\) switch' (Birnbaumer et al., 1990).

1.2.5. G protein classification.

The classification of G proteins is based upon the identity of the \( G_\alpha \) subunits. \( G_\alpha \) subunits share ~45-80% amino acid similarity and they have been divided into 4 classes on the basis of amino acid sequence comparison: \( G_1\alpha \), \( G_2\alpha \), \( G_3\alpha \) and \( G_12\alpha \) (Table 1.1). Most \( G_\alpha \) subunits are widely expressed with a few exceptions including \( G_0\alpha \) and \( G_{par}\alpha \) which are found in sensory organs and \( G_{15}\alpha \) and \( G_{4}\alpha \) which are predominately expressed in hematopoietic cells and neuronal cells respectively.

1.2.6. Molecular structure of \( G_\alpha \) subunits and their intramolecular associations.

In the recent past the analysis of the structure and function of \( G_\alpha \) subunits has been greatly enhanced by high resolution crystallographic studies (Noel et al., 1993; Lambright et al., 1994; Coleman et al., 1994; Wall et al., 1995; Lambright et al., 1996; Iiri et al., 1998). These studies have defined which residues contact the guanine nucleotide and the key regions which undergo conformational changes as the \( G_\alpha \) subunit goes from the inactive to the active form.

The \( G_\alpha \) subunit consists of two distinct domains. The first, a 'GTPase domain' that consists of five \( \alpha \) helices surrounding a six stranded \( \beta \) sheet. This contains the guanine nucleotide-binding pocket and also serves as the site for interaction with receptors, effectors and \( G_\beta \gamma \). The second is an entirely helical region termed the 'helical
Table 1.1

The G-protein $\alpha$ subunit subfamilies.

Key to table; AC adenylyl cyclase, PLC phospholipase C, $K_{Ca}$ $Ca^{2+}$ activated potassium channel, $K_{ATP}$ ATP-sensitive potassium channel, $K_{ir}$ inward rectifier, CaN N-type voltage operated calcium channel, CaL L-type voltage operated calcium channel, ↑ indicates stimulation and ↓ indicates inhibition (Gilman 1987; Birnbaumer, 1992).

<table>
<thead>
<tr>
<th>Family</th>
<th>Members</th>
<th>Effect</th>
</tr>
</thead>
</table>
| $\alpha_s$ ($G_s$) | $G_s\\alpha_S, G_s\\alpha_L, G_{o(f)\\alpha}$ | -AC ↑  
-K$^+$(K$_{Ca}$) and $Ca^{2+}$ channel (CaL) ↑ (G$_s$$\alpha$) |
| $\alpha_i$ ($G_i$) | $G_i\\alpha, G_{i2}\\alpha, G_{i3}\\alpha, G_{o1}\\alpha, G_{o2}\\alpha, G_{p\\alpha}, G_{i1\\alpha}, G_{i2\\alpha}, G_s\\alpha$ | -cAMP phosphodiesterase ↑ (G$_{p\\alpha}$$\alpha$)  
-cGMP phosphodiesterase ↑ (G$_i$$\alpha$)  
-AC ↓ (G$_i$$\alpha, G_s$$\alpha, G_s$$\alpha$)  
-K$^+$ channel (K$_{ir}$ K$_{ATP}$) ↑ (G$_i$$\alpha$)  
-$Ca^{2+}$ channel (CaL CaN) ↓ (G$_s$$\alpha$)  
-PLC$\beta$ ↑(G$_s$$\alpha$) |
| $\alpha_q$ ($G_q$) | $G_q\\alpha, G_{i1}\\alpha, G_{i4}\\alpha, G_{i3}\\alpha, G_{i6}\\alpha$ | -PLC$\beta$ ↑ |
| $\alpha_{12}$ ($G_{12}$) | $G_{i2}\\alpha, G_{i3}\\alpha$ | Regulate Na$^+$/H$^+$ exchange |
domain', the function of which is not as clearly defined, yet it may play a role in positioning a key residue (Arg178) within the catalytic cleft which is required for GTPase activation (Markby et al., 1993; Conklin and Bourne, 1993). Most Gα subunits contain palmitate linked to the protein on the cysteine residue nearest the amino terminal also in addition to this post-translational modification some Gα subtypes are also acylated at an amino terminal glycine (Wedegaertner et al., 1995). Palmitoylation and myristoylation of Gα occur independently. Myristoylation is an irreversible covalent modification and this attachment appears to be necessary for membrane binding and βγ interaction (Casey, 1994). Palmitoylation however is reversible and it has been suggested that changes in the degree of palmitoylation may lead to variations in Gα membrane affinity and modulation of signalling (Bigay et al., 1994; Grasey et al., 1994), although an alternative hypothesis has been proposed by Hepler et al. (1996) which suggests that the cysteine residues themselves are more important in signal generation.

Gα and Gβγ interactions appear to occur at two distinct interfaces: the first involves both the switch I and switch II regions and β strands 2 and 3 of the Gα subunit, and residues from five of the seven β sheets of the Gβ subunit and is termed the ‘switch interface’. The second interface is formed between the N-terminal of Gα and the side of the β-propeller domain of Gβγ termed the ‘N-terminal interface’ (Wall et al., 1995; Lambright et al., 1996). The high resolution crystal structure of Gα,β and Gβγ (Lambright et al., 1996) shows the switch interface as involving extensive hydrophobic interactions between six hydrophobic residues on Gα and four on Gβγ with this interaction being further stabilised by two salt bridges and a network of hydrogen bonds. The N-terminal interface is less extensive than the switch interface but still involves two salt bridges, several hydrogen bonds, and four hydrophobic interactions between Gα and Gβγ side chains, this interaction serves to determine the location of the N-terminus of Gα as well as stabilising the heterotrimeric complex. The last member of the heterotrimeric complex Gγ is found tightly complexed with the Gβ subunit (see below for discussion of Gβγ dimer). However, as yet no direct interactions have been observed between the Gα and Gγ subunits.
Conformational changes that occur in Go after activation can be inferred by comparisons between the high resolution crystal structures GTP-Go, free GDP-Go and GDP-Go bound forms (Noel et al., 1993; Lambright et al., 1994; Lambright et al., 1996; Iiri et al., 1998). The differences between the free and heterotrimeric forms of Go-GDP appear to be most pronounced for the residues that interact with Gβγ, with the most dramatic changes occurring in the switch II region. The α2 helix is taut and compact in Go-GTP however, in free Go-GDP this helix adopts a loosely configured helix allowing the helix to swing outwards exposing and positioning the residues to interact with Gβ. It appears that the binding of Gβ stabilises the flexible switch I and II regions.

Activated seven transmembrane receptors interact at many sites with the G protein causing structural changes to occur within Go which ultimately cause displacement of GDP. High affinity interaction of the Go subunit with the receptor is dependent upon the presence of the heterotrimer, and this interaction appears to require lipid modifications of Go and Gy (Casey, 1995; Milligan et al., 1995). Critical sites for G protein-receptor interaction are the extreme C-terminus and regions of the α5 helix of Go (Conklin and Bourne, 1993; Bourne, 1997). One of these sites, the extreme C-terminus, also appears to be involved in defining the specificity of the G protein-receptor interaction (Conklin et al., 1993). Onrust et al. (1997) identified other key sites within seven transmembrane receptors for receptor-G protein interaction including the ERY sequence in TM III of rhodopsin (analogous to the conserved DRY sequence found in all 7TM receptors), and the i3 loop connecting TM V and TM VI which was shown to be located close to a prominent cavity between the Go and Gβ subunits. Recently Iiri et al. (1998) have proposed a mechanism of action to explain how a heptahelical receptor induces the conformational changes required in Go for GDP/GTP exchange. This mechanism includes the three major sites for receptor-G protein interaction mentioned above. The study was based around the biochemical phenotype of two pathological states (testotoxicosis and type I pseudohypoparathyroidism) both resulting from a mutation in the β6/α5 loop of Gsα causing its constitutive activation. As mentioned above the C-terminal of Go is important for receptor interaction and perhaps this interacts with the crevice formed
by TMs III, VI and VII separating from one another after receptor activation (Farrens et al., 1996; Lambright et al., 1996; Bourne, 1997). As the C-terminal tail is located at the opposite end of the α5 helix to the β6/α5 loop this provides a system whereby the receptor can act at distance to deform the β6/α5 loop and activate Go. Next the prominent cavity between the Go and Gβ subunits provides an opportunity for activated regions of the receptor to tilt Go and Gβγ away from each other providing an exit site for GDP. However, these events alone are not enough to generate Go-GTP, next the receptor must destabilise the guanine nucleotide binding site to promote release of bound GDP. GTP efficiently replaces GDP because the γ-phosphate stabilises the energetically unfavourable state of Go induced by the receptor and causes Go dissociation from the receptor and Gβγ. This is achieved by axial rotation of helix α2 towards the guanine nucleotide and away from Gβγ, thus stabilising Go-GTP and making key residues for Gβγ interaction unavailable. The final stage in the generation of activated Go is the formation of a salt bridge between helices α2 and α3. This involves a conserved arginine (Arg231) in helix α2 and a conserved glutamate in helix α3. This intramolecular interaction serves to lock together the α2 and α3 helices allowing Go to hold GTP tightly thus, maintaining the activated conformation.

Following activation Go-GTP interacts and regulates the function of a variety of effector molecules. α helices 2, 3, and 4 and the loops connecting them to the subsequent β sheets appear to make a surface for effector interaction. As the effector binding region includes the α2 helix it is unlikely that the Go subunit can bind to the effector protein and the Gβγ subunits simultaneously (Hamm and Gilchrist, 1996). Also the critical rearrangement of helices α2 and α3 during Go activation, means that the conformation of these regions are likely to be dramatically different when involved in effector binding compared to Gβγ binding (Iiri et al., 1998).

1.2.7. Molecular structure of the Gβγ dimer.

Gβγ is a tightly complexed dimeric molecule which only dissociates under denaturing conditions and can be thought of as a functional monomer. Gβγ serves to increase the
affinity of the Go subunit for its receptor and regulate the function of a wide variety of effector molecules (Birnbaumer, 1992). The five mammalian β subunits are between 53% and 90% identical to each other. The Gβ subunit is predicted to contain two types of structure, an amino-terminal region thought to form an amphipathic α helix followed by seven 43 amino acid repeating units (Simon et al. 1991). The repeating units in Gβ are examples of WD repeats, a motif found in a variety of proteins with a potential macromolecular complex forming role (Neer, 1995b). The WD repeats define the overall structure of Gβ by forming a seven spoked β propeller, this together with the α helical region is termed the ‘β superbarrel’. Gγ subunits are the most divergent of the three subunits which form the G protein sharing only 27% to 75% sequence homology. These subunits are small proteins (6-9 kDa) which all undergo post-translational modifications at their carboxyl-termini. Some have been shown to be isoprenylated at a carboxyl-terminal cysteine and if this process is blocked the Gβγ dimer will form but the dimeric complex will not interact correctly either with the lipid membrane or the Go subunit. The structure of Gγ is predicted to be largely α helical, with the selectivity for different Gβ subunits being determined by a stretch of 14 amino acids located centrally (Neer, 1995a). The Gγ subunit is found associated with the amino-terminal region of the Gβ subunit.

1.2.8. Regulators of G protein signalling.

As discussed above a large variety of receptors initiate their intracellular signalling by interacting with heterotrimeric guanine nucleotide binding proteins. The GTPase activation cycle of G proteins consists of two key stages. First, the receptor catalyses the exchange of GTP for GDP, this serves to activate the G protein signalling pathway by promoting the dissociation of the GTP-Go and Gβγ subunits. Second, inactivation occurs by the hydrolysis of the bound GTP to GDP by the α subunit. The intrinsic rate of hydrolysis of GTP by Go is slow (Kcat 1-5 min⁻¹). Hydrolysis of GTP serves to inactivate the signal as the Gβγ subunits have a higher affinity for the GDP-Go species than the GTP-Go form and effectors have much lower affinity for GDP-Go than GTP-Go. So signal termination for both Go and Gβγ occurs through GTP hydrolysis. Regulatory mechanisms exist to limit the duration or sensitivity of G
protein signalling. For example, negative regulation exists at the level of the receptor where phosphorylation by protein kinases and receptor-binding proteins, like arrestins contribute to long term receptor desensitisation (Premont et al., 1995; Gurevich et al., 1995; Ferguson et al 1996). This form of signal regulation is thought to be a common feature of G protein signalling pathways.

Evidence for another form of regulation comes from a number of converging sources. Heterotrimeric G proteins are members of a superfamily of GTPases including Ras and EF-Tu. Ras and EF-Tu are dependent on GTPase activating proteins (GAP) to stimulate their GTPase activities. Although the intrinsic GTPase activity of purified heterotrimeric G proteins is greater than that of Ras or EF-Tu, the levels of activity are not as great as those reported in living cells. This suggests that GAP proteins must also exist for Ga subunits. Effector molecules, PLCβ and γ subunit of cGMP phosphodiesterase, have been shown to act as GTPase activators for Gq11α and Giα respectively (Berstein et al., 1992b; Arshavsky and Bownds, 1992) and these findings explained, in part, the discrepancies between the observed activity rate of Ga subunits in-vitro and in-vivo. The search for Ga GAP proteins then concentrated on other effector molecules, but these did not seem to influence the GTPase activities of Ga proteins. Also Ga GTPase-activating activities had been detected which appeared to be separable from effector proteins (Angleson and Wensel, 1993). The evidence discussed above together with genetic studies of S. cerevisiae, C. elegans and A. nidulans, identifying SST2, EGL-10 and FlbA genes (Koelle, 1997; Dohlman and Thorner, 1997; Iyengar, 1997) revealed a new family of proteins that serve to limit G protein signalling, termed regulators of G protein signalling (RGS proteins). Multiple homologues of SST2 and EGL-10 are present in higher eukaryotes. Using the yeast two-hybrid system a human RGS protein that interacts with G13α was identified and termed GAIP (Ga interacting protein). Regions of protein homology between the RGS family members were assessed and a ~130 amino acid core domain, the RGS domain, was used to defined the RGS superfamily. Additional mammalian RGS family members were identified by a number of complementary methods including screening for rat brain cDNAs that could functionally substitute for SST2 when expressed in yeast, PCR hybridisation and database searching for the RGS domain
and the family currently has 16 members (RGS 1-15 and GAIP) (De Vries et al., 1995; Druey et al., 1996; Koelle and Horvitz, 1996; Siderovski et al., 1996).

RGS family members have now been shown to potently stimulate the rate of GTP hydrolysis (>40 fold) by several Gα proteins (Berman et al., 1996a; Hunt et al., 1996; Watson et al., 1996; Chatterjee et al., 1997; He et al., 1998). Selective binding of the transition-state conformation of Gα, elicited in the presence of both GDP and AlF4−, has now been demonstrated for many members of the RGS family (Berman et al., 1996b; Hunt et al., 1996; Watson et al., 1996). The selective binding of the RGS proteins to the transition state Gα is consistent with the theory that RGS proteins accelerate GTP hydrolysis by preferentially binding to and stabilising G proteins in the transition state for the hydrolysis reaction.

Comparisons of the crystal structures of Ras and Gα has allowed the generation of a single model for the activation of both these GTPases (Markby et al., 1993). Ras-GAP and the helical domain of Gα both introduce an Arg residue into the catalytic cleft that helps to steady the transition state, by stabilising the developing negative charge on the γ-phosphate leaving group. Recently it has been postulated that if Gα has a 'tethered' GAP that interacts with the GTP-binding pocket, then the RGS proteins may stabilise the switch regions which also undergo considerable conformational change during GTP hydrolysis, so that the conformation of RGS-Gα-GTP complex approximates to that of Ras-GAP bound to Ras-GTP (Dohlman and Thorner, 1997). The specificity of RGS proteins remains to be fully evaluated at this point, but initial studies suggest that divergent RGS proteins stimulate the GTPase activity of a number of overlapping Gα subunits. So it appears at present that the regulation of Gα signalling is not a simple one RGS protein-one Gα subunit interaction. Therefore, the specificity of RGS action may be restricted by expression patterns or agonist induction. Alternatively additional regulatory factors or post-translational modifications may be required to impose specificity upon RGS function.

The role of RGS9 in phototransduction is the best characterised example of the importance of RGS proteins in G protein signalling cascades to date. Rapid
termination of the light response in photoreceptors requires other mechanisms in addition to the relatively slow intrinsic GTPase of \( G_\alpha \), which hydrolyses bound GTP and returns the G protein to the inactive state on a time scale of tens of seconds (Dratz et al., 1987; Arshavsky et al., 1989; Arshavsky and Bownds, 1992). RGS9 not only plays a vital role in obtaining the correct kinetics of \( G_\alpha \) GTPase, in conjunction with the \( \gamma \) subunit of cGMP PDE, to correlate with the photoresponse recovery of intact rod cells (Arshavsky and Pugh, 1998; He et al., 1998; Tsang et al., 1998). It has also recently been reported that RGS9 links the cGMP-PDE system and the GC system, indicating dual control over cytoplasmic cGMP concentration which is key to phototransduction (Seno et al., 1998).

1.3. Muscarinic receptors.

Muscarinic acetylcholine receptors belong to the superfamily of G protein-coupled receptors. It was noted in 1914 that two types of response are elicited by acetylcholine, one of which was mimicked by muscarine and one by nicotine (Dale, 1914; Dale and Ewin, 1914). This provided the platform for the classical definition of muscarinic and nicotinic receptors, nicotinic receptors being activated by nicotine and inhibited by curare and muscarinic receptors being activated by muscarine and antagonised by atropine. Nicotinic receptors have a central pore through which sodium and potassium ions pass, evoking rapid responses that are as fast as the channel opening rate (ms). Muscarinic responses are slower and more diverse with the speed of responses on stimulation ranging from hundreds of ms to seconds.

It became apparent in the early 1980s that one muscarinic receptor subtype could not account for muscarinic receptor-mediated actions of acetylcholine. The anti-ulcer drug pirenzepine demonstrated differences in binding affinities of muscarinic receptors from different tissue preparations, suggesting the existence of at least 2 receptor subtypes, \( M_1 \) and \( M_2 \) (Hammer et al., 1980). Discrimination of binding of the antagonist 4-diphenylacetoxy-methyl piperidine methiodide (4-DAMP) to \( "M_1" \) receptors in heart and ileum (Barlow et al, 1976) reinforced the idea of muscarinic receptor subtypes. The production of further selective antagonists lead to the
identification of the M₁, M₂, and M₃ receptor populations from binding studies in rat cerebral cortex, myocardium and salivary gland respectively (Michel and Whiting, 1988; Lazareno and Roberts, 1989). Molecular biological techniques have defined muscarinic receptor subtypes (Kubo et al., 1986; Bonner et al., 1987, 1988; Peralta et al., 1987; reviewed by Hulme et al., 1990). In the defined nomenclature of these subtypes, proteins encoded by m₁ genes appear to correspond with M₁ receptors in neuronal tissue; m₂ gene product to M₂ receptors found in heart and cerebellum; m₃ to M₃ receptors in smooth muscle; m₄ to M₄ receptors in rabbit lung; and the m₅ receptor mRNA has been located in discrete regions of the rat CNS (Vilaro et al., 1990; Caulfield, 1993). Muscarinic receptor distribution has been assessed by both mRNA and protein distribution studies using either northern blotting and immunological detection respectively (see Table 1.2). However, no absolute expression levels have been defined as many contradictions exist between different studies, different methods and between the different mammalian species investigated. One major discrepancy is the high levels of m₃ and m₅ mRNA detected in the brain, whereas immunological studies in the brain do not show a corresponding high level of M₃ and M₅ receptor expression (Wei et al., 1994).

Muscarinic receptors have been assigned to the GPCR superfamily on the basis of membrane topology, possessing 7 transmembrane helices joined by alternating intracellular and extracellular loops (Hulme et al., 1990) (See Figure 1.2 for generalised structural diagram. This diagram shows a degree of separation between TM V and TM VI due to the proposed flexibility in this region, for further detail refer to Section 1.6. Receptor Dimerization). It is believed that the third cytoplasmic loop is critically involved in G protein coupling. Chimeric studies have been used to define the domains involved in muscarinic receptor G protein coupling (Wess et al., 1993; Brann et al., 1995). The muscarinic receptors M₁, M₃, and M₅ preferentially couple to phospholipase C-mediated phosphoinositide hydrolysis, while M₂ and M₄ preferentially couple to the inhibition of adenylyl cyclase (Caulfield, 1993). This difference in second messenger generation between acetylcholine muscarinic receptor subtypes is mediated by a large third intracellular loop (i₃). The i₃ loop displays very little sequence identity among the five different subtypes, except for the membrane
Table 1.2.

Acetylcholine muscarinic receptor distribution.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>M₁</th>
<th>M₂</th>
<th>M₃</th>
<th>M₄</th>
<th>M₅</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total brain</td>
<td>31</td>
<td>32</td>
<td>1.5</td>
<td>19</td>
<td>-</td>
</tr>
<tr>
<td>Hippocampus</td>
<td>47</td>
<td>19</td>
<td>10</td>
<td>19</td>
<td>0.9</td>
</tr>
<tr>
<td>Pons/medulla</td>
<td>5</td>
<td>70</td>
<td>5</td>
<td>6</td>
<td>0.8</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>2</td>
<td>75</td>
<td>5</td>
<td>3</td>
<td>0.6</td>
</tr>
<tr>
<td>Sympathetic ganglia</td>
<td>18</td>
<td>71</td>
<td>0.5</td>
<td>3</td>
<td>-</td>
</tr>
<tr>
<td>Vas deferens</td>
<td>66</td>
<td>30</td>
<td>1.3</td>
<td>1</td>
<td>0.7</td>
</tr>
<tr>
<td>Urinary bladder</td>
<td></td>
<td>86</td>
<td>11</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lung</td>
<td>3-6</td>
<td>30-40, 92</td>
<td>3-4, 3</td>
<td>40-60</td>
<td>0-1</td>
</tr>
<tr>
<td>Ileum</td>
<td>2.5, 18</td>
<td>69, 70</td>
<td>3.5, 3</td>
<td>12</td>
<td>-</td>
</tr>
<tr>
<td>Submaxillary gland</td>
<td>36</td>
<td>12</td>
<td>42</td>
<td>7</td>
<td>1.6</td>
</tr>
<tr>
<td>Heart</td>
<td>0.5</td>
<td>88, 92</td>
<td>1</td>
<td>0.5</td>
<td>-</td>
</tr>
</tbody>
</table>

Proportions of muscarinic receptor subtypes in rat and rabbit tissues (i.e. M₁, M₂, M₃, M₄ and M₅ ≥ 100%). A comparison of immunoreactivity in rabbit peripheral tissues and mRNA levels in the rat central nervous system. Numbers in italics indicate the proportion of the individual receptor subtype in the given tissue extrapolated from the mRNA levels measured. After: Kukkonen J. 1997.
Figure 1.2. Generalised structure of human acetylcholine muscarinic receptors.
proximal regions. This region is one of the most divergent regions between subtypes, in contrast the rest of the secondary sequence shares a high degree of sequence homology (Hulme et al., 1990). Ligand recognition and binding to muscarinic receptors is believed to occur in a pocket formed by the seven TM domains (Hulme et al., 1990; Wess, 1993) and which is consistent with other members of the GPCR superfamily. Mutagenesis studies and molecular modelling in conjunction propose that the agonist binding site is located in a narrow cleft defined by several TM helices about 1.5 nm from the cell surface (Wess 1993; Schwartz, 1994). One conserved aspartate residue located in TM III is believed to be important in ion-ion interactions for the positively charged head group which is present in nearly all muscarinic ligands, and to date this residue has been found in all receptors that bind biogenic amines. This aspartate residue is part of a ammonium-aspartate pair which is encased in a cluster of aromatic residues forming a cation-\pi interaction which is believed to play a role in ligand binding (Wess et al., 1992; Dougherty, 1996). Mutation of this site has been shown to abolish or reduce the efficacy of receptor G protein coupling in many biogenic amine receptors, e.g. dopamine (Mansor et al., 1991).

Functional studies with hybrid m2/m3, m2/m5 muscarinic receptors have illustrated that the first 16-21 amino acids of i3 largely determine G protein coupling (Wess et al., 1989, 1990). The C-terminal of the i3 and the i2 are also important in this process (Wang et al., 1991). Studies of the residues in the i3 loop near TM V show a conservation of amino acids in this region between the odd and the evenly numbered muscarinic receptor subtypes. The use of chimeras formed from this region has shown that the ability to inhibit adenyl cyclase can be conferred to the m3 receptor by exchanging the i3 loop of the native receptor for that of the m2 receptor subtype. Similarly the i3 of the m3 receptor can elicit phosphoinositide hydrolysis via a PTx-insensitive G protein in the m2 receptor. This illustrates the importance of the third intracellular loop of muscarinic receptors for the modulation of intracellular events.

Post-translational modifications such as glycosylation, disulphide bridge formation, palmitoylation and phosphorylation have been suggested to affect the properties of G protein-coupled receptors. N-glycosylation is the addition of sugar residues to the N-
terminal consensus sequence. Removal of these consensus sequences and
deglycosylation has been investigated in recombinant porcine M<sub>2</sub> receptors (Van
Koppen and Nathanson, 1990) and in porcine brain muscarinic receptors (Ohara et al.,
1990). Both of these studies reported no significant differences in receptor expression,
ligand binding or the responses mediated by these deglycosylated receptors compared
to normal glycosylated muscarinic receptors. Nine conserved cysteine residues are
found in the muscarinic receptor family, and they have been mutated to serine
residues in the M<sub>i</sub> receptor to evaluate the importance of such residues (Savarese et
al., 1992). Mutation of Cys98 or Cys178 abolished QNB binding and carbachol-
stimulated IP-hydrolysis and this is probably due to disulphide bridge disruption
(Curtis et al., 1989; Kurtenbach et al., 1989). However, the mutation of the other
cysteine residues either had no effect or altered the affinity and/or the efficacy of
carbachol. Mutation of the C-terminal cysteines, the putative palmitoylation sites
located at Cys435 in the rat M<sub>1</sub> receptor and Cys457 in the porcine M<sub>2</sub> receptor did
not alter receptor binding or function (Van Koppen and Nathanson, 1991; Savarese et
al., 1992). Muscarinic receptors also display consensus sequence sites for receptor
phosphorylation and these sites are located mainly in i3 and the C-terminus.
Muscarinic receptors have been shown to be phosphorylated by several kinases
including PKA, PKC, G protein-receptor kinases and caseine kinase 1α (Wess, 1993;
Tobin et al., 1996). Phosphorylation with G protein-receptor kinases has been
associated with short-term homologous desensitisation, and PKC phosphorylation
with heterologous desensitisation (Wess, 1993).

1.4. GTPγS Binding. General Background.

The activation of G proteins by seven transmembrane G protein-linked receptors can
be measured directly in membrane preparations. The activation of G proteins in
membrane preparations can be assessed on two levels 1) the rate of nucleotide
hydrolysis or 2) GDP/GTP exchange (Gilman, 1987; Conklin and Bourne, 1993). This
can be achieved by measuring the hydrolysis of [γ<sup>32</sup>P]-GTP to GDP + 32Pi or by
measuring the binding of a non-hydrolysable GTP-analogue (Wieland and Jakobs,
1994; Gierschik et al., 1994). The assay outlined by Gierschik et al. (1994) is more
difficult since it requires the separation of radioactive phosphate from labelled GTP by chromatography or by adsorption to charcoal and also this method measures the inactivation of Go subunits. Agonist-stimulated [³⁵S]-GTPγS binding has been more widely used as the separation of free from bound radioactivity is much simpler, using the technique of vacuum filtration, and this methodology measures the activation of Go subunits. Agonist occupation of the receptor causes GDP dissociation from the G protein α subunit and, if found in excess, the Gα will bind the [³⁵S]-GTPγS trapping the activated G protein α subunit. [³⁵S]-GTPγS binding has been widely employed to investigate the action of members of the G protein-coupled receptor superfamily including muscarinic (Lazareno and Birdsall, 1993a, 1993b), adenosine A₁ (Lorenzen et al., 1993) dopamine D₂ (Gardner et al., 1996) μ opioid receptors (Traynor and Nahorski, 1995) and the type 1α metabotropic receptor (Akam et al., 1997). Previous studies have shown that agonist-stimulated binding of [³⁵S]-GTPγS requires the presence of GDP, NaCl and Mg²⁺ (Gierschik et al., 1989; Hilf and Jakobs, 1992; Lorenzen et al., 1993; Pauwels et al., 1997, 1998; Williams et al., 1997; Breivogel et al., 1998). When these conditions are manipulated for a particular receptor system this assay system offers the ability to distinguish between agonist and antagonist and allows a quantitative determination of potency and efficacy of both ‘partial’ and ‘full’ agonists for a given GPCR in a given cellular system (Lazareno et al., 1993; Thomas et al., 1995; Lorenzen et al., 1993; Gardner et al., 1996). Also the relatively new concept of inverse agonism (Lefkowitz et al., 1993; Milligan et al., 1995), where a receptor exhibiting spontaneous/constitutive activity can be inhibited by an antagonist, can be gauged in a quantitative manner using this assay system (Costa and Hertz, 1989; Thomas et al., 1995; Stanton and Beer, 1997; Akam et al., 1996). This assay has been validated as a functional assay for GPCR activation by comparison with other functional studies such as second messenger generation (Lazareno et al., 1993a).

1.5. Constitutive activity and inverse agonism.

Because a guanine nucleotide will bind within milliseconds to the Gα subunit, the complex of agonist, receptor and G protein is normally transitory. When GTP binds to
the Gα subunit the affinity of the receptor for the G protein is decreased, and the two
dissociate resulting in the low affinity state of the receptor. However, guanine
nucleotides can be removed from cell membranes by washing and omitted under
certain experimental conditions leaving the receptor associated with the G protein and
this results in the high affinity state of the receptor. Therefore, the affinity state of the
receptor is affected by guanine nucleotides and hence association with the G protein.
Agonists, but not antagonists, can distinguish between the high and low receptor
affinity states, and the proportion and relative affinity of ligands for these two states
has been observed to vary with the intrinsic activity of agonists. These early
pharmacological observations led to the development of the ternary complex model,
which defined the active form of the receptor with a ternary complex involving
hormone (H), receptor (R) and G protein (G) (DeLean et al., 1980).

However, more recently observations have indicated that the ternary complex model
may be incomplete. A mutant β2-adrenoceptor was identified that shows constitutive
activation of G protein in the absence of agonist (Samama et al., 1993). This study
also illustrated a dramatic increase in the measured affinity for agonist, but not for
antagonist. This leads to a hypothesis that the mutation within the receptor allows it to
adopt an active configuration, possibly by removing a usually constraining
interaction. The transition from inactive to active receptor is believed to be due to an
isomerisation step. The conformational change producing the active receptor is
thought to be agonist driven rather than caused as previously proposed. This is
achieved by the selective binding of the agonist to R* driving the equilibrium,
describing the isomerisation between R and R*, towards R*. Thus the ternary
complex model has been extended to accommodate these findings. In the modified
ternary complex model, the process of receptor activation occurs in two steps: (1)
conversion of inactive to active receptor conformation, and (2) binding of the
activated receptor to G protein. The ligand is thought to influence both steps by
facilitating the conversion to the active receptor conformation, and stabilising the
ternary complex of agonist-bound activated receptor and G protein. Figure 1.3 shows
both the classical and the modified ternary complex models.
The ternary complex model.

A: The classical form of the model, H, hormone, R, Receptor, G, G protein. B: The 'modified' ternary complex model which includes an isomerisation step between the R inactive and R* active receptor conformations which is described by the equilibrium constant J. R and R* interact with H and G in a similar fashion. After DeLean et al., 1980 and Lefkowitz et al., 1993.
In the simplest modified ternary complex model receptors are thought to exist in equilibrium between two states, R (inactive), and R* (active). An agonist must bind with a higher affinity to the active conformation of its receptor (R*). In G protein-linked receptors, the active receptor conformation is the one that binds most tightly to the G protein transition state (G\(\alpha\beta\gamma\) - no nucleotide). In the absence of nucleotides when the transition state is long-lived, a fraction of the receptors bind agonist with high affinity. When nucleotide is added, it binds to the vacant site on the \(\alpha\) subunit and the transition state is lost. With the loss of the transition state, the association between receptor and G protein is weakened and with it the high affinity binding of agonist to receptor.

Antagonists may work by causing the receptor to bind with equal affinity to both transition and non-transition state G proteins so the equilibrium remains unchanged. However, the system is now insensitive to nucleotides. In this hypothesis the antagonist is preventing the ligand binding on the receptor, and thus preventing the agonist acting. For this model the antagonist has equal affinity for the R and R* states of the receptor. Alternatively, certain antagonists may have a higher affinity for the R state, thereby inducing a reversal of the isomerisation step. This moves the equilibrium towards the inactive configuration and uncouples the receptor from the G protein. Evidence for this comes from the findings that antagonist binding to receptors can inhibit basal responses in the absence of agonist. In a study of the 5-HT\(_2\) receptor it has been shown that there are inverse agonists and neutral antagonists, demonstrating with inverse agonists binding preferentially to the uncoupled form of the receptor whilst neutral antagonists had equal affinity for both conformations of the receptor, and agonists had higher affinity for the G protein-coupled form of the receptor (Saunders-Bush et al., 1994; Westphal et al., 1995). In this model the inverse agonist had a greater affinity for the inactive, R state of the receptor. Thus, the addition of guanine nucleotides to washed membranes would, theoretically, increase the affinity of inverse agonists for the receptor. This is the opposite of the effect observed with agonists, which have reduced affinity in the presence of guanine nucleotides. Neutral antagonists show no change in affinity for receptors in the presence and absence of guanine nucleotides.
Therefore, in the generalised two state receptor model, the receptor is in equilibrium between R and R*. Agonists and inverse agonists work by binding to the receptor and shifting the position of the equilibrium. Agonists shift the equilibrium towards R*, neutral antagonists bind with equal affinity to both R and R*, thus maintaining the equilibrium, and inverse agonists bind most tightly to R which shifts the equilibrium towards R.

Because the receptor is in equilibrium between R and R* there will be a certain amount of basal activity of the receptor cascade due to the presence of R*, varying between receptor species due to the varying levels of constraint. It is theoretically possible to overexpress a receptor to such an extent that there is enough R* present to result in maximal activation of an effector in the absence of agonist (Milligan, 1995). This prediction has been supported by the demonstration that an increase in the level of expression of the β2-adrenoceptor in cell lines resulted in elevation of basal cAMP levels (Lefkowitz et al., 1993, Samama et al., 1993). Overexpression of the β2-adrenoceptor in myocardial tissue in transgenic mice elevated basal levels of cAMP and increased cardiac contractility. In the transgenic mice the basal level of cAMP and cardiac contractility was maximal, and the β2-adrenoceptor agonist isoprenaline caused no further increase in cAMP or cardiac contractility (Bond et al., 1995). In most cells the number of available G proteins appears to limit the number of receptors that can form a high affinity complex with an agonist. For example, in fibroblasts, when the number of muscarinic receptors is increased the fraction of receptors that can form a high affinity complex with an agonist is reduced (Mei et al., 1989).

In limiting the number of conformational states, to two, the extended ternary complex model fails to encompass a wide variety of current pharmacological data, therefore, an alternative three state model is gaining credence (Bouaboula et al., 1997; Leff et al., 1997; Leff and Scaramellini, 1998; Gether and Kobilika, 1998; Bouaboula et al., 1999). In this model it is assumed that the receptor is in equilibrium among three conformations; R°, R+ and R-. In the R° state, the receptor is uncoupled form the G protein, whereas both the R+ and R- forms are able to bind the G protein. R+/G
represents the classical active conformation, selected for by agonists, and R'/G is representative of the inactive conformation which now captures the G protein and prevents its participation in further signal generation. The three state model is supported by recent evidence involving the selective removal of G proteins by the CB1 and CB2 receptor subtypes (Bouaboula et al., 1997; Bouaboula et al., 1999), and the upregulation of G,α by long-term action of the CB2 inverse agonist SR 144528 (Bouaboula et al., 1999). The need for a constantly evolving model system to explain receptor activity serves to illustrate how much we have to learn about receptor signalling and the exact nature of drug efficacy (Stephenson, 1956; Schwartz and Ijzerman, 1998).

The third intracellular loop (i3) and the sixth transmembrane domain (TM VI) appear to play an important role in determining the equilibrium between the active and inactive receptor species. This is supported by both the high incidence of constitutively activated receptors generated by introducing mutations in the i3 and TM VI regions of GPCRs (Cottechia et al., 1990; Kjelsberg et al., 1992; Ren et al., 1993; Samama et al., 1993; Parent et al., 1996) and a number of disease states, in which mutations in these key regions of G protein-coupled receptors result in constitutive activity, have been identified (Clapham, 1993; Coughlin, 1994). For example, a single amino acid mutation in the sixth transmembrane domain (Asp 578 to Gly) in the luteinising hormone (LH) receptor results in constitutive generation of cAMP in the testis which ultimately leads to precocious puberty of affected males (Shenker et al., 1993), whilst mutations in the third intracellular loop of the thyrotropin receptor have been identified in subsets of patients with hyperfunctioning thyroid adenomas (Parma et al., 1993). The high incidence of constitutive activating mutations (CAMs) found in the i3 and TM VI regions of GPCRs are supported by studies of the luteinising hormone and follicle-stimulating hormone receptors which show that constraint of TM VI is required for the inactive receptor conformation, however mutations in the i3 can overcome this conformational constraint and give rise to the active receptor conformation by moving TM VI (Kudo et al., 1996). The potential role(s) for the i3 and TM VI in the conformational equilibria of G protein-coupled receptors are discussed further below.
1.6. Receptor dimerization.

Agonist-induced receptor dimerization is critical to the function of several non-GPCR receptor families having single membrane spanning domains such as the receptor tyrosine kinases and growth hormone receptors (Wells, 1996). This dimerization process allows auto-phosphorylation to occur, which subsequently serves to recruit adaptor and effector molecules. There is an increasing body of evidence which suggests that receptor dimerization may play a role in GPCR signalling, and that this event may even need to be integrated into current receptor theory.

Muscarinic acetylcholine receptors have been observed in oligomeric structures when evaluated by photoaffinity labelling (azido-4NMPB) followed by SDS-PAGE (Avissar et al., 1983). This study illustrated that different tissues display differential levels of monomeric and dimeric receptor species, with the cortex and hippocampus only showing a single low molecular weight species and the medulla pons, cerebellum and cardiac atrium showing both high and low molecular weight species. These observations allowed the authors to go on to demonstrate multimeric changes in the presence of guanine nucleotides and transition elements. In the rat medulla pons interconversion from a high-affinity to a low-affinity state induced by the addition of guanine nucleotides corresponded to a shift to the monomeric receptor species. In contrast, the application of transition metals to cortical membranes, to shift the receptors to the high-affinity state, caused the appearance of the dimeric receptor structure in this tissue.

More evidence for in-vivo intramolecular interactions within muscarinic receptors came more recently from studies on muscarinic M₂ receptors purified from porcine atria which appeared to contain, in addition to the monomer, trimeric and tetrameric homoligomers (Wreggett and Wells, 1995). Further evidence for muscarinic receptor associations came from co-expression studies of gene fragments containing TMD I-V and TMD VI-VII of M₂ and M₃ receptors in COS-7 cells. Co-expression of the truncated receptor portions produced functional receptors displaying the ligand binding properties of the wild type receptor (Maggio et al., 1993a). Further co-
transfection studies using chimeric $\alpha_{2C}$-adrenoceptor and $M_3$, muscarinic receptors also demonstrated that functional intramolecular interactions can occur between GPCRs (Maggio et al., 1993b), and this led to the proposal of a functional role for the third intracellular loop in intramolecular associations. Later this idea was supported by using cDNA constructs for $\alpha_{2C}/M_3$ and $M_3/\alpha_{2C}$ chimeras with shortened i3 loops, where it appeared that the length of the i3 was critical in the regulation of receptor dimerization (Maggio et al., 1996).

This body of evidence for dimerization in muscarinic receptors is strongly supported by many studies of the $\beta_2$-adrenoceptor. Similar to those outlined above, the first evidence for receptor dimerization came from observed associations between 'split' $\beta_2$-adrenergic receptors co-expressed in Xenopus oocytes (Kobilka et al., 1988). More recently, Hebert et al. (1996) showed that $\beta_2$-adrenoceptors form homodimers in transfected Sf9, LTK' and CHW cells. Multimeric complexes have often been suggested to result from non-specific aggregation, however, in this study a membrane permeant crosslinking agent increased the proportion of the dimeric species visualised after electrophoresis. This indicates that $\beta_2$-adrenoceptor homodimers are pre-formed in the intact cell and that they are resistant to SDS and reducing agents. Glycophorin A forms SDS-resistant oligomers and this is mediated by a dimerization motif and subsequent sequence comparisons between glycophorin A and the $\beta_2$-adrenoceptor revealed the existence of a similar dimerization motif in TM VI of the $\beta_2$-adrenoceptor (Hebert et al., 1996). A synthetic peptide derived from this region decreased the appearance of the dimer and the capacity of the agonist isoprenaline to stimulate adenylyl cyclase, however pre-treatment with isoprenaline could protect against the effects of the TM VI peptide. A final noteworthy result from this study is that the inverse agonist timolol decreased the appearance of the dimer presumably by stabilising the monomeric form. As discussed above the $\beta_2$-adrenoceptor has been mutated to generate a constitutively active receptor species (Samama et al., 1993; Pei et al., 1994). Further studies of this mutant illustrate that constitutive activity in the $\beta_2$-adrenoceptor appears to alter the receptor conformation (Gether et al., 1997), especially the position of the sixth transmembrane domain (Javitch et al., 1997). The
conformational change in TM VI associated with β₂-adrenoceptor activation may be playing a role in the dimerization process.

Similar results illustrating the importance of transmembrane VI in dimerization have been reported for the dopamine D₂ receptor (Ng et al., 1996). As well as the muscarinic receptor family and the β₂-adrenoceptor higher, molecular weight species have been observed for the D₁, D₂ and D₃ dopamine receptor family members (Ng et al., 1994a; Ng et al., 1994b; Nimchinsky et al., 1997), the substance P receptor (Schreurs et al., 1995) and the mGluR1 receptor (Pickering et al., 1993) and these appear to be comprised of multiples of a monomeric species.

The increasing evidence of oligomeric GPCRs together with the high incidence of constitutively activating mutations reported in the i3 and TM VI regions of GPCRs and the potential role of these regions in dimerization, leads to the idea that perhaps a dynamic equilibrium between monomeric and dimeric receptor species could be an integral part of the extended ternary complex model. The consequence of GPCR receptor dimerization still remains to be fully resolved, however now this area is becoming increasingly recognised as significant (Gether and Kobilka, 1998; Milligan, 1998)

1.7. Adenylyl Cyclase isoforms.

Adenylyl cyclase (AC) hydrolyses ATP to generate PP₁ and cAMP; cAMP can then be rapidly hydrolysed by cyclic nucleotide phosphodiesterases (PDEs) to give rise to 5'-AMP. The Gₛ-class of G proteins have been shown to stimulate adenylyl cyclase with the Gᵣ-class of G proteins serving to inhibit adenylyl cyclase. Ten isoforms of adenylyl cyclase have been reported to date (I-X), of which eight have been fully characterised, and it has been shown that all eight are stimulated by Gₐα and by diterpine forskolin.

Examination of the amino acid sequences of the 10 mammalian adenylyl cyclase family members reveals 12 hydrophobic regions in conserved positions which are
presumed to represent transmembrane regions. These regions are arranged in two sets of six contiguous membrane-spanning domains separated by a large intracellular hydrophilic sequence. The proposed structure possesses a short variable amino terminus (N), followed by the first six transmembrane spans (M₁), a large intracellular domain containing 360-390 amino acids (C₁a and C₁b), the second set of six transmembrane spans (M₂) and a second large intracellular region containing 225-330 amino acids (C₂a and C₂b). Sequence comparisons of the adenylyl cyclase family members reveals that the overall similarity is approximately 60%, with the most highly conserved sequences located in the C₁a and C₂a domains (Sunahara et al., 1996). This proposed structure for mammalian adenylyl cyclases are similar to those of a number of ion channels and other pore forming molecules, including the P-glycoproteins and the cystic fibrosis transmembrane conductance regulator (Krupinski et al., 1989). Other structural similarities have been noted between mammalian adenylyl cyclases and guanylyl cyclases. Homodimeric membrane-bound and heterodimeric soluble guanylyl cyclases contain sequences homologous to the C₁a and C₂a domains found in adenylyl cyclases (Chinkers and Garbers, 1991). These regions are known to be the catalytic domains of receptor guanylyl cyclases and therefore the C₁a and/or the C₂a domains are presumed to represent the catalytic site(s) of adenylyl cyclase.

Gβγ subunits have also been shown to influence the activity of adenylyl cyclase isoforms. Types II and IV are stimulated by βγ subunits with the stimulatory effect being dependent upon coincident activation by Gα. Type I however, is inhibited by the action of Gβγ, whilst the other adenylyl cyclase isoforms do not appear to be influenced by this signalling molecule. The concentrations of Gβγ required to effect changes in adenylyl cyclase activity (5-100 nM) are much greater then those required for Gα subunits. As well as adenylyl cyclase activity being mediated by GTP-Gα and Gβγ,Ca²⁺-calmodulin can also affect changes in the level of adenylyl cyclase activity. Types I, III and VIII can be stimulated by Ca²⁺-calmodulin and types V and VI are inhibited by micromolar Ca²⁺ concentrations independent of the presence of calmodulin (Tang and Gilman, 1992; Birnbaumer and Birnbaumer, 1995; Cooper et al., 1995; Sunahara et al., 1996). Changes in adenylyl cyclase activity serves to alter
the intracellular cAMP concentration, which mediates the activity of protein kinase A (PKA). cAMP binds to the regulatory subunits of PKA and releases the active catalytic subunits. Although feedback inhibition is commonly used within signal transduction cascades only the activities of AC V and AC VI have been seen to be regulated PKA-mediated phosphorylation.

Distinct expression patterns exist for mammalian adenylyl cyclases and this together with the overlapping patterns of regulation suggests that adenylyl cyclases have evolved to permit extensive signal integration and cross-talk between distinct signalling cascades.

1.8. Phosphoinositide specific phospholipase C isoforms.

Many extracellular signalling molecules including hormones, neurotransmitters and growth factors bind to their cell surface receptors and elicit intracellular signalling cascades by the activation of a specific phospholipase C. The activated PLC serves to hydrolyse a minor membrane phospholipid, phosphatidylinositol 4,5-bisphosphate (PIP\(_2\)), and this reaction generates two distinct signalling molecules diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (Ins(1,4,5)P\(_3\)). DAG is the physiological activator of protein kinase C (PKC) and Ins(1,4,5)P\(_3\) mediates Ca\(^{2+}\) release from intracellular stores. This bifurcating pathway is known to have an important role in many cellular processes for example smooth muscle contraction and fertilisation, consequently the activity of PLC is strongly regulated in cells by numerous distinct mechanisms linking PLC isoforms to various receptors.

10 mammalian PLC isoforms have currently been identified, and these are all reported as single polypeptides. These can be divided into three types \(\beta\), \(\gamma\) and \(\delta\) on the basis of sequence homology, with the \(\delta\) isoforms being significantly smaller (M, 85,000) than the PLC-\(\beta\) and PLC-\(\gamma\) isoforms (M, 140,000-155,000). Overall sequence homology is low but two regions X (approx. 70 amino acids) and Y (approx. 240 amino acids) thought to constitute the PLC catalytic domain display high sequence homology (displaying ~60% homology and ~40% homology respectively). Another feature that
all the PLC isoforms have in common is a N-terminus of approximately 300 amino acid residues, and within this region the three types of PLC possess a pleckstrin homology (PH) domain which precedes the X domain. The catalytic activities of the three PLC isoforms are dependent upon the presence of Ca\(^{2+}\) and the C-terminal region of the Y domain is proposed as a site of Ca\(^{2+}\) binding on the basis that this region is homologous to the amino acid sequence of the Ca\(^{2+}\) binding domains of PKC and cytosolic phospholipase A\(_2\) (Clark et al., 1991). A region of variation among the three types of PLC is the sequence that separates the X and Y regions, in the \(\beta\) and \(\delta\) isoforms this is a short amino acid sequence (50-70 residues), whereas the \(\gamma\) isoforms have a long sequence of \(~400\) amino acids encoding two SH2 (Src homology) and one SH3 domains. Also within this region PLC-\(\gamma\) isoforms contain an additional PH domain that is split by the Src homology domains.

1.8.1. PLC-\(\beta\).

The G\(\alpha\) subunits of the four members of the G\(_q\) class of heterotrimeric G proteins can activate PLC-\(\beta\) (Sternweis and Smrcka, 1992). There are many receptors which activate the G\(_q\)\(\alpha\)-PLC-\(\beta\) pathway including the muscarinic acetylcholine receptors subtypes M\(_1\), M\(_3\) and M\(_5\). The GTP\(\gamma\)S-activated G\(_q\)\(\alpha\) or G\(_{11}\)\(\alpha\) subunits stimulate PLC-\(\beta\) isoforms with the rank order of potency PLC\(\beta\)1 > PLC\(\beta\)3 > PLC\(\beta\)2 (Noh et al., 1995; Lee and Rhee, 1995). The members of the G\(_q\) class of G proteins are palmitoylated at the N-terminus, but it has been shown that the state of palmitoylation is unimportant in the activation of PLC\(\beta\). However, the cysteine residues at the N-terminus, the sites of palmitoylation, confer a hydrophobic character to the G\(\alpha\) protein which appears to play an important role in the activation of PLC\(\beta\) (Hepler et al., 1996).

Coupling of the M\(_1\) receptors to the activation of PLC-\(\beta\) has been studied in detail by reconstituting the M\(_1\) receptor, PLC-\(\beta\) and G\(_{q11}\)\(\alpha\) in phospholipid vesicles (Blank et al., 1991; Berstein et al., 1992a; Nakamura et al., 1995; Biddlecome et al., 1996). Upon addition of the muscarinic agonist carbachol the activity of PLC-\(\beta\) is strongly stimulated (up to 90 fold), and this effect can be mediated by all members of the G\(_q\)\(\alpha\) class. PLC-\(\beta\) has been shown to act as a GTPase activating protein (GAP) for the
members of the $G_q\alpha$ class of G proteins (Berstein et al., 1992b; Biddlecome et al., 1996), stimulating the GTPase of $G_q\alpha$ by >50 fold in the reconstituted system. The results from these studies indicate that the receptor, $G_q\alpha$, and PLC-β1 in the presence of agonist serve to regulate the amplitude and duration of the PLC signal. The different isoforms of PLCβ have differing sensitivities to $G_{\beta\gamma}$ compared with activated $G_{\gamma1}\alpha$ subunits, with $G_{\beta\gamma}$ sensitivity being observed to be PLCβ3 > PLCβ2 > PLCβ1 (Rhee and Bae, 1997). The ability of $G_{\beta\gamma}$ to activate PLC-β has now been demonstrated for a variety of receptors. The concentrations of $G_{\beta\gamma}$ necessary for activation are much larger than those required for $G_\alpha$ subunit-mediated activation of PLC-β, but despite this difference, the maximal activation mediated by both of these signalling molecules is similar. The region of the PLC-β that interacts with $G_{\beta\gamma}$ is distinct from the C-terminal region downstream of the Y domain which is the site of $G_q\alpha$ interaction. The site of $G_{\beta\gamma}$ interaction with PLC-β2 has been localised to the region Glu435 to Val641, near the X and Y domains (Kuang et al., 1996). So from this structural information it would appear that $G_q\alpha$ and $G_{\beta\gamma}$ may independently modulate a single PLC-β.

1.8.2. PLC-γ.

The PLC-γ family is comprised of two members $\gamma1$ and $\gamma2$. Growth factor receptors cause the hydrolysis of PIP$_2$ by the activation of PLC-γ. Growth factor receptor binding results in the activation of the intrinsic protein tyrosine kinase activity of the receptor and this phosphorylates critical tyrosine residues within the receptor itself and numerous other proteins including PLC-γ. Autophosphorylation creates high affinity binding sites for many SH2 domain-containing proteins including PLC-γ. The receptor-PLC association is mediated by a high affinity interaction between one of the PLC-γ SH2 domains and a specific tyrosine autophosphorylated site. Mutation of this tyrosine residue in PDGF, EGF and NGF receptors prevents PLC-γ association and subsequent Ins(1,4,5)P$_3$ generation (Noh et al., 1995). The major sites for PLC-γ1 phosphorylation by all growth factor receptors appear to be identical (Tyr771, Tyr783, Tyr1254) (Rhee and Bae, 1997). After this initial interaction between PLC-γ and the growth factor receptor the now tyrosine phosphorylated PLC-γ1 appears to associate
with the cytoskeleton. The purpose of this cytoskeletal association is unknown but it may serve to bring the enzyme into contact with its substrate and/or other regulatory proteins.

Protein tyrosine kinase-independent activation of PLC-γ isoforms can be mediated by several lipid-derived second messengers. Arachidonic acid (AA) stimulates PLC-γ in the presence of tau or tau-like proteins, and phosphatidic acid (PA) has also been shown to be an allosteric modulator of PLC-γ (Jones and Carpenter, 1993; Hwang et al., 1996). Therefore, receptors which activate PLD or cPLA₂ may influence PLC-γ indirectly.

1.8.3. PLC-δ.

PLC-δ appears to be the evolutionary precursor for this family of enzymes. Lower eukaryotes such as yeast and slime moulds contain only δ-type PLC isoforms and this suggests that PLC-β and PLC-γ evolved from the δ-type PLC. Four distinct PLC-δ isoforms have been identified (δ1-δ4). The exact mechanism by which these PLC isoforms are activated by membrane receptors is still uncertain, but recent evidence suggests that a novel G protein G₉ may be involved. The G₉α subunit (75-80 kDa) appears to be a multifunctional protein as in addition to guanine nucleotide binding it also possesses transglutaminase activity (Nakaoka et al., 1994). This novel G protein has been shown to associate with agonist bound α₁-adrenoceptors and the G₉α subunit activates purified PLC-δ1 and forms a complex with PLC-δ1 in cells after α₁-adrenergic receptor stimulation (Feng et al., 1996). These findings appear to suggest that G₉α couples α₁-adrenoceptors to PLC-δ1, yet further studies are required to identify other interactions between GPCRs, G₉α and all the PLC-δ isoforms. The activation of PLC-δ isoforms may alternatively be via receptor-mediated activation of other PLC isozymes or mechanisms which elevate [Ca²⁺]ᵢ as the PLC-δ isozymes are the most sensitive to Ca²⁺.
1.9. Inositol phosphate metabolites and their role in cell signalling.

In the early 1980s it was established that neurotransmitter-stimulated calcium release correlated well with enhanced hydrolysis of the membrane phospholipid phosphatidylinositol 4,5-bisphosphate (PIP$_2$) (Michell, 1982), and a year later it was shown that inositol 1,4,5-trisphosphate triggered the rapid release of calcium in permeabilised pancreatic cells (Streb et al., 1983). Now it has been well established that many receptors activate PLC isoforms which serve to hydrolyse the minor membrane phospholipid PIP$_2$ to give rise to sn 1,2-diacylglycerol (DAG) and D-myoinositol 1,4,5-trisphosphate (Ins(1,4,5)P$_3$), and that both DAG and Ins(1,4,5)P$_3$ act as second messenger molecules (Berridge, 1993). DAG is the established physiological regulator of protein kinase C (PKC) which can phosphorylate and therefore modulate the activities of a number of key cellular proteins (see discussion below). Ins(1,4,5)P$_3$ plays a role in the liberation of calcium from intracellular stores which in turn affects a diverse array of cellular processes. DAG is metabolised by DAG kinase which serves to terminate PKC activation. Ins(1,4,5)P$_3$ metabolism regulates three important aspects of cell signalling; 1) its rapid metabolism by two key enzymes (5-phosphatase and 3-kinase) regulates the intracellular level of Ins(1,4,5)P$_3$ and hence the cellular response to receptor-stimulation; 2) Ins(1,4,5)P$_3$ metabolism is an integral part of inositol recycling which is essential for the synthesis and maintenance of the inositol phospholipids; 3) The action of the 3-kinase results in the generation of inositol 1,3,4,5-tetrakisphosphate which may play an important signalling role itself (Irvine, 1991). So as the metabolism of Ins(1,4,5)P$_3$ plays an important role in intracellular signalling the control of 5-phosphatase and 3-kinase also represent potential sites for signal regulation. The 3-kinase is dependent upon the Ca$^{2+}$ concentration in the cytosol and can be phosphorylated by PKC and Ca$^{2+}$-CaM-dependent protein kinase II suggesting that this Ins(1,4,5)P$_3$ metabolising enzyme is modulated by feedback mechanisms.
1.9.1. Inositol 1,4,5-trisphosphate and calcium signalling.

Ins(1,4,5)P$_3$ is a second messenger that controls a number of cellular processes by modulating internal calcium signals. This is achieved by Ins(1,4,5)P$_3$ binding to the Ins(1,4,5)P$_3$ receptor that is located in particular regions of the endoplasmic reticulum (ER). The Ins(1,4,5)P$_3$ receptor is a heterotetramer composed of 4 ~310 kDa subunits which combine to form a relatively nonselective cationic pore (Mikoshiba, 1993). Anchoring the Ins(1,4,5)P$_3$ receptor to the ER is mediated by typical membrane-spanning domains located within the C-terminal region of the receptor. The Ins(1,4,5)P$_3$, ATP and Ca$^{2+}$ binding sites are all located in the large cytoplasmic N-terminal region of the receptor and serve to integrate the signals which mediate channel opening. Regulation of the tetrameric Ins(1,4,5)P$_3$ receptor is highly complex in that it can bind multiple Ins(1,4,5)P$_3$ molecules (the matter of cooperativity is still unresolved) and displays biphasic sensitivity to both Ins(1,4,5)P$_3$ and cytoplasmic Ca$^{2+}$. Therefore, at low and high Ca$^{2+}$ concentrations the Ins(1,4,5)P$_3$ receptor is relatively insensitive to Ins(1,4,5)P$_3$ and the sensitivity of the receptor to Ins(1,4,5)P$_3$ is greatest in the physiological range between 0.1 μM and 1.0 μM (Clapham, 1995). The role of Ins(1,4,5)P$_3$ in Ca$^{2+}$ release is now thought to be to prime the cell for calcium oscillations by sensitising the Ins(1,4,5)P$_3$ receptor rather than acting completely independently to elicit release (Berridge, 1997a). It now appears that it is the dual regulation of the Ins(1,4,5)P$_3$ receptor by Ins(1,4,5)P$_3$ and Ca$^{2+}$ that provides the basis for Ca$^{2+}$ signalling in a range of cell types. The Ca$^{2+}$ sensitivity of the Ins(1,4,5)P$_3$ receptor means that the release of Ca$^{2+}$ from one receptor excites neighbouring receptors thereby propagating the release of Ca$^{2+}$ throughout the cytoplasm. This regulation of the Ins(1,4,5)P$_3$ receptor by Ca$^{2+}$ enables the process of calcium-induced calcium release (CICR) to occur, which accounts for the ability of the receptors to propagate calcium waves or calcium spikes (Berridge, 1993; Berridge, 1997a). With the understanding that Ins(1,4,5)P$_3$ receptors are activated through the process of CICR, the phenomenon of quantal calcium release (Bootman, 1994), in which low concentrations serve to release fractions of the calcium pool seems hard to explain as the Ca$^{2+}$ signals should be "all-or-nothing". Yet recent evidence obtained at the subcellular level serves to explain this apparent dichotomy. The solution proposed
to address the paradox between CICR and quantal calcium release suggests that the calcium signalling system is composed of independent elementary events and that these events are composed from fundamental events termed ‘blips’ (Bootman et al., 1997). Elementary events, termed ‘puffs’, are likely to reflect localised groups of channels which are linked together as functional units during the development of typical (global) calcium signals. In a variety of cells it has been shown that the elementary events are characterised by a rapid rise in localised $\text{Ca}^{2+}$ followed by a slower recovery period as the $\text{Ca}^{2+}$ diffuses away. The biphasic regulation of the Ins(1,4,5)P$_3$ receptor by $\text{Ca}^{2+}$ means that these elementary events will be strongly influenced by both the positive and negative feedback effects of $\text{Ca}^{2+}$. Thus, the negative effects of $\text{Ca}^{2+}$ will limit the amount of $\text{Ca}^{2+}$ released during each elementary event. In turn this will determine the amplitude of the global calcium signal, as these elementary events combine to produce the well characterised spatiotemporal patterns of calcium signalling. The existence of autonomous elementary events in signalling, providing highly localised pulses of $\text{Ca}^{2+}$, means that this ubiquitous cation can regulate physiological processes such as exocytosis and ion channel activation or they can contribute to a global elevation of $\text{Ca}^{2+}$ to activate more distant effector systems.

The global release of ionised calcium ($\text{Ca}^{2+}$) from the ER serves to increase the intracellular [Ca$^{2+}$] from $\sim$100 nM to $\sim$1 μM. This transient increase in ionised calcium release is involved in a range of cellular processes including fertilisation, cell growth, transformation, secretion, smooth muscle contraction, sensory perception and neuronal signalling. How $\text{Ca}^{2+}$ regulates such diverse processes appears to be related to both the frequency and amplitude of the calcium signal generated (Berridge, 1997a; Berridge, 1997b).

### 1.10. The Protein Kinase C superfamily.

After discussing the role of Ins(1,4,5)P$_3$ in the cell it seems important not to overlook the role of the other signalling molecule generated after the hydrolysis of PIP$_2$, diacylglycerol (DAG). In addition to the rapid generation of DAG by PLC, PLD activation can also produce DAG albeit more slowly, thus, multiple receptor pathways
ultimately generate DAG. DAG serves to activate the members of the protein kinase C (PKC) superfamily and these kinases typically phosphorylate serine or threonine residues located in basic sequence regions of a diverse array of substrates (Newton, 1995). Genetic analysis has defined a superfamily of mammalian PKC isozymes that currently arises from twelve distinct genes (Mellor and Parker, 1998). The PKC family is a heterogeneous family of single polypeptide phospholipid-dependent kinases which can be divided into four smaller subfamilies based upon their enzymic properties, structure and cofactor regulation. In addition to phosphatidylinerine (PS) the conventional PKCs (cPKCs), α, β, γ also require Ca\(^{2+}\) and diacylglycerol as cofactors. DAG serves to increase the specificity of the enzyme for PS and also shifts the affinity for Ca\(^{2+}\) into the physiological range. The ε, η, δ and θ PKC isoforms are grouped together and termed the novel PKCs; these kinases require DAG in the presence of PS for activity. However, in contrast to the conventional PKCs their activation appears to be Ca\(^{2+}\)-insensitive. The third category, consisting of the τ and ζ isoforms are termed the atypical PKCs. Atypical PKCs are Ca\(^{2+}\) insensitive, like the novel PKCs, but in contrast to this subfamily they do not require DAG for maximal activity (Newton, 1995; Jaken, 1996; Mellor and Parker, 1998). A fourth and final group consists of the recently discovered PKC related kinases (PRK), these enzymes appear to be activated by RhoA (Mellor and Parker, 1998). A multiplicity of functions have been ascribed to PKC due to the wide range of substrates this kinase can phosphorylate in vitro after phorbol ester stimulation and the high incidence of PKC consensus phosphorylation sites in cellular proteins. However, the exact functions of different PKC isoforms remains to be elucidated but a key regulators are likely to be the subcellular localisation of both enzyme and substrate and the unique patterns of activation.

1.11. Thesis aims.

The aims of this study were to investigate the activation of specific G proteins produced by stimulation of recombinant muscarinic receptor subtypes expressed as homogeneous populations in Chinese hamster ovary (CHO) cells. By profiling agonist-stimulated G protein activation mediated by single muscarinic receptor
subtypes expressed in the same cellular background direct comparisons between different receptor subtypes could be made. This may lead to an understanding of differences in the response elicited by the different muscarinic receptor subtypes and the magnitude of such responses.

In this study the activation of G proteins was measured by two direct methods both involving $[^{35}\text{S}]$-GTPγS binding in membranes and then using either filtration-based or immunoprecipitation-based procedures to gauge the final activity levels. Functional assays such as Ins(1,4,5)P$_3$ accumulation, $^{45}\text{Ca}^{2+}$ release from permeabilised cell suspensions; and cAMP accumulation in cell suspensions were used to infer the role of G proteins in more distal signalling events.

The M$_1$ and M$_3$ subtypes are traditionally believed to couple to G$_{q/11}$, and M$_2$ and M$_4$ subtypes are traditionally defined as activators of G$_{i}$-like G proteins (Caulfield, 1993). However, many studies have either shown or implied that muscarinic acetylcholine receptors are capable of interacting with a wider range of G proteins. For example, the M$_1$ and M$_3$ receptor subtypes have been shown to interact with both pertussis toxin-sensitive and -insensitive G proteins (Lazareno et al., 1993a; Offermans et al., 1994a; Burford and Nahorski, 1996). Also M$_2$ and M$_4$ muscarinic receptor stimulation has been shown to mediate phosphoinositide hydrolysis, detected as total inositol phosphate accumulation (Ashkenazi et al., 1987, 1989; Peralta et al., 1988; Caulfield, 1993) and the M$_4$ receptor subtype has been shown to mediate a rise in intracellular cAMP (Jones et al., 1991; Dittman et al., 1994; Migeon and Nathanson, 1994). The differences observed in the G protein-coupling of muscarinic acetylcholine receptor subtypes may be due to the cell type or the receptor expression level in the clonal cell lines utilised. Therefore, the major aim of this study was to clarify differences in the activation of G proteins after receptor-stimulation by using the M$_1$, M$_2$, M$_3$, and M$_4$ receptor subtypes expressed in a common cellular background and by matching the receptor expression levels for the M$_1$/M$_3$ and the M$_2$/M$_4$ receptor subtypes. With these parameters matched another aim of this study was to generate complementary experimental approaches to enable clear and logical conclusions to be drawn. Thus, the experimental evidence obtained from membrane filtration-based $[^{35}\text{S}]$-GTPγS
binding was to be assessed at the level of second messenger generation and specific
Gα-[35S]-GTPγS binding to develop detailed information on the initial stages of the
muscarinic acetylcholine receptor signalling.
Chapter Two.

Experimental Methodology.
2.1. Cell culture techniques.

Chinese hamster ovary cells (CHO-K1) transfected with cDNA encoding human m1, m2, m3 or m4 muscarinic receptors (CHO-M1, CHO-M2, CHO-M3, CHO-M4, respectively) were obtained from Dr N. Buckley (National Institute for Medical Research, Mill Hill, London). Further cells transfected with cDNA encoding human m2 muscarinic receptors (CHO-SLM2), at a relatively higher expression level were obtained from Dr S. Lazareno (National Institute for Medical Research, Mill Hill, London). CHO cell clones were grown in αMEM (Minimum Essential Medium) supplemented with 10% newborn calf serum, 100 IU/ml of penicillin, 100μg/ml of streptomycin and 2.5μg/ml of fungizone (amphotericin B). Cells were maintained at 37°C in 5% CO₂ humidified air. Cells were routinely split 1 in 10, three times weekly and they were not utilised beyond passage 40.

If pre-treatment with pertussis toxin (PTx) was required, PTx was added to the tissue culture medium at a final concentration of 100 ng ml⁻¹ (unless otherwise stated) for 20-24 hours prior to harvesting and subsequent experimentation.

2.2. Preparation of cell membranes.

The CHO-transfects were grown to confluency and then washed with 10ml HBS-EDTA (10mM HEPES, 0.9% NaCl, 0.2% EDTA, pH 7.4). The HBS-EDTA was removed and a further 5ml of HBS was placed in the flasks and left for approximately 15 min or until the cells had lifted. The cells were then removed from the flasks and centrifuged at 200g for 4 min to allow a loose pellet to form. The supernatant was discarded and 1ml/175cm³ flask of wash buffer 1 (10mM HEPES, 10mM EDTA, pH 7.4) was added to the pelleted cells. These were homogenised using a polytron homogeniser (speed 6, 4x5 seconds bursts separated by approx. 30 seconds) on ice. The homogenate was centrifuged at 40,000g (18,300 rpm Sorvall RC5) for 15 min at 4°C and the resulting supernatant was discarded. The pelleted cells were homogenised and re-centrifuged as described above in wash buffer 2 (10mM HEPES, 0.1mM EDTA, pH 7.4). The final pellet of cell membranes were resuspended in 10mM
HEPES, 0.1mM EDTA, pH 7.4 at a final concentration of 1mg ml⁻¹, protein concentration was determined by the method of Lowry et al. (1951) (see section on Protein Determinations below), using Bovine serum albumin as a standard and “snap frozen” in liquid nitrogen and maintained at -80°C for future use.

2.3. [³H]-NMS radioligand binding to stably transfected CHO-cells.

Both N-methyl-[³H]-scopolamine ([³H]-NMS) saturation and displacement experiments were carried out for CHO-m1-m4 membranes.

Cells were harvested using HBS-EDTA. Membranes of CHO cells were prepared by homogenisation of the cell clones in ice-cold wash buffer 1 using 4×5 sec bursts of a Polytron tissue disrupter, speed 6. The homogenate was centrifuged at 40,000g for 15 min at 4°C. The resulting supernatant was discarded and the pellet resuspended in wash buffer 2. The pellet was resuspended using 4×5 sec bursts of the Polytron and spun as before. The resulting pellet was resuspended at 1mg ml⁻¹ in wash buffer 2. For more detail see above.

A range of concentrations of [³H]-NMS from 0.07-3nM was used in the absence and presence of atropine (1µM; to define the non-specific binding) to construct saturation binding curves, as described by Lambert et al., (1989). Muscarinic antagonist displacement curves were performed in membrane preparations in binding assay buffer (10mM HEPES, 100mM NaCl, 10mM MgCl₂, pH 7.4) for 1h at 37°C, using [³H]-NMS (specific activity approx. 83Ci/mmol) at or below the Kᵣ determined for the receptor sub-types. Reactions were terminated by rapid vacuum filtration through Whatman GF/B filters followed by 2×4 ml washes with ice-cold binding assay buffer. Filters were removed to scintillation vials containing 5ml scintillant and radioactivity was quantified >12 hours later by liquid scintillation spectrometry. Total membrane binding was always <20 % of [³H]-NMS added.
2.4. [³⁵S]-Guanosine 5'-[γ-thio]triphosphate (³⁵S]-GTPγS) radioligand binding mediated by human muscarinic receptors stably expressed in CHO cells.

Prior to the assay, CHO cells stably expressing the M₁- M₄ receptors were harvested according to the section entitled ‘Preparation of Cell Membranes’ (above).

Following the protocol described by Lazareno et al. (1993a), 100μl of test drug was mixed in a final volume of 1ml, containing 100μl GDP (10μM final), 100μl mAChR membrane preparation (1 mg ml⁻¹) and 100μl of [³⁵S]-GTPγS. Binding was determined using approximately 70 pM final [³⁵S]-GTPγS (specific activity 1000-1400 Ci/mmol from NEN) diluted in assay buffer (10mM HEPES, 100mM NaCl, 10mM MgCl₂, pH 7.4). GDP was included in the reaction mixture to allow a reduction in the basal binding of the radiolabel thus producing a higher agonist signal-to-noise and the inclusion of Mg²⁺ is thought to stabilise the transitional activation state of the G protein α subunit (see introduction). The reaction was initiated by addition of the membrane suspension to all tubes, except the blank where assay buffer replaced the membrane suspension. All samples were vortexed and placed in a shaking water bath at 30°C for 30 min (unless otherwise stated). The assay is terminated by filtering the tube contents through wetted GF/B filters using a Brandel cell harvester followed by 2×4 ml washes with ice-cold assay buffer, to separate bound and free radioactivity. The filters were placed in 5ml of liquid scintillant and left to extract for >12 hours, these were then counted using liquid scintillation spectroscopy.

2.5. Permeabilised cell preparation.

CHO-transfects were grown to confluency as described in the section entitled ‘Cell Culture’. To each confluent flask (175 cm³) 15 ml of HBS-EDTA (pH 7.4) was added to remove all traces of media, this was washed over the cell surface and then discarded. Then 5 ml of HBS-EDTA (pH 7.4) was added to each flask and left for 10-
15 min or until the cells had lifted. The cells were then removed from the flasks and centrifuged at 200g for 5 min to allow a loose pellet to form. The supernatant was discarded and the resulting pellet was manually agitated to resuspend the cells. To this 2ml of Cytosol-Like Buffer (CLB) (pH 7.2)/175cm³ flask was added and mixed together by gentle agitation. The resuspended cells were then spun at 200g for 5 min. This washing process was repeated twice before finally resuspending the cells in 1.2ml of CLB/ 175cm³ flask and subjecting them to the permeabilising agent.

2.6. [³H]-Adenine release from permeabilised CHO-cells.

[³H]-adenine metabolite release was utilised to evaluate CHO-cell permeabilisation by a number of different detergents. CHO-cells were grown to confluence before [³H]-adenine was added for 24h at a final concentration of 1.5 mCi ml⁻¹. Cells were permeabilised as described in ‘Permeabilised Cell Preparation’ using either β-Escin (50 µg ml⁻¹), saponin (100 µg ml⁻¹) or digitonin (500 µg ml⁻¹) as the permeabilising agent. The permeabilising agent was manually agitated with the cells for either 2 or 5 min. The cells were centrifuged at 14,000g for 5 min and the resulting supernatant was transferred to a scintillation vial. Radioactivity release by cells was assayed by the addition of 5 ml of scintillation fluid and quantified using liquid scintillation spectroscopy. TCA (500 µl) was added to the pellet and left to extract on ice for 30 min. The pellet was then centrifuged at 14,000g for 5 min and the supernatant was assayed by the addition of 5 ml of scintillation fluid, and quantified using liquid scintillation spectroscopy. This allowed the calculation of the radioactivity that had not been released from the cytosol by the action of the permeabilising agent. The radioactivity remaining after both these procedures (i.e. that incorporated in cellular constituents) was assayed in the resulting pellet by the addition of 500 µl of 0.1 M NaOH. The pellet was left to solubilise overnight, then transferred to a scintillation vial with 5 ml of scintillation fluid.
2.7. Mobilisation of intracellular Ca\(^{2+}\) stores in permeabilised CHO-cells after muscarinic receptor activation.

Release of calcium from intracellular stores was used as a downstream indicator of receptor-G protein interaction and calcium mobilisation studies were performed by modifying the methodology of Wojcikiewicz et al. (1990b).

Cells were permeabilised as described above using β-Escin, Saponin or Digitonin as the permeabilising agents at final concentrations of 50 μg ml\(^{-1}\), 100 μg ml\(^{-1}\) or 500 μg ml\(^{-1}\) respectively. \(^{45}\)Ca\(^{2+}\) was added at 2 μCi/ml and, the cells were incubated at room temperature for 15 min with occasional gentle agitation. Concentration response relationships were constructed by incubating \(^{45}\)Ca\(^{2+}\) prelabelled cells with CLB (pH 7.2) containing stimuli. Reactions were initiated by adding 50 μl of the prelabelled permeabilised cell suspension to 50 μl of CLB containing agonist. After 1 min at room temperature the samples were centrifuged at 16,000g for 2 min to separate cells and reaction buffer. Silicon oil (500 μl) (Dow Corning 550/556 1:1 v/v) was added to the centrifuged reaction mixture to allow separation of the aqueous phase and spun at 16,000g for a further 2 min. The released radiolabelled calcium was removed by aspiration of aqueous phase from the silicon oil. The remaining radioactivity (i.e. that not released from intracellular stores) was assayed by the addition of 1.1 ml of scintillation fluid and quantified using liquid scintillation spectroscopy. Release of \(^{45}\)Ca\(^{2+}\) in the presence of agonist was calculated from the amount remaining in the cell pellet, expressed as a percentage of \(^{45}\)Ca\(^{2+}\) released from the control tubes containing ionomycin (20 μM).

2.8. \(^{35}\)S-GTP\(_\gamma\)S binding in permeabilised CHO-cells after muscarinic receptor activation.

\(^{35}\)S-GTP\(_\gamma\)S binding to activated G proteins was using a modification of the methods of Lazareno et al. (1993a; 1993b) and Wieland et al. (1995). Cells were prepared as described above. After harvesting, each 175 cm\(^3\) confluent flask was resuspended in 2
ml of CLB pH 7.2 using β-Escin as the permeabilising agent at a final concentration of 50 μg ml⁻¹.

Permeabilised cells (20 μl) were incubated in the presence of 180 μl of assay buffer (CLB) including 1.2 nM [³⁵S]-GTPγS (1250 Ci/mmol) 10 μM GDP (unless otherwise stated) at 30 °C for 1 hour (unless otherwise stated). Incubations were terminated by rapid filtration through Whatman GF/B filters using a manifold. 5 ml of harvesting buffer (10 mM HEPES, 100 mM NaCl, 10 mM MgCl₂, pH 7.2) was used to wash the reaction tubes three times. The filters were placed in scintillation vials and 5 ml of scintillation fluid was added, these were left to extract for at least 12 hours. The resulting radioactivity was counted using liquid scintillation spectroscopy. Non-specific binding was defined using 100 pM GTPγS and subtracted from the final reaction values.

2.9. Methacholine-stimulated inositol 1,4,5-trisphosphate generation in permeabilised CHO-cells.

2.9.1. Generation of Ins(1,4,5)P₃ in permeabilised cells.

Cells were permeabilised as described above. After harvesting, each 175 cm³ confluent flask was resuspended in 2.5 ml of Cytosol Like Buffer (CLB) and β-Escin was used as the permeabilising agent at a final concentration of 50 μg ml⁻¹. Cells in suspension (50 μl) were added (at time zero) to tubes containing 50 μl of CLB (pH 7.2) in the presence or absence of stimuli and then incubated in a waterbath at 37°C. The reactions were terminated by the addition of 50 μl of 1.5M trichlororacetic acid (TCA), and the reaction tubes were placed on ice for approximately 1 hour. TCA served to lyse the cells and denature the cellular protein content by a reduction in pH, preventing further enzyme activity.

Samples were centrifuged at 14,000 rpm for 2 min. Supernatant (125 μl) was removed and added to fresh tubes, with 25 μl of 60 mM EDTA (pH 7.0) and 200 μl of a 1:1 mixture of 1, 1, 2-trichlorotrifluoroethane (Freon) and tri-n-octylamine. The EDTA
serves to aid separation of the organic and aqueous phases after the addition of Freon/tri-n-octylamine and to sequester divalent cations that may interfere with the Ins(1,4,5)P₃ radioassay. Freon/tri-n-octylamine removed the TCA and thus neutralised the extract. The resulting mixture was vortex mixed thoroughly, left to stand at room temperature for 15 minutes and then centrifuged at 14,000 rpm for 5 min to achieve the separation of the two phases. The lower phase contained the Freon/tri-n-octylamine and extracted TCA while the upper aqueous phase contained the generated inositol phosphates. The upper phase (70 µl) was removed from each sample and mixed with 20 µl of 60 mM NaHCO₃ in fresh tubes to correct the pH to approx. pH 7.0 which was optimal for the binding assay. The samples were then maintained at 4°C for up to 1 week until the binding assays were performed.

2.9.2. Ins(1,4,5)P₃ binding assay.

The Ins(1,4,5)P₃ binding assay (Challiss et al., 1990) is a competition assay, whereby radiolabelled Ins(1,4,5)P₃ at a single concentration competes for the same Ins(1,4,5)P₃ binding sites present in bovine adrenal cortical membranes as the Ins(1,4,5)P₃ generated in each sample or present in each standard.

Assay tubes contained 30µl of standard/sample, 30 µl of Tris (100 mM)/EDTA (4 mM) pH 8.0 and 30 µl of [³H]-Ins(1,4,5) P₃ (at a final concentration of approximately 0.8 nM; specific activity 35 Ci/mmol). Incubations were initiated by the addition of 30 µl of Ins(1,4,5)P₃ binding protein at 4°C. After approximately 1 hour the reaction was terminated by rapid filtration through pre-wetted Whatman GF/B filters. These were washed twice with 4 ml of ice cold wash buffer (Tris, EDTA, NaHCO₃ pH 7.8). Filters were placed in scintillation vials containing 5 ml of liquid scintillant and left for at least 12 hours before radioactivity was detected by liquid scintillation spectrometry.
2.10. **Muscarinic agonist-stimulated inhibition of cAMP accumulation.**

Cells were grown to confluence in 175 cm³ flasks, the growth medium removed and the cells washed and harvested in HBS-EDTA, pH 7.4. These cells were pelleted by centrifugation at 200g for 5 min, then washed in 10 ml Krebs-Henseleit Buffer (KHB) and gently mixed prior to centrifugation at 200g for 5 min. The resulting washed cells were then gently resuspended by agitation in 3 ml KHB to produce an even cell suspension.

The cells were then incubated for 10 min in 150 µl of KHB in the presence of 10µM forskolin and the presence or absence of muscarinic stimuli. The reaction was stopped by the addition of 100µl ice-cold TCA (1.5M) and the cells were left on ice for approximately 30 min to allow total cyclic AMP extraction. Samples were centrifuged at 14,000 rpm for 2 min and 125 µl of supernatant was removed and added to fresh tubes. 25 µl of 60 mM EDTA (pH 7.0) and 200 µl of a 1:1 mixture of 1, 1, 2-trichlorotrifluoroethane (Freon) and tri-n-octylamine were added. The samples were vortex mixed and left to stand for about 15 min before re-centrifugation at 14,000 rpm. Aqueous phase (70 µl) was neutralised with ~20µl 60mM NaHCO₃, to correct the sample to ~pH 7.0 and samples were stored at 4°C for up to 1 week.

2.11. **Cyclic AMP determination.**

Cyclic AMP was quantified using a competitive binding assay, as described by Brown et al. (1971). Known concentrations of cAMP were made up in blank buffer (Freon/tri-n-octylamine extracted-1.5M TCA alone), and 50µl of sample, standard, blank buffer or cAMP (5µM; to define the non-specific binding) was added to microfuge tubes. To each tube was added 100µl [³H]-cAMP (~4nM; specific activity 29 Ci/mmol), followed by 150µl bovine adrenal cortical membrane preparation to initiate the reaction (see Brown et al., 1971). Tubes were capped, mixed and incubated at 4°C for approximately 90 min after which equilibrium had been
achieved. Bound and free cAMP were separated by the addition of 250μl ice-cold activated charcoal (0.25g/50ml) and bovine serum albumin (0.1g/50ml) made up in 50mM Tris-HCl, 4mM EDTA (pH 7.4). After 7-8 min standing, the samples were centrifuged at 16,000g for 4 minutes. Supernatant (400μl) was removed, placed in a scintillation vial and radioactivity was detected by liquid scintillation spectrometry.

2.12. G Protein Western Blotting.

Western blotting membrane samples (as described in ‘Preparation of Cell Membranes’) were mixed with an equal volume of sample buffer (100 mM Tris, 200 mM dithiothreitol, 2% SDS, 0.1% bromophenol blue, 10% glycerol) and boiled for 5 min. Samples were electrophoresed on a 12%, 0.75mm thick SDS-PAGE minigel, with a 5% stacking gel, at around 100V for approximately 1 hour (running buffer 25 mM Tris, 250 mM glycine, 0.1% SDS, pH 8.0). Transfer to nitro-cellulose was achieved using a semi-dry apparatus with a transfer buffer consisting of 20 mM Tris, 150 mM glycine, 0.037% SDS and 10% MeOH, at 0.65 mA/cm². The primary antibodies (rabbit polyclonal) were purchased from Calbiochem and Santa Cruz; these were used in 1% milk at a dilution of 1:1000. The secondary antibody was purchased from Sigma and used in 1% milk at 1 in 1000. The ECL reagent kit from Amersham was used (according to manufactures instructions) to develop the blot.

2.13. Immunoprecipitation of [35S]-GTPγS-bound specific Gα subunits in response to muscarinic receptor activation.

2.13.1. Preparation of cell membranes.

CHO-cell transfects were grown to confluence as described in ‘Cell Culture’. Each 175cm³ confluent flask was washed with 10-15 ml HBS-EDTA, then the cells were lifted by the addition of 5 ml of HBS-EDTA for approx. 10-15 min. CHO-cell transfects expressing the same receptor were pooled and centrifuged at about 200g for 5 min. Pelleted cells were homogenised in the presence of hypo-osmotic lysis buffer (10 mM EDTA, 10 mM HEPES, pH 7.4). Disruption of the cells was achieved by
using a polytron homogeniser (4x5 sec bursts speed 7). The homogenate was then centrifuged at 1000 rpm for 5 min and the resulting supernatant further centrifuged at 20,000 rpm for 30 min in a Sorvall RC5. The final pellet was resuspended in freezing buffer (0.1 mM EDTA, 10mM HEPES, pH 7.4) at a protein concentration of around 5-9 mg/ml and frozen rapidly in liquid nitrogen. Membranes were then stored at -80°C until used.

2.13.2. \[^{35}\text{S}]\text{-GTP}\gamma\text{S binding after muscarinic receptor activation.}\)

\[^{35}\text{S}]\text{-GTP}\gamma\text{S binding to G-proteins and the subsequent immunoprecipitation used a modification of the methods of Friedman et al. (1993) Wang et al. (1995) and Burford et al. (1998).}\)

Frozen membrane aliquots were diluted in assay buffer (10 mM HEPES, 100 mM NaCl, 10 mM MgCl\textsubscript{2}, pH 7.4) to give a final protein concentration of 75 µg/50 µl. These were incubated in the presence of 50 µl of assay buffer containing 1 nM \[^{35}\text{S}]\text{-GTP}\gamma\text{S (1250 Ci/mmol) and 1 or 10 µM GDP (unless otherwise stated) at 30 °C for 2 min (unless otherwise stated). Incubations were terminated by adding 900 µl of ice-cold assay buffer and placing the Eppendorf tubes on ice. The cell membranes were separated from the reaction mixture by centrifugation at 20,000g for 6 min (Eppendorf 5417 R). The membrane pellets were solubilised by the addition of 50 µl of ice-cold solubilisation buffer containing SDS (100 mM Tris, 200 mM NaCl, 1 mM EDTA, 1.25% Igepal CA 630, 0.2% SDS pH 7.4) and vortex mixing. Once the protein had been solubilised an equal volume of solubilisation buffer without SDS was added to each Eppendorf tube.

2.13.3. Immunoprecipitation of \[^{35}\text{S}]\text{-GTP}\gamma\text{S bound G proteins with antisera to specific G\textalpha{} proteins.}\)

The solubilised cellular membranes were pre-cleared with normal rabbit serum (1:100 dilution) and 30 µl of protein A beads (protein A sepharose bead suspension 30% w/v in TE buffer (10 mM Tris, 10 mM EDTA, pH 8.0)) for 1 hour at 4°C. The protein A
beads and any insoluble material were collected by centrifugation at 20,000g for 6 min. Supernatant (100 µl) was transferred to a fresh Eppendorf tube containing the G protein antisera (1:100 dilution) and samples were vortex mixed and rotated for 1.5 hours at 4 °C. Protein A sepharose bead suspension (70 µl; 30% w/v in TE buffer pH 8.0) was added to each sample tube and the samples were again vortex mixed and rotated for 1.5 hours at 4 °C. Protein A beads were then pelleted at 16,000g in an Eppendorf 5417 R and the supernatant removed by aspiration. The beads were then washed with 500 µl solubilisation buffer without SDS, pelleted and aspirated to remove supernatant as described above, this process was carried out 3 times in total. After the final wash the sepharose beads were mixed with 1.1ml of scintillation cocktail and vortex mixed. Radioactivity was detected by liquid scintillation spectroscopy. Non-specific binding was determined in the presence of 10 µM GTPγS and was subtracted from the absolute cpm values obtained.

### 2.14. Protein Determinations.

Protein concentrations in both membranes and permeabilised cells were determined by a modification of the method of Lowry et al. (1951). Protein standards were prepared using bovine serum albumin (BSA) diluted in 0.1 M NaOH to give a range of protein concentrations from 0 to 200 µg ml⁻¹. 500µl of standards across an appropriate range were aliquoted into test tubes in duplicate, also protein samples were diluted in 0.1 M NaOH to give a final volume of 500µl. 2.5 ml of solution A was added to each test tube, this consisted of 50 ml of solution B (2% Na₂CO₃ in 0.1 M NaOH) + 0.5 ml of solution C (1% CuSO₄) and 0.5 ml of solution D (2% Na⁺ K⁺-tartrate). After 10 min 100 µl of solution E (a 1:3 dilution of Folin-Ciocalteu’s phenol reagent in distilled water) was added to each test tube and mixed. After 20 min the absorbance was measured at 750 nm and the sample protein concentration was determined relative to the protein standards.
Chapter Three.

Characterisation of Recombinant Acetylcholine Muscarinic Receptors and the G protein Complement in CHO-K1 cells.
3.0. Introduction.

The muscarinic acetylcholine receptor family has been characterised by both traditional pharmacological and molecular biological methods. Initially in 1914 Sir Henry Dale found that both fast and slow responses to acetylcholine could be mimicked by nicotine and muscarine (Dale 1914). This lead to the initial classification of acetylcholine receptors into nicotinic and muscarinic. Then in 1980 it became obvious that a single muscarinic receptor subtype could not account for all the muscarinic receptor-mediated actions of acetylcholine. Pharmacologically distinguishable subtypes of the mAChR were observed in the binding preparations from different tissues by the detection of substantial differences in affinity for the anti-ulcer drug pirenzepine. This observation led to the provisional classification into two muscarinic receptor subtypes, M₁ and M₂ (Hammer et al., 1980; Gil and Wolfe, 1984). Further distinguishable binding with 4-diphenylacetoxy-methyl piperidine methiodide (4-DAMP) allowed discrimination of binding to M₂ receptors in heart and ileum (Barlow et al., 1976) and suggested a further sub-division of muscarinic receptors. Development of a series of polymethylene tetraamines allowed the identification of methoctramine as a cardiac muscarinic selective antagonist (Melchiorre et al., 1987). Methoctramine was then utilised to dissect the family further in studies of rat cortex, heart and submaxillary gland (Michel and Whiting, 1988; Lazareno and Roberts, 1989) identifying three pharmacologically distinct receptor subtypes to be defined and designated M₁, M₂, and M₃.

With the emergence of molecular biological approaches the problems encountered in receptor classification were addressed at the genomic level. Initially the sequence of cDNA encoding a mAChR was derived from a porcine cerebrum library. The receptor was expressed in Xenopus oocytes producing a functional M₁ muscarinic receptor (Kubo et al., 1986). Then from 1986-1989 the muscarinic acetylcholine family m1-m5 were evaluated at the genetic level and primary structural information was published (Kubo et al., 1986a, b; Bonner et al., 1987, 1988; Peralta et al., 1987a, b; reviewed by Hulme et al., 1990).
The cloning of the five members of the muscarinic acetylcholine family has allowed their expression in a range of cell types (Buckley et al., 1989; Hulme et al., 1990; Dorje et al., 1991; Caulfield, 1993; Rinken, 1995). This has allowed each of the receptor subtypes to be studied in isolation, allowing the biochemical and pharmacological characterisation of individual muscarinic subtypes. Recombinant receptor expression systems have overcome some of the problems inherent in studies based in tissues where multiple subtypes are often co-expressed which prevented the study of muscarinic receptors in a homogenous population.

The identification of the primary sequences of the muscarinic receptor family has permitted the evaluation of a number of traditional and novel pharmacological agents. Yet despite the advances molecular biology has made in defining muscarinic receptor family members there still exists a lack of highly selective pharmacological agents. No single selective muscarinic antagonist can adequately distinguish among the muscarinic receptor subtypes. This means that to define a muscarinic receptor subtype pharmacologically the dissociation constants of a number of selective antagonists need to be evaluated.

With the emergence of primary sequence data the acetylcholine muscarinic receptor family members were seen to possess 7 transmembrane spanning domains. The superfamily of 7 transmembrane domain receptors are now known to transduce their signals by interaction with guanine nucleotide binding proteins (G proteins), and this is true of the acetylcholine muscarinic receptor subtypes (Caulfield, 1993; Eglen et al., 1996). The G protein family is sub-divided into 4 classes $G_1$, $G_2$, $G_3$ and $G_4$ on the basis of the $\alpha$ subunit of the heterotrimer (Gilman, 1987; Pennington, 1994). The odd numbered muscarinic receptor subtypes ($M_1$, $M_3$ and $m_5$) are reportedly coupled to members of the $G_2$ family, with the even numbered muscarinic receptor subtypes ($M_2$ and $M_4$) reportedly coupling to $G_1$ family members (Caulfield 1993). Specific antisera to $G\alpha$ subunits have been utilised in a wide variety of studies to identify and locate specific G proteins (McLaughlin et al., 1991; Clark et al., 1993; Maier et al., 1995; Grant et al., 1997). By using antisera to $G\alpha$ subunits changes in G protein levels associated with pathological states such as diabetes and obesity have been profiled.
(Livingstone et al., 1991; Strassheim et al., 1991). Also agonist-mediated down-regulation of Gq has been evaluated with α subunit specific antiserum (Mullaney et al., 1993). The functional applications of antisera to Gα subunits are wide ranging (Milligan, 1994), and the development of this technology has allowed the characterisation, at both the quantitative and qualitative level, of the G protein complement in a wide variety of cells.

Chinese hamster ovary-cell clones transfected with cDNA encoding the Hm1, Hm2, Hm3 and Hm4 receptors were a gift from Dr N. Buckley and further cells expressing the Hm2 receptor at a slightly higher density were a generous gift from Dr S. Lazareno. The initial aim of this study was to characterise the muscarinic receptor subtype expressed in the individual CHO-cell clones, then to assess their relative receptor expression levels, their affinity profiles for reported selective antagonists and the G-protein complement in each clone.

3.1 Methods.

For details of all the methodologies employed throughout this Chapter refer to Chapter 2 ‘Experimental Methodology’ especially the sections entitled ‘Preparation of Cell Membranes’, ‘[^3H]-NMS Radioligand Binding to Stably Transfected CHO cells’, ‘G Protein Western Blotting’ and ‘Protein Determinations’.

3.2. Data Analysis.

[^3H]-NMS saturation binding data for each individual experiment were analysed by Scatchard analysis to determine maximal binding capacity (B_max), equilibrium dissociation constant (K_D) and Hill coefficient (n_H) values. Competition binding experiments were analysed using the computer program Prism 2 (dose-response curve (variable slope)), which generates IC_{50} and Hill coefficient values. IC_{50} values were adjusted to inhibition dissociation constant values (K_i) by utilising the concentration of the competing radioligand and the equation of Cheng and Prusoff (1973). K_i values
reported were generated using the $K_D$ values reported in Table 3.3.2. All data are shown as means ± s.e.mean for ‘n’ separate experiments.

3.3. Results.

Receptor-Characterisation in transfected CHO-cells.

$[^3H]-NMS$ saturation binding data were obtained according to the Methods section entitled ‘$[^3H]-NMS$ Radioligand Binding to Stably Transfected CHO Cells’. Equilibrium binding was achieved after 60 min and could be maintained for 120 min without any significant loss of binding (data not shown).

Saturation binding experiments were performed on cell membranes prepared from cells at a variety of passage levels throughout the duration of this study. The saturation binding data generated using CHO-cell membranes are shown in Tables 3.3.1. and 3.3.2. $[^3H]-NMS$ binding to the transfected CHO cells expressing the Hml-Hm4 receptors show that $[^3H]-NMS$ binding to the different muscarinic receptor subtypes was non-selective with dissociation constants between 0.1-0.2 nM. All of the Hill coefficient values obtained were close to unity suggesting that each clonal cell line expressed a single receptor subtype with a single affinity.

$B_{\text{max}}$ determinations in CHO-M$_1$ cells showed that they expressed the Hml receptor at relatively high levels when compared to the other transfected cell lines used in this study. It could be seen that this cell line possessed the highest density of receptors of all the clonal cell lines utilised, with receptor expression levels ranging from $2.39 ± 0.19$ to $2.72 ± 0.17$ pmol mg$^{-1}$ protein. The CHO-M$_3$ cells also showed a high level of receptor expression, comparable to that of the CHO-M$_1$ cells. The receptor expression level of M$_3$ receptors in the CHO-M$_3$ cell line was in the range $2.43 ± 0.18$ to $2.56 ± 0.03$ pmol mg$^{-1}$ protein over the duration of the study. With both the $G_{q/11}\alpha$ linked muscarinic receptors displaying similar expression levels it allowed direct comparison of downstream signalling events after muscarinic receptor stimulation.
The Hm2 receptor was maintained in two cell lines termed CHO-M2 and CHO-SLM2. The original clone CHO-M2 provided by Dr N. Buckley was subcloned to gain a clone with a higher receptor density by Dr S. Lazareno. The CHO-M2 expressed the Hm2 receptor at levels ranging from 0.54 ± 0.05 to 0.61 ± 0.10 pmol mg\(^{-1}\) protein whereas the CHO-SLM2 cell line gave approx. 50% higher receptor levels of Hm2 receptor between 0.82 ± 0.09 to 0.97 ± 0.04 pmol mg\(^{-1}\) protein.

The CHO-SLM2 Hm2 receptor cell line expressed the receptor at a level approaching that seen in the other G\(_i\alpha\) coupled receptor cell line studied, CHO-M4. CHO-M4 cells expressed an intermediate density of Hm4 receptors, with the receptor expression level obtained in the region of 1.29 ± 0.15 to 1.52 ± 0.23 pmol mg\(^{-1}\) protein.

The two different receptor densities of the Hm2 receptor (the CHO-M2 and CHO-SLM2 clones) allowed the comparison of signalling responses elicited by a single receptor subtype expressed at varying levels to be evaluated and the higher expressing clone allowed a closer comparison to the Hm4 receptor subtype. This means we also had a system to evaluate the differences between the G\(_i\alpha\) activating muscarinic receptor subtypes.

All the cell lines studied appeared to maintain stable levels of receptor expression throughout the study period when assessed by \([\text{H}^3]\)-NMS binding. The stability of expression across all the cell lines utilised can be seen in Figure 3.3.1a and for further detail these data can be seen in tabulated form in Table 3.3.1. Also a representative binding experiment can be seen in Figure 3.3.1b.

Saturation binding data generated using permeabilised CHO-cells are shown in Table 3.3.3. \([\text{H}^3]\)-NMS binding to the permeabilised transfected CHO cells expressing the Hm1-Hm4 receptors illustrate that \([\text{H}^3]\)-NMS binding is again non-selective for the muscarinic receptor subtypes. They all display similar dissociation constants around 0.1 nM, this is as expected from the membrane results and reported data of others (Hulme, 1990, Richards and Van Giersbergen, 1995). As in the membrane binding data the Hill coefficient values suggest binding to a single affinity receptor.
population. The maximal binding capacity values for the permeabilised CHO-cells were similar to those obtained in the membrane based experiments. The CHO-M1 and CHO-M3 cells appeared to express the Hml and Hm3 receptors respectively at high levels, with receptor densities of 2.72 ± 0.17 and 2.55 ± 0.15 pmol mg⁻¹. CHO-SLM2 cells expressed a low density of Hm2 receptors (1.00 ± 0.12 pmol mg⁻¹). Bmax determinations showed that the CHO-M4 cells expressed an intermediate density of Hm4 receptors (1.38 ± 0.15 pmol mg⁻¹). From these data it can be seen that no differences in receptor densities across the CHO-cell clones were observed in the distinct cellular preparations.

[³H]-NMS showed apparently equivalent binding affinity to the receptor subtypes being assessed, this allowed the evaluation of three selective antagonists in competition experiments with a single concentration of [³H]-NMS (approximately 0.3 nM). Each of the selective antagonists pirenzepine, methoctramine and tropicamide produced inhibition binding curves in the cell lines described above with Hill coefficient values approaching unity. This provided further evidence that each of the cell lines used in this study possessed a homogeneous receptor population of a single receptor subtype (refer Table 3.3.4). This result was as expected due to the lack of endogenous muscarinic acetylcholine receptors in CHO cells.

The data shown in Table 3.3.4 illustrate that each of the muscarinic acetylcholine receptor subtypes could be defined pharmacologically. Pirenzepine showed a selectively higher affinity for the Hml receptor subtype, Caulfield (1993) reported a higher affinity of pirenzepine for the M1 receptor subtypes over M2, M3 or M4 receptor subtypes. Pirenzepine showed a 60 fold and 57 fold selectivity over the Hm2 subtype expressed in the CHO-SLM2 and CHO-M2 cell lines respectively, when compared to the Hml subtype in this study. The Hm3 muscarinic receptor subtype expressed in CHO-M3 cells displayed a 34 fold lower affinity for pirenzepine when compared to the Hml expressing cells. Little selectivity (2-3 fold) was seen when the Hml subtype was compared to the Hm4 receptor subtype. In this study pirenzepine showed a rank order of affinity when assessed by [³H]-NMS displacement studies of Hml > Hm4 >> Hm3 > Hm2.
Methoctramine in this study showed a significantly higher affinity for the Hm2 receptor, expressed in both the CHO-SLM2 and CHO-M2 cell lines, when compared to the other muscarinic receptors. Methoctramine bound with a 4 fold lower affinity to the Hm4 receptor subtype expressed in the CHO-M4 cells, and a 5 fold lower affinity to the Hm1 expressed in CHO-M1 cells when compared to the Hm2 receptor subtype. The difference in affinity of methoctramine for different acetylcholine muscarinic receptor subtypes was more pronounced when comparing the Hm2 subtype to the Hm3 expressed in CHO cells. Methoctramine displayed a 20 fold lower affinity for the Hm3 receptor subtype when compared to the Hm2. From these experimental data methoctramine showed a rank order of affinity when assessed by $[^3H] \text{-NMS}$ displacement studies of Hm2 > Hm4 > Hm1 > Hm3; this is in agreement with published data (Caulfield, 1993).

Tropicamide is proposed to be an M4 selective antagonist (Lazareno et al., 1990a, b). From the data in Table 3.3.4 it can be seen that tropicamide showed some selectivity for the Hm4 receptor subtype expressed in the CHO-M4 cells. In the clonal cell lines studied tropicamide appeared to show 11 fold selectivity over the Hm1 receptor subtype expressed in CHO-M1 cells. When compared to the Hm2 and Hm3 receptor subtypes the selectivity was less, showing 3 and 2 fold selectivity over the M2 and M3 subtypes respectively. From these experimental data tropicamide showed an order of affinity of Hm4 ≥ Hm3 > Hm2 > Hm1. This is in agreement with published data (Lazareno et al., 1990 b).

**Gα Protein-Identification in transfected CHO-cells.**

Gα protein expression in the transfected CHO cells was assessed by Western Blotting according to the Methods section entitled ‘G Protein Western Blotting’. Comparisons across all the transfected cell lines and against untransfected CHO-K1 cells were made. The data obtained using this experimental methodology are shown in Figures 3.3.2 to 3.3.6.
Initial Western blot experiments performed upon cellular membranes prepared from CHO cells transfected with the human muscarinic acetylcholine receptors m1-m4 examined the presence of different Go proteins and gave a crude quantitative indicator of protein levels. Clear, well defined bands were detected when antisera to the Gi3/0, Gq11 and Go isoforms were utilised. Anti Gl2/3 and Go also produced bands in the expected molecular weight region. The bands produced by the anti Gl2/3 and anti-Go antisera were not as strong as those obtained when using the anti-Gi3/0, anti-Gq11 and anti-G. This may reflect a change in the intracellular abundance of relative Go subunits or it may be due to differing affinities of the antibodies for their antigenic sequences. For detail of the antibodies utilised refer to Table 3.3.5.

Antisera directed against the Gi3/0 and Gl2/3 isoforms of the G family gave single bands which migrated well below the position of the 43 kDa marker. The molecular weights of these highly homologous subunits did not allow their separation under the conditions utilised outlined in the Methods section called 'G Protein Western Blotting'. The Gq11 antisera also produced a single band near to the 43 kDa molecular marker, this can be seen in Figure 3.3.5.

An antiserum directed against Go gave two bands in the region of 45 kDa indicative of the expression of both the long and short forms of Go in crude rat brain homogenate, untransfected CHO-K1 cells and CHO-cell transfected with Hm1-Hm4. The Western blot shown in Figure 3.3.6 shows that a clearly defined band exists in a higher molecular weight position, and a faint second band is located below this in a lower molecular weight position. This means that the long form of Go is found in higher abundance in our clonal cell lines than that of the short form. The other noteworthy thing about this Western blot is that the ratio of the long to short form of Go is increased in the transfected and untransfected CHO-cells with respect to the crude rat brain homogenate used as a control.

Figure 3.3.4 shows the results of using anti-Go antibody against our clonal cell lines. This is a Go subunit found typically in cells of a neuronal phenotype, which can be seen by the strong band produced when this antiserum interacted with the crude rat...
brain homogenate. Yet $G_\alpha$ was also detected in the transfected and untransfected CHO-K1 cells. In both the CHO-cells and the crude rat brain homogenate two faint bands could be seen below the 43 kDa marker. This may be indicative of the presence of both $G_{\alpha 1}$ and $G_{\alpha 2}$.

The Western blotting procedure revealed that the untransfected and transfected CHO-K1 cells showed no differences in the expression pattern of $G\alpha$ subunits. Across all the transfected cells no large differences in the level of any one specific $G\alpha$ protein appeared. However, due to the potentially differing affinities of the antibodies for their respective antigenic sequences no conclusions can be drawn about the relative abundance of specific $G\alpha$ subunits in these CHO transfects. This means that the transfection procedure, whereby a non-native receptor is expressed in a cell by the addition of plasmid DNA incorporating the cDNA for the selected receptor has no effect, in this case, on the G protein complement of the host cell.

So it can be seen from these results that the untransfected CHO-K1 cells and the Hm1-Hm4 transfected CHO-cells expressed all $G\alpha$ proteins tested for including $G_\alpha$, and that there is no difference in the relative abundance of any single $G\alpha$ protein tested for across the transfected CHO-cells.
Figure 3.3.1a.

$[^3]H\text{-NMS}$ binding to CHO-cell membranes, illustrating receptor expression levels over time.

CHO-cell membranes were incubated in the absence or presence of atropine for 1 h to obtain the total level of saturable binding. Data are expressed as pmol $[^3]H\text{-NMS}$ bound mg$^{-1}$ protein and are shown as means ± s.e.mean for ≥ 4 separate experiments.
Figure 3.3.1b.

\[^{1}\text{H}]-\text{NMS}\) binding to \(\text{CHO-SLM}_{2}\) cell membranes, illustrating receptor expression level.

CHO-cell membranes were incubated in the absence or presence of atropine for 1 h to obtain the total level of saturable binding. The data shown are representative of 30 further experiments and further experiments demonstrated no significant differences in the receptor expression levels (Table 3.3.2). All experiments were performed in duplicate.
G Protein Western Blotting.

Figure 3.3.2. Photograph of Western Blot using anti $G_{11\alpha}/G_{12\alpha}$.

Transfected and untransfected CHO-cell membranes (1 mg ml$^{-1}$) were mixed with an equal volume of 2 x sample buffer and subjected to SDS-PAGE and the proteins then transferred to nitrocellulose membranes for immunoblotting. $G\alpha$ proteins were identified in CHO-cell membranes (20 $\mu$g) expressing human muscarinic acetylcholine receptors using 1:1000 dilution of anti-$G_{11/2\alpha}$. Lane 1 Crude Rat Brain homogenate, Lanes 2-5 CHO-M$_1$ to CHO-M$_4$ membranes respectively and Lane 6 untransfected CHO-K1. The blot was taken such that the top and bottom edges correspond to the 73 and 32.3 kDa standards respectively. The photograph shown here is representative of the results obtained in $\geq$ 3 similar experiments.
**G Protein Western Blotting.**

Figure 3.3.3. Photograph of Western Blot using anti $G_{i3}\alpha/G_{0}\alpha$.

Membranes from transfected and untransfected CHO-cell (1 mg ml$^{-1}$) were subjected to SDS-PAGE and the proteins then transferred to nitrocellulose membranes for immunoblotting according to the methods section entitled ‘G Protein Western Blotting’. Anti-$G_{i3}\alpha/G_{0}\alpha$ (1:1000 dilution) identified in $\alpha$ subunits in CHO-cell membranes (20 $\mu$g) expressing human muscarinic acetylcholine receptors, all samples displayed an apparently single polypeptide of 40 kDa. Lane 1 Crude Rat Brain homogenate, Lanes 2-5 CHO-M$_1$ to CHO-M$_4$ membranes respectively and Lane 6 untransfected CHO-K1. The blot was taken such that the top and bottom edges correspond to the 73 and 32.3 kDa standards respectively. The photograph shown here is representative of the results obtained in $\geq$ 3 similar experiments.
Detection of $G_\alpha$ in transfected and untransfected CHO-cell membranes. Samples were subject to the immunoblotting procedure outlined in the methods section entitled ‘G Protein Western Blotting’. $G_\alpha$ proteins were identified in CHO-cell membranes (20 µg) expressing human muscarinic acetylcholine receptors using 1:1000 dilution of anti-$G_\alpha$. Lane 1 Crude Rat Brain homogenate, Lanes 2-5 CHO-M₁ to CHO-M₄ membranes respectively and Lane 6 untransfected CHO-K1. The blot was taken such that the top and bottom edges correspond to the 73 and 32.3 kDa standards respectively. The photograph shown here is representative of the results obtained in ≥ 3 similar experiments.
G Protein Western Blotting.

Figure 3.3.5. Photograph of Western Blot using anti Gq/11α.

Immunodetection of Gq/11α in transfected and untransfected CHO-cell membranes. Gα proteins were identified in CHO-cell membranes (20 μg) expressing human muscarinic acetylcholine receptors using anti-Gq/11α (1:1000 dilution). All samples appeared to display a single polypeptide of about 42 kDa. Lane 1 Crude Rat Brain homogenate, Lanes 2-5 CHO-M1 to CHO-M4 membranes respectively and Lane 6 untransfected CHO-K1. The blot was taken such that the top and bottom edges correspond to the 73 and 32.3 kDa standards respectively. The photograph shown here is representative of the results obtained in ≥ 3 similar experiments.
**G Protein Western Blotting.**

Figure 3.3.6. Photograph of Western Blot using anti Gα.

Membranes from transfected and untransfected CHO-cell membranes were subjected to SDS-PAGE and the proteins then transferred to nitrocellulose membranes for immunoblotting (for further detail see methods section entitled 'G Protein Western Blotting'). Gα proteins were identified in CHO-cell membranes (20 μg) expressing human muscarinic acetylcholine receptors by utilising a 1:1000 dilution of anti-Gα. Lane 1 Crude Rat Brain homogenate, Lanes 2-5 CHO-M\textsubscript{1} to CHO-M\textsubscript{4} membranes respectively and Lane 6 untransfected CHO-K1. The blot was taken such that the top and bottom edges correspond to the 73 and 32.3 kDa standards respectively. The photograph shown here is representative of the results obtained in ≥ 3 similar experiments.
Table 3.3.1.

Receptor expression levels in clonal cell lines used throughout study.

<table>
<thead>
<tr>
<th>Experimentation date</th>
<th>CHO-M₁</th>
<th>CHO-M₂</th>
<th>CHO-SLM₂</th>
<th>CHO-M₃</th>
<th>CHO-M₄</th>
</tr>
</thead>
<tbody>
<tr>
<td>November 1995</td>
<td>2.59 ± 0.04</td>
<td>0.60 ± 0.08</td>
<td>0.90 ± 0.01</td>
<td>2.46 ± 0.09</td>
<td>1.39 ± 0.06</td>
</tr>
<tr>
<td>May 1996</td>
<td>2.61 ± 0.02</td>
<td>0.54 ± 0.05</td>
<td>0.88 ± 0.10</td>
<td>2.50 ± 0.12</td>
<td>1.49 ± 0.10</td>
</tr>
<tr>
<td>September 1996</td>
<td>2.45 ± 0.15</td>
<td>0.61 ± 0.10</td>
<td>0.94 ± 0.02</td>
<td>2.43 ± 0.18</td>
<td>1.29 ± 0.15</td>
</tr>
<tr>
<td>March 1997</td>
<td>2.63 ± 0.20</td>
<td>0.65 ± 0.01</td>
<td>0.97 ± 0.04</td>
<td>2.56 ± 0.03</td>
<td>1.52 ± 0.23</td>
</tr>
<tr>
<td>August 1997</td>
<td>2.72 ± 0.17</td>
<td>0.57 ± 0.07</td>
<td>0.82 ± 0.09</td>
<td>2.55 ± 0.15</td>
<td>1.49 ± 0.10</td>
</tr>
<tr>
<td>February 1998</td>
<td>2.39 ± 0.19</td>
<td>N/A</td>
<td>0.91 ± 0.02</td>
<td>2.52 ± 0.10</td>
<td>1.51 ± 0.10</td>
</tr>
</tbody>
</table>

[³H]-NMS binding results from experiments carried out throughout the duration of this study. Experiments were performed according to Methods section entitled ‘[³H]-NMS Radioligand binding to stably transfected CHO-cells’. Membranes were prepared on the dates indicated and receptor expression levels, expressed as pmol mg⁻¹, did not vary significantly through time. All data are expressed as the means ± s.e.mean of values which were obtained from individual experiments.
[$^3$H]-NMS saturation binding in CHO cell membranes and in permeabilised CHO cell clones.

Table 3.3.2. [$^3$H]-NMS binding in cell membranes

<table>
<thead>
<tr>
<th>Cell line</th>
<th>$K_D$ (log molar values)</th>
<th>$B_{max}$ (pmol mg$^{-1}$)</th>
<th>$nH$</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHO-M$_1$</td>
<td>-9.82 ± 0.06</td>
<td>2.57 ± 0.15</td>
<td>0.98 ± 0.07</td>
<td>(24)</td>
</tr>
<tr>
<td>CHO-M$_2$</td>
<td>-9.87 ± 0.10</td>
<td>0.60 ± 0.06</td>
<td>1.10 ± 0.05</td>
<td>(28)</td>
</tr>
<tr>
<td>CHO-SLM$_2$</td>
<td>-9.78 ± 0.09</td>
<td>0.91 ± 0.05</td>
<td>0.93 ± 0.09</td>
<td>(30)</td>
</tr>
<tr>
<td>CHO-M$_3$</td>
<td>-9.90 ± 0.07</td>
<td>2.50 ± 0.11</td>
<td>0.92 ± 0.12</td>
<td>(24)</td>
</tr>
<tr>
<td>CHO-M$_4$</td>
<td>-9.73 ± 0.04</td>
<td>1.45 ± 0.12</td>
<td>0.95 ± 0.04</td>
<td>(30)</td>
</tr>
</tbody>
</table>

Table 3.3.3. [$^3$H]-NMS binding in permeabilised CHO cells

<table>
<thead>
<tr>
<th>Cell line</th>
<th>$K_D$ (log molar values)</th>
<th>$B_{max}$ (pmol mg$^{-1}$)</th>
<th>$nH$</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHO-M$_1$</td>
<td>-9.79 ± 0.10</td>
<td>2.72 ± 0.17</td>
<td>0.95 ± 0.12</td>
<td>(5)</td>
</tr>
<tr>
<td>CHO-M$_2$</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>CHO-SLM$_2$</td>
<td>-9.81 ± 0.08</td>
<td>1.0 ± 0.12</td>
<td>0.90 ± 0.05</td>
<td>(6)</td>
</tr>
<tr>
<td>CHO-M$_3$</td>
<td>-9.71 ± 0.15</td>
<td>2.55 ± 0.15</td>
<td>0.84 ± 0.06</td>
<td>(5)</td>
</tr>
<tr>
<td>CHO-M$_4$</td>
<td>-9.88 ± 0.03</td>
<td>1.38 ± 0.15</td>
<td>0.93 ± 0.18</td>
<td>(6)</td>
</tr>
</tbody>
</table>

$K_D$ (log molar values) represents the dissociation constant from individual experiments. $nH$ represents the Hill number and $B_{max}$ represents the maximal binding capacity in pmol mg$^{-1}$ observed. All data are expressed as the means ± s.e.mean of values obtained from all the saturation binding experiments performed over the duration of this study (i.e. those included in Table 3.3.1). Numbers in parentheses represent the number of experiments performed.
Table 3.3.4.
Antagonist binding activities derived from [3H]-NMS displacement experiments in CHO-cell membranes.

$K_i$ (log molar values) represents the inhibition constant for the individual compounds derived by using the Cheng-Prusoff equation and the [3H]-NMS $K_d$ values from Table 3.2.2. $nH$ represents the hill coefficient obtained from graphical analysis and numbers in parentheses represent the number of individual experiments. All data are expressed as the means ± s.e.mean of values which were obtained from individual experiments.

<table>
<thead>
<tr>
<th></th>
<th>CHO-M₁</th>
<th>CHO-M₂</th>
<th>CHO-SLM₂</th>
<th>CHO-M₃</th>
<th>CHO-M₄</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_i$</td>
<td>-8.07 ± 0.04</td>
<td>-6.29 ± 0.05</td>
<td>-6.31 ± 0.14</td>
<td>-6.54 ± 0.02</td>
<td>-7.81 ± 0.06</td>
</tr>
<tr>
<td>Pirenzepine</td>
<td>nH</td>
<td>0.91 ± 0.03</td>
<td>0.96 ± 0.08</td>
<td>0.92 ± 0.02</td>
<td>0.88 ± 0.10</td>
</tr>
<tr>
<td></td>
<td>n</td>
<td>(3)</td>
<td>(3)</td>
<td>(3)</td>
<td>(3)</td>
</tr>
<tr>
<td>$K_i$</td>
<td>-7.33 ± 0.04</td>
<td>-7.98 ± 0.01</td>
<td>-8.03 ± 0.07</td>
<td>-6.72 ± 0.05</td>
<td>-7.42 ± 0.04</td>
</tr>
<tr>
<td>Methoctramine</td>
<td>nH</td>
<td>0.91 ± 0.11</td>
<td>1.10 ± 0.03</td>
<td>0.99 ± 0.07</td>
<td>1.04 ± 0.10</td>
</tr>
<tr>
<td></td>
<td>n</td>
<td>(4)</td>
<td>(5)</td>
<td>(5)</td>
<td>(4)</td>
</tr>
<tr>
<td>$K_i$</td>
<td>-7.12 ± 0.08</td>
<td>-7.62 ± 0.03</td>
<td>-7.65 ± 0.08</td>
<td>-7.89 ± 0.06</td>
<td>-8.15 ± 0.07</td>
</tr>
<tr>
<td>Tropicamide</td>
<td>nH</td>
<td>0.89 ± 0.09</td>
<td>0.99 ± 0.09</td>
<td>1.02 ± 0.05</td>
<td>0.94 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>n</td>
<td>(3)</td>
<td>(4)</td>
<td>(4)</td>
<td>(3)</td>
</tr>
</tbody>
</table>
Table 3.3.5.

 Antibodies used in western blotting and immunoprecipitation of various Ga subunits.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Antigen sequence</th>
<th>Region of Ga</th>
<th>Specificity</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>371723</td>
<td>KNNLKDCGLF</td>
<td>C-terminal</td>
<td>Gi1α, Gi2α</td>
<td>Calbiochem</td>
</tr>
<tr>
<td>371726</td>
<td>KNNLKECGLY</td>
<td>C-terminal</td>
<td>Gi3α, Goα</td>
<td>Calbiochem</td>
</tr>
<tr>
<td>AS/7</td>
<td>KENLKDCGLF</td>
<td>C-terminal</td>
<td>Gi1α, Gi2α</td>
<td>N.E.N</td>
</tr>
<tr>
<td>EC/2</td>
<td>KNNLKECGLY</td>
<td>C-terminal</td>
<td>Gi3α, Goα</td>
<td>N.E.N</td>
</tr>
<tr>
<td>SC823</td>
<td>KEAIETEVAAMSNLVPVNE</td>
<td>N-terminal</td>
<td>Gsα</td>
<td>Santa Cruz</td>
</tr>
<tr>
<td>SC392</td>
<td>CFAAVKDTILQNLKEYNLV</td>
<td>C-terminal</td>
<td>Gq/11α</td>
<td>Santa Cruz</td>
</tr>
<tr>
<td>SC387</td>
<td>KMVCDVSVRMEDTEPFAEL</td>
<td>N-terminal</td>
<td>Goα</td>
<td>Santa Cruz</td>
</tr>
<tr>
<td>SC262</td>
<td>KNNLKECGLY</td>
<td>C-terminal</td>
<td>Gi1-3α</td>
<td>Santa Cruz</td>
</tr>
</tbody>
</table>

Details of the antigenic sequences used to generate the antisera used for western blotting and immunoprecipitation studies. The antisera utilised predominantly for western blotting against G\textsubscript{i1α}/G\textsubscript{i2α} and G\textsubscript{i3α}/G\textsubscript{qα} were supplied by Calbiochem and for the immunoprecipitaion studies antisera against G\textsubscript{i1α}/G\textsubscript{i2α} and G\textsubscript{i3α}/G\textsubscript{qα} were supplied by N.E.N. The antisera supplied by Santa Cruz were used for both western blotting and immunoprecipitation studies.
3.4. Discussion.

All the clonal cell lines used through the duration of this study were found to stably express muscarinic receptors (Figure 3.3.1.). The expression levels of receptors appear to be much higher than those initially reported by Buckley et al., (1989) who originally supplied the clones utilised. The values obtained by Buckley et al., (1989) were 180 ± 43, 121 ± 63, 1981 ± 423 and 903 ± 241 fmol mg⁻¹ for the Hm1, Hm2, Hm3 and Hm4 expressing clones respectively. Our values were 2.39 ± 0.19 to 2.72 ± 0.17 pmol mg⁻¹ for the CHO-M₁ cells and 0.54 ± 0.05 to 0.61 ± 0.10, 2.43 ± 0.18 to 2.56 ± 0.03 and 1.29 ± 0.15 to 1.52 ± 0.23 pmol mg⁻¹ for the CHO-M₂, -M₃ and -M₄ cells respectively. This may be due to the accidental selection of higher expressing subclones from the original transfected cells. This could occur easily if the original stock was a heterogeneous population, but it appears that despite this possibility the CHO-cell clones maintained their respective expression levels over time. No differences in Bₘₐₓ values (determined by [³H]-NMS saturation binding) for the transfected CHO-cells were identified in either cell membranes or permeabilised cells. This result is surprising due to the totally different nature of the cell preparation between permeabilised cells and membranes in which the structure of the cell membrane would be expected to be vastly different. This is reflected by the fact that many signalling pathways (e.g. inositol phospholipids) cannot be assessed in membrane preparations due to the loss of vital soluble proteins. The loss of soluble proteins during membrane preparation also makes the lack of difference in Bₘₐₓ values between these preparations unusual. This would lead to an overall loss of protein making the ratio of the level of receptors to total protein higher, therefore for each mg of protein the number of receptors would be increased leading to higher Bₘₐₓ values being obtained. This may not have occurred in these experiments due to the method of membrane preparation utilised. The different binding buffers utilised in the membrane and permeabilised binding may also have concealed any small differences present (Hulme et al., 1990).

The clonal cell lines utilised in this study have been characterised on the basis of each muscarinic receptor subtype possessing a distinct pharmacological profile based upon
the rank order of affinity for a number of selective muscarinic antagonists (Buckley et al., 1989; Hulme et al., 1990; Lazareno et al., 1990; Dorje et al., 1991; Caulfield, 1993; Rinken, 1995; Eglen et al., 1996). The selective antagonists used in this study pirenzepine, methoctramine and tropicamide all produced Hill coefficient values approaching unity in each of the CHO-cell clones suggesting interactions with homogeneous populations of muscarinic receptors.

Pirenzepine binds preferentially to the M₁ muscarinic receptor subtype (Hammer et al., 1980) compared to the M₂, M₃ and m₅ receptors (Dorje et al., 1991; Caulfield, 1993). However, there exists a significant overlap between affinities of pirenzepine for M₁ and M₄ receptors (Lazareno et al., 1990; Caulfield and Brown, 1991). The rank order of affinity, obtained by the method of [³H]-NMS displacement, in this study for pirenzepine was Hm₁ > Hm₄ > Hm₃ > Hm₂. The pirenzepine affinity profile is consistent with that obtained by Buckley et al. (1989) and Dorje et al. (1991) in CHO-cell membranes expressing muscarinic receptors. The affinity values for pirenzepine binding at the M₁, M₂, M₃ and M₄ receptors expressed in CHO-cells were closely correlated to the range of antagonist affinities collated in an extensive review by Caulfield (1993). Pirenzepine binding to the M₁ receptor expressed in CHO-cells bound with a pKᵦ of 8.07 which is in agreement with the values collated by Caulfield (1993) where pirenzepine was reported to bind to m₁/M₁ receptors with a pKᵦ (negative logarithm of dissociation constant) in the range 7.9-8.2 (data collated from various studies). This is also the case for the pirenzepine values for the other muscarinic receptor subtypes evaluated. The pKᵦ values for pirenzepine binding to M₂, M₃ and M₄ receptors in CHO-M₂, CHO-SLM₂, CHO-M₃ and CHO-M₄ cells respectively, were 6.29, 6.31, 6.54, and 7.81. Compared to the pKᵦ values for pirenzepine binding to m₂/M₂, m₃/M₃ and m₄/M₄ of 6.3-6.5, 6.7-7.1 and 6.2-7.1 (Caulfield 1993).

Methoctramine, initially synthesised and evaluated by Melchiorre et al. (1987), is a M₂ selective antagonist (Michel and Whiting, 1988; Lazareno and Roberts, 1989), possessing high affinity for the m₂ muscarinic subtype and low affinity for the M₃ subtype. Methoctramine produced an affinity profile of Hm₂ > Hm₄ > Hm₁ > Hm₃ in
the CHO-cell clones this is in agreement with published data (Dorje et al., 1991).
Methoctramine bound to the M₁, M₂, M₃ and M₄ receptors in CHO-M₁, CHO-M₂, 
CHO-SLM₂, CHO-M₃, and CHO-M₄ cells respectively with pKₐ values of 7.33, 7.98, 
8.03, 6.72 and 7.42. The range of pKₐ values collated from the literature by Caulfield 
for the m₁/M₁, m₂/M₂, m₃/M₃, and m₄/M₄ receptors were 7.1-7.6, 7.8-8.3, 6.3-6.9 and 
7.8-8.1 respectively. The affinity value for the M₄ receptor subtype is slightly weaker 
than the affinity profile predicts, but the rank order of affinities still suggests that the 
CHO-cell clones express the predicted muscarinic receptor subtype.

Tropicamide is proposed to be an M₄-specific antagonist (Lazareno et al., 1990; 
Lazareno et al., 1993; Lazareno and Birdsall 1993; TiPS Receptor and Ion Channel 
Nomenclature Supplement 1994). In this study affinity values for tropicamide binding 
to M₁, M₂, M₃, and M₄ receptors expressed in CHO-M₁, CHO-M₂, CHO-SLM₂, CHO-
M₃, and CHO-M₄ cells respectively were evaluated. pKₐ values of 7.89, 7.62, 7.65, 
7.12 and 8.15 were obtained for tropicamide binding in CHO-M₁, CHO-M₂, CHO-
SLM₂, CHO-M₃, and CHO-M₄ cells respectively. The order of affinity values H₄ ≥ 
H₃ > H₂ > H₁ are in agreement with those obtained by Lazareno et al., (1990) 
in the study of M₄ binding sites in rabbit lung and NG108-15 cells, however, the 
affinity values for the M₁, M₂ and M₃ subtypes obtained are in closer agreement with 
those of Rinken (1995) where tropicamide only appears to be M₄ selective upon 
receptor solubilisation. Variations in affinity estimates are seen between different 
studies by different groups. So the differences seen in tropicamide affinity may be due 
to variations in experimental conditions.

As mentioned above in the discussion of the tropicamide results, selective muscarinic 
antagonist binding affinities tend to vary by approximately 3 fold between different 
studies by different groups (Caulfield, 1993). These variations are likely to be due to 
changes in assay conditions (Hulme, 1990). Therefore, using a single selective 
antagonist for comparison of binding affinities can produce errors in defining the 
muscarinic receptor subtype present. Hence the only definitive way to evaluate the 
presence of a particular muscarinic receptor subtype is to assess the rank order of 
affinity for a number of selective antagonists.
In this study we chose to use three reportedly selective muscarinic antagonists to characterise the recombinant receptors. Comparison of the affinity profiles obtained for pirenzepine, methoctramine and tropicamide obtained for the clonal cells studied indicate that they express the expected muscarinic acetylcholine receptor subtypes. For further clarity other selective muscarinic antagonists could be utilised, for example to dissect the M$_3$ subtype 4-DAMP could have been utilised (Barlow et al., 1976; Caulfield, 1993) and himbacine to further distinguish the M$_3$ and M$_4$ subtypes (Dorje et al 1991; Caulfield, 1993). The antagonist binding properties of the cloned receptors are in close agreement with the binding properties seen at receptors endogenously present in tissues (Lazareno et al., 1990; Caulfield, 1993). This means that the pharmacological data can be seen to be physiologically relevant. Also it appears that antagonist affinities determined by radioligand binding are in agreement with those obtained in corresponding functional studies, indicating that the characteristics of the antagonist binding sites reflect the corresponding coupled receptors (Caulfield, 1993; Richards, 1991). So we conclude that our CHO-M$_1$, CHO-M$_2$, CHO-SLM$_2$, CHO-M$_3$ and CHO-M$_4$ cells stably express the M$_1$, M$_2$, M$_3$, and M$_4$ receptors respectively.

Using the specific G$\alpha$ antisera we performed western blots to identify the G$\alpha$ complement of each of the cell clones. This showed that no differences, for the G$\alpha$ proteins tested for, were seen between the untransfected CHO-K1 cells and the CHO-cells transfected with cDNA encoding the Hm1, Hm2, Hm3 and Hm4 muscarinic receptor subtypes. Therefore the introduction of an exogenous receptor into a CHO-cell appears to have no effect on the relative expression of G$\alpha$ subunits. The stability of G$\alpha$ protein expression after transfection has been observed for the G$_{q1}$,G$\alpha$ subunit in CHO-K1 cells expressing the Hm1 receptor gene (Mulaney et al., 1993). G$\alpha$ protein immunoblotting studies have been carried out to illustrate alterations in G$\alpha$ protein expression both positively (Livingstone et al., 1991; Strassheim et al., 1991; Mullaney et al., 1995; Grant et al., 1997) and negatively (McLaughlin et al., 1991; Clark et al., 1993), so this methodology would be expected to detect any change in G$\alpha$ protein levels (For review see Pennington, 1994).
Muscarinic receptor subtypes may interact with multiple G protein subtypes (Matesic et al., 1989; Matesic et al., 1991) so it is important to define the presence of G\(\alpha\) subunits in our recombinant cell lines prior to investigating their interaction with the muscarinic receptor subtypes. CHO-K1 cells have been widely used to express exogenous receptors and in a limited number of studies the G protein complement of the parental and transfected cells has been investigated (Gerhardt and Neubig, 1991; Mullaney et al., 1993). In the study by Gerhardt and Neubig the presence of the G\(_i\) isoforms G\(_{i2}\alpha\) and G\(_{i3}\alpha\) was confirmed in CHO-K1 cells expressing the \(\alpha_{2A}\)-adrenoceptor by utilising antisera to G\(_{i1}\alpha/G_{i2}\alpha\) and G\(_{i3}\alpha\). Mullaney et al., (1993) identified the presence of G\(_{q11}\alpha\), G\(_{i2}\alpha\) and G\(_s\alpha\) in CHO-K1 cells transfected with the M\(_1\) muscarinic receptor. However, there cannot be a definitive list of G\(\alpha\) proteins expressed in CHO-K1 cells as this will vary depending on the source of the cells and also changes in expression may been seen through time as the cells are passaged and therefore undergo a restricted selection.

The CHO-K1 transfected cell lines obtained from Dr N. Buckley and Dr S. Lazareno illustrated the presence of immunoreactivity when each of the G\(_{i3}\alpha\), G\(_{i12}\alpha\), G\(_s\alpha\), G\(_{q11}\alpha\) and G\(_s\alpha\) antisera were utilised. Immunoblotting of membranes allowed the identification of G\(_{i3}\alpha\), G\(_s\alpha\), G\(_s\alpha\) (long and short forms). Yet we can only speculate that all four molecular species identified by antisera to G\(_{i12}\alpha\) and G\(_{q11}\alpha\) are present in these cells. The resolution of the G\(_i\) isoforms requires a lower gel bis-acrylamide concentration (Milligan, 1993) than we used in this study. The resolution of the G\(_q\alpha\) and G\(_{i1}\alpha\) isoforms can be obtained by utilising more specific antisera or by altering the SDS-PAGE conditions by either increasing the acrylamide concentration to 13 %, or including 6 M urea (Blank et al., 1991; Milligan et al., 1993).

The cell lines CHO-M\(_1\), CHO-M\(_2\), CHO-SLM2, CHO-M\(_3\) and CHO-M\(_4\) cells stably express the M\(_1\), M\(_2\), M\(_2\), M\(_3\) and M\(_4\) receptors respectively and all these cells possess equivalent levels of G\(\alpha\) proteins that react with anti- G\(_{i30}\alpha\), G\(_{i12}\alpha\), G\(_s\alpha\), G\(_{q11}\alpha\) and G\(_s\alpha\). In the next chapter the interaction between the muscarinic acetylcholine receptor subtypes and G proteins is assessed.
Chapter Four.

$[^{35}\text{S}]\text{-GTP}^\gamma\text{S}$ Binding in Membranes
Prepared from CHO-cell clones
Expressing Recombinant Human
Acetylcholine Muscarinic Receptors.
4.0. Introduction.

Agonist stimulation of G protein coupled receptors (GPCRs) results in the activation of heterotrimeric G proteins, promoting the dissociation of GDP and the subsequent binding of GTP. The resulting activated G protein α subunit, bound to GTP, dissociates from the βγ subunits and modulates the activities of effector molecules such as adenylyl cyclase (AC) and phospholipase C (PLC). Then the GTP bound to the α subunit is hydrolysed by the action of an intrinsic GTPase yielding GDP and Pi. The GDP bound α subunits reassociate with βγ subunits and terminate the signal (Gilman, 1987; Neer, 1995). The intrinsic GTPase activity of the Gα subunit, and consequently the duration of signalling, is also modulated by the action of a further group of signalling proteins termed the regulators of G protein signalling (RGS proteins) (Koelle, 1997). This emerging family of proteins appear to act as GTPase activating proteins (GAPs) for G protein α subunits by stabilising the transition state required for the hydrolysis of GTP (Dohlman and Thorner, 1997). So the concerted actions of the Gα subunit GTPase, the RGS proteins and in some instances the effector protein (Burstein et al., 1992; Tsang et al., 1998) results in an inactive GDP-containing heterotrimeric G protein ready for the next cycle of activation.

The activation cycle of heterotrimeric G proteins is the first stage in often complex signal transduction cascades and as such has provided a key area of examination in the activity of GPCRs. To study this initial step of G protein activation by agonist-liganded receptors in a quantitative manner two main experimental approaches have been employed, the measurement of receptor-stimulated guanosine 5'-O-(γ-thio)triphosphate (GTPγS) binding by G proteins (Wieland and Jakobs, 1994) and receptor-stimulated hydrolysis of [γ32P]-guanosine 5'-triphosphate in membrane preparations (Gierschik et al., 1994). Both approaches allow the assessment of GPCR activity at the level of G protein activation rather than measurements made at more distal signalling events e.g. cAMP accumulation and inositol phosphate accumulation.

The measurement of receptor-stimulated guanosine 5'-O-(γ-thio)triphosphate binding by G proteins allows quantitative information about the level of GDP/GTP exchange...
after GPCR-stimulation to be evaluated. This is achieved by the binding of the radiolabelled GTP-analogue \( ^{35}S \)-GTP\( \gamma \)S to activated G protein \( \alpha \) subunits. This compound dissociates slowly from G\( \alpha \) subunits and is resistant to hydrolysis by the G protein \( \alpha \) subunit. Agonist-stimulated \( ^{35}S \)-GTP\( \gamma \)S binding to G\( \alpha \) subunits can be measured by using a membrane preparation containing the receptor of interest in the presence of radiolabelled GTP\( \gamma \)S. \( ^{35}S \)-GTP\( \gamma \)S will bind to the activated G\( \alpha \) subunit population and this can be separated from free \( ^{35}S \)-GTP\( \gamma \)S by rapid vacuum filtration. Utilising this methodology \( ^{35}S \)-GTP\( \gamma \)S binding can be used to define agonist potency and intrinsic activity. \( ^{35}S \)-GTP\( \gamma \)S binding has been employed in numerous studies to investigate agonist efficacy at many diverse receptor subtypes, including muscarinic M\( _1 \)-M\( _4 \) (Lazareno et al., 1993a, 1993b; Burford et al., 1995b), adenosine A\( _1 \) (Lorenzen et al., 1993), \( \mu \) opioid (Traynor and Nahorski 1995; Cohen et al., 1996; Selley et al., 1996), dopamine D\( _2 \) (Gardner et al., 1997), 5-hydroxytrptamine 5HT\( _{1A} \), 5HT\( _{1D} \) (Thomas et al., 1995; Newman-Tancredi et al., 1996; Stanton and Beer, 1997; Pauwels et al., 1997, 1998), somatostatin sst\( _{4} \) (Williams et al., 1997) and cannabinoid CB\( _1 \) and CB\( _2 \) (Breivogel et al., 1998).

These studies have illustrated that certain experimental conditions are required to observe agonist-stimulated \( ^{35}S \)-GTP\( \gamma \)S binding. \( Mg^{2+} \) is required to observe \( ^{35}S \)-GTP\( \gamma \)S binding and at higher concentrations (millimolar range) to observe agonist-stimulated \( ^{35}S \)-GTP\( \gamma \)S binding (Hilf et al., 1989; Hilf et al., 1992).

The second important requirement to maximise receptor-stimulated \( ^{35}S \)-GTP\( \gamma \)S binding is the inclusion of GDP in the reaction mixture. GDP has been shown to decrease basal \( ^{35}S \)-GTP\( \gamma \)S binding to a greater extent than agonist-stimulated \( ^{35}S \)-GTP\( \gamma \)S, allowing the signal-to-noise to be optimised. The requirement for micromolar concentrations of GDP to enhance agonist effects in membrane preparations has been reported widely in most systems studied to date (Hilf et al., 1989; Gierschik et al., 1991; Wieland et al., 1992; Lorenzen et al., 1993). GDP concentration has been shown to play an important role in determining the maximal effect and apparent potency of receptor ligands assessed by \( ^{35}S \)-GTP\( \gamma \)S binding. The modulation of intrinsic activity by GDP has been observed in the adenosine A\( _1 \), 5HT\( _{1A} \) and
cannabinoid receptor systems (Lorenzen et al., 1993; Pauwels et al., 1997, 1998; Breivogel et al., 1998). The effect of GDP concentration on agonist efficacy is proposed to involve the activity state of G proteins.

A third requirement to enhance agonist-stimulated \[^{35}\text{S}\]-GTP\(\gamma\)S binding is NaCl. The effect of Na\(^+\) to reduce agonist efficacy could be seen in cardiac membranes, 150 mM NaCl reduced the basal binding of \[^{35}\text{S}\]-GTP\(\gamma\)S binding and produced a 10 fold increase in the \(EC_{50}\) value for carbachol, without significant effect upon the extent of absolute \[^{35}\text{S}\]-GTP\(\gamma\)S binding (Hilf et al., 1989; Hilf and Jakobs 1992). The sst\(_5\) receptor subtype showed that Na\(^+\) in this system had a similar effect, with the agonists SRIF-28, SRIF-14 and L-362,855 displaying lower \(EC_{50}\) values in the presence of 50 mM Na\(^+\) than in the presence of 150 mM Na\(^+\) (Williams et al., 1997). Both systems showed that Na\(^+\) decreased basal \[^{35}\text{S}\]-GTP\(\gamma\)S binding and agonist-stimulated \[^{35}\text{S}\]-GTP\(\gamma\)S binding.

According to Costa et al. (1990; 1992) the efficacy of a ligand depends upon the ability of a given ligand to induce coupling of the receptor to the G protein. So GDP or NaCl concentrations, which serve to alter the position of the binding equilibrium between the receptor and G protein, will affect the efficacy of a ligand in an inversely proportional manner to the given efficacy of the specified ligand.

As discussed in Chapter Three I have characterised the muscarinic receptor subtypes and assessed the G protein complement in the cell lines utilised in this study. The next step was to elucidate the receptor subtype-G protein interactions and to evaluate these interactions in a quantitative manner. In this series of experiments the recombinant human muscarinic receptor subtypes expressed in CHO-cells were evaluated at the level of G protein activation by utilising \[^{35}\text{S}\]-GTP\(\gamma\)S binding.

4.1 Methods.

For detail on the methodologies used throughout this Chapter see Chapter Two 'Experimental Methodology' with reference to the sections entitled 'Preparation of
Cell Membranes’, ‘[^35S]-GTPγS Radioligand Binding Mediated by Human Muscarinic Receptors Stably Expressed in CHO-cells’, ‘[^3H]-NMS Radioligand Binding to Stably Transfected CHO cells’, and ‘Protein Determinations’.

4.2. Data Analysis.

[^35S]-GTPγS radioligand binding data were analysed using a least-sum-of-squares non-iterative curve fitting program, Prism 2 (dose-response curve (variable slope)) to determine IC$_{50}$, EC$_{50}$ and Hill coefficient (nH) values.

[^35S]-GTPγS isotope dilution data for each individual experiment were analysed by both linear (Scatchard analysis) and non-linear approaches to determine maximal binding capacity (B$_{max}$) and equilibrium dissociation constant (K$_D$) values, again these parameters were obtained using Prism 2.

[^3H]-NMS competition binding experiments were analysed by Prism 2 (dose-response curve (variable slope)), which generates IC$_{50}$ and Hill coefficient values. IC$_{50}$ values were adjusted to inhibition dissociation constant values (K$_{b}$) by utilising the concentration of the competing radioligand and the method of Leff and Dougall (1993). K$_{b}$ values reported were generated using the K$_D$ values reported in Table 3.3.2. All data are shown as means ± s.e.mean for ‘n’ separate experiments.

4.3. Results.

Effect of time and GDP concentration upon methacholine-stimulated[^35S] GTPγS binding in CHO-cell membranes expressing recombinant muscarinic receptors.

Time-course studies in the presence of 10 μM GDP were carried out initially in the presence and absence of 1 mM methacholine and optimum methacholine-stimulated[^35S] GTPγS binding over basal was observed after a 30 min incubation (Figure 4.3.1) in CHO-M2, CHO-SLM2 and CHO-M4 cell membranes. This time point also provided
large stimulations over basal in the CHO-M1 and CHO-M3 cell lines (Figure 4.3.1). All further experiments were therefore incubated for 30 min. Methacholine-stimulated $[^{35}\text{S}]-\text{GTP\gamma S}$ binding was also evaluated in non-transfected parental CHO-cells, these cells produced no response to methacholine for up to 120 min. Untransfected CHO-K1 cells gave rise to basal $[^{35}\text{S}]-\text{GTP\gamma S}$ binding values of $8577 \pm 769$ cpm and methacholine-stimulated values of $8182 \pm 538$ cpm at 30 min (n=3). This indicates that the CHO-K1 cell line possesses no endogenous muscarinic acetylcholine receptors. (Data shown in Figure 4.3.2.).

Methacholine-stimulated $[^{35}\text{S}]-\text{GTP\gamma S}$ binding was not observed in any of the CHO-cell clones in the absence of GDP (data not shown). Co-incubation of increasing concentrations of GDP in the presence of 1 mM methacholine and approx. 70 pM $[^{35}\text{S}]-\text{GTP\gamma S}$ illustrated that the inclusion of GDP in the reaction mixture was required to increase the ratio of agonist response to basal activity for the M2 and M4 muscarinic acetylcholine receptor subtypes. Increasing GDP concentration from $1 \times 10^{-9}$ to $1 \times 10^{-4}$ M lead to a dose-dependent decrease in total $[^{35}\text{S}]-\text{GTP\gamma S}$ binding in the CHO-M2, CHO-SLM2 and CHO-M4 cell lines (Figure 4.3.4.b, Figure 4.3.5.b and Figure 4.3.7.b). The reduction in $[^{35}\text{S}]-\text{GTP\gamma S}$ binding is more pronounced in the absence of methacholine than in its presence, producing an augmentation in the percentage of methacholine-stimulated $[^{35}\text{S}]-\text{GTP\gamma S}$ binding over basal as the GDP concentration is increased (Figure 4.3.4.a, Figure 4.3.5.a and Figure 4.3.7.a). This is illustrated by the percentage increase in $[^{35}\text{S}]-\text{GTP\gamma S}$ binding over basal values observed in the CHO-M2, CHO-SLM2 and CHO-M4 cell membranes in the presence of GDP at a final concentration of 0.1 μM and 10 μM. The M2 expressing CHO-M2 cell line produced increases over basal $[^{35}\text{S}]-\text{GTP\gamma S}$ binding of $116 \pm 4$ % and $214 \pm 5$ % (n=3), and the CHO-SLM2 cell line produced values of $112 \pm 3$ % and $255 \pm 34$ % (n=3) in the presence of 0.1 and 10 μM GDP respectively. The M4 expressing cell line, CHO-M4, elicited percentage increases over basal $[^{35}\text{S}]-\text{GTP\gamma S}$ binding of $141 \pm 2$ % and $328 \pm 56$ % in the presence of 0.1 and 10 μM GDP respectively (n=3). In further experiments a final concentration of 10 μM GDP was utilised in further studies as this produced a large stimulation over basal.
Altering the GDP concentration from $1 \times 10^{-7}$ to $1 \times 10^{-5}$ M lead to a dose-dependent decrease in both the basal and agonist-stimulated $[^{35}\text{S}]-\text{GTP}_{\gamma}\text{S}$ binding in the CHO-M$_1$ and CHO-M$_3$ cell lines. The differential reduction in both the basal and agonist-stimulated $[^{35}\text{S}]-\text{GTP}_{\gamma}\text{S}$ binding in these cell lines leads to an increase in the percentage of methacholine-stimulated $[^{35}\text{S}]-\text{GTP}_{\gamma}\text{S}$ binding over basal as the GDP concentration is increased (Figure 4.3.3.a, b and Figure 4.3.6.a, b). At 0.1 and 10 μM GDP the M$_1$ expressing CHO-cells produced increases over basal $[^{35}\text{S}]-\text{GTP}_{\gamma}\text{S}$ binding of 47.8 ± 10.8 % and 71.4 ± 6.1 % and the M$_3$ expressing CHO-cells elicited increases over basal $[^{35}\text{S}]-\text{GTP}_{\gamma}\text{S}$ binding of 29.2 ± 3.6 % and 36.5 ± 4.0 %. So increasing the concentration of GDP in the CHO-M$_1$ and CHO-M$_3$ cell lines appears to maximise the ratio of methacholine-stimulated $[^{35}\text{S}]-\text{GTP}_{\gamma}\text{S}$ binding to basal binding. To maintain identical experimental conditions across the muscarinic receptor subtypes, and maximise the signal-to-noise level for $[^{35}\text{S}]-\text{GTP}_{\gamma}\text{S}$ binding, a final concentration of 10 μM GDP was utilised in further studies of all the CHO-cell lines. This produces statistically significant stimulations over basal (P<0.05, paired Student's t-test) in all the CHO-cell lines. The differential effects of GDP concentration are discussed later in this Chapter.

Using binding conditions of 30 min incubations at 30°C with the inclusion of 10 μM GDP the CHO-M$_1$, CHO-M$_2$, CHO-SLM$_2$, CHO-M$_3$ and CHO-M$_4$ cell membranes produced maximal methacholine-stimulated $[^{35}\text{S}]-\text{GTP}_{\gamma}\text{S}$ binding as cpm above basal values of; 7923 ± 1180 (n=3), 16960 ± 790 (n=3), 16835 ± 1686 (n=3), 3266 ± 434 (n=3) and 18131 ± 266 (n=3), with basal values of 9202 ± 583, 13383 ± 969, 19177 ± 1210, 7926 ± 243 and 12935 ± 556 respectively (Figure 4.3.1). Under these conditions atropine (10 μM) completely inhibited methacholine-stimulated $[^{35}\text{S}]-\text{GTP}_{\gamma}\text{S}$ binding for all the muscarinic receptor subtypes examined (data not shown).

**Effect of pertussis toxin pre-treatment on methacholine-stimulated $[^{35}\text{S}]-\text{GTP}_{\gamma}\text{S}$ binding in CHO-cell membranes expressing recombinant muscarinic receptors.**

Pertussis toxin (PTx) pre-treatment (100 ng ml$^{-1}$ 18-20 h, for details see the Experimental Methodology section entitled ‘Cell Culture Techniques’) resulted in a
75-85% decrease in the basal $[^{35}\text{S}]-\text{GTP}_{\gamma}\text{S}$ binding in the CHO-M$_2$, CHO-SLM$_2$ and CHO-M$_4$ cell lines. The maximal methacholine-stimulated $[^{35}\text{S}]-\text{GTP}_{\gamma}\text{S}$ binding in the presence of pertussis toxin was reduced by greater than 99% compared to responses observed in membranes from non-PTx-treated cells. The methacholine-stimulated $[^{35}\text{S}]-\text{GTP}_{\gamma}\text{S}$ binding in absence and presence of 100 ng ml$^{-1}$ PTx in the CHO-M$_2$, CHO-SLM$_2$ and CHO-M$_4$ cell lines were 13776 ± 801, 339 ± 134, 17361 ± 1012, 153 ± 34, 18408 ± 1111 and 91 ± 34 cpm above basal respectively, see Figure 4.3.8 (n=4). Agonist-stimulated $[^{35}\text{S}]-\text{GTP}_{\gamma}\text{S}$ binding was no longer statistically significant when compared to basal levels after pertussis toxin pre-treatment in the CHO-M$_2$, CHO-SLM$_2$ and CHO-M$_4$ cell lines (P > 0.05 paired Student’s t-test). Pertussis toxin at a final concentration of 100 ng ml$^{-1}$ is maximally effective as increasing the concentration to 300 ng ml$^{-1}$ produces no further decrease in the agonist-stimulated $[^{35}\text{S}]-\text{GTP}_{\gamma}\text{S}$ binding elicited by 1 mM methacholine (Figure 4.3.9).

Pertussis toxin pre-treatment (100 ng ml$^{-1}$) resulted in decreases in both the basal and agonist-stimulated $[^{35}\text{S}]-\text{GTP}_{\gamma}\text{S}$ binding in the CHO-M$_1$ and CHO-M$_3$ cell lines. Pertussis toxin concentrations of 30 ng ml$^{-1}$, 100 ng ml$^{-1}$ and 300 ng ml$^{-1}$ all elicited equivalent reductions in methacholine-stimulated $[^{35}\text{S}]-\text{GTP}_{\gamma}\text{S}$ binding, illustrating that all the $G_i$-like G proteins had been successfully inactivated (Figure 4.3.9). However, the use of 100 ng ml$^{-1}$ toxin to inactivate the $G_i$-like G proteins did not prevent statistically significant methacholine-stimulated increases over basal levels mediated by both the M$_1$ and M$_3$ receptor subtypes (Figure 4.3.8). PTx-treatment of CHO-M$_1$ cells resulted in a significant reduction in methacholine-stimulated $[^{35}\text{S}]-\text{GTP}_{\gamma}\text{S}$ binding above basal levels after 30 min incubation at 30°C in the presence of 10 µM GDP from 6853 ± 829 to 3321 ± 282 (P < 0.05 paired Student’s t-test (n=3)); a reduction of approximately 52% of the methacholine-stimulated $[^{35}\text{S}]-\text{GTP}_{\gamma}\text{S}$ binding observed in untreated membranes. After PTx pre-treatment methacholine-stimulated $[^{35}\text{S}]-\text{GTP}_{\gamma}\text{S}$ binding remained significantly higher than basal levels of $[^{35}\text{S}]-\text{GTP}_{\gamma}\text{S}$ binding in the presence of PTx (P < 0.05 paired Student’s T-test, refer Figure 4.3.8). PTx pre-treatment of CHO-M$_3$ cells also resulted in a significant reduction in methacholine-stimulated $[^{35}\text{S}]-\text{GTP}_{\gamma}\text{S}$ binding above basal levels after 30 min incubation at 30°C in the presence of 10 µM GDP from 4053 ± 323 to 2830 ± 286 (P
< 0.05 paired Student's t-test (n=3); a reduction of approximately 30 % of the methacholine-stimulated [35S]-GTPγS binding. Again as in the CHO-M1 expressing membranes the CHO-M3 cell membranes still elicited significantly higher methacholine-stimulated [35S]-GTPγS binding in the presence of PTx when compared to basal levels (P < 0.05 paired Student's T-test, refer Figure 4.3.8). This enabled the further investigation of the M1 and M3 receptor subtypes at the level of [35S]-GTPγS binding when only a non-pertussis toxin sensitive pool of G proteins is available.

Time-course analysis carried out in the presence and absence of 1 mM methacholine with the inclusion of 10 μM GDP showed a reduction in the methacholine-stimulated [35S]-GTPγS binding over basal as noted previously, but the time profile of activation followed a similar pattern in the presence and absence of PTx (Figure 4.3.10). The CHO-M1 cell membranes gave rise to 7923 ± 1180 cpm above a basal value of 9202 ± 583 at 30 min in the absence of PTx compared to a stimulation of 3951 ± 490 over a basal value of 4079 ± 159 in the presence of PTx, corresponding to a 50 % reduction in methacholine-stimulated [35S]-GTPγS binding and an equivalent fall in basal [35S]-GTPγS binding of approximately 55% (n=3). The CHO-M3 cell membranes gave rise to 3266 ± 434 cpm above a basal value of 7926 ± 243 at 30 min in the absence of PTx compared to a stimulation of 2510 ± 131 above a basal value of 3877 ± 297 in the presence of PTx (Figure 4.3.10). This corresponds to a 24 % reduction in methacholine-stimulated [35S]-GTPγS binding in the presence of PTx, which is smaller than that seen with the CHO-M1 cell line. The reduction in basal [35S]-GTPγS binding was equivalent to that seen in the CHO-M1 cell line giving a value of approximately 51% (n=3).

Quantitation of G proteins activated by agonist stimulation in CHO-cell membrane preparations expressing recombinant muscarinic receptors.

Isotope-dilution analysis of methacholine-stimulated [35S]-GTPγS binding was performed by incubating CHO-cell membranes with a single concentration of [35S]-GTPγS (70 pM) and increasing concentrations of unlabelled GTPγS in the absence and presence of methacholine (1mM). The difference in binding determined in the
presence versus the absence of agonist provides an estimate of the G protein receptor population activated by the specified muscarinic receptor subtype. The stimulation of $[^{35}\text{S}]-\text{GTP} \gamma \text{S}$ binding by methacholine via the $M_1$, $M_2$, $M_3$, and $M_4$ receptor subtypes was to a single high affinity site. The results were analysed by both linear and non-linear regression methods to obtain $B_{\text{max}}$ and $K_D$ values, the data obtained were comparable and are shown in Table 4.3.1 and Figure 4.3.11. Scatchard analysis of these data suggested that methacholine activates a population of G proteins with a $K_D$ of $3.37 \pm 0.92$ nM and a $B_{\text{max}}$ of $1957 \pm 302$ fmol mg$^{-1}$ via the $M_1$ receptor subtype expressed in CHO-M$_1$ cells ($n=4$). Further Scatchard analyses of the data obtained from the other muscarinic receptor subtypes gave rise to activated G protein populations with definable $K_D$ and $B_{\text{max}}$ values of $1.18 \pm 0.08$ nM and $1469 \pm 108$ fmol mg$^{-1}$ for the $M_2$ receptor expressed in CHO-M$_2$ cells ($n=3$); $1.27 \pm 0.03$ nM and $1669 \pm 224$ fmol mg$^{-1}$ for the $M_2$ receptor expressed in CHO-SLM$_2$ cells ($n=3$); $4.85 \pm 0.47$ nM and $1540 \pm 154$ fmol mg$^{-1}$ for the $M_3$ receptor expressed in CHO-M$_3$ cells ($n=4$); $1.12 \pm 0.06$ nM and $2254 \pm 157$ fmol mg$^{-1}$ for the $M_4$ receptor expressed in CHO-M$_4$ cells ($n=3$).

**Pharmacological characterisation of agonist-stimulated $[^{35}\text{S}]-\text{GTP} \gamma \text{S}$ binding in CHO-cell membranes expressing recombinant muscarinic acetylcholine receptors.**

Using the characterised assay conditions, full concentration-response analyses were carried out using the muscarinic acetylcholine receptor ‘full’ agonist methacholine and the muscarinic receptor ‘partial’ agonists arecoline and pilocarpine in all five cell lines. Although the increases in $[^{35}\text{S}]-\text{GTP} \gamma \text{S}$ binding stimulated by 1 mM methacholine in the CHO-M$_1$ and CHO-M$_3$ cell membranes are small in comparison to the levels stimulated in the CHO-M$_2$, CHO-SLM$_2$, and CHO-M$_4$ cell membranes the level of stimulation elicited by all five receptor expressing cell lines was sufficient to investigate the actions of receptor agonists at the level of G protein activation.

The muscarinic acetylcholine receptor agonist methacholine acted as a ‘full’ agonist at all the receptor subtypes. When assessed at the level of $[^{35}\text{S}]-\text{GTP} \gamma \text{S}$ binding
methacholine caused maximal effects at all four receptors. The log M EC\textsubscript{50} values for methacholine at the M\textsubscript{1}, M\textsubscript{2}, M\textsubscript{3} and M\textsubscript{4} receptor subtypes expressed in CHO-M\textsubscript{1}, CHO-M\textsubscript{2}, CHO-SLM\textsubscript{2}, CHO-M\textsubscript{3} and CHO-M\textsubscript{4} were -4.86 ± 0.07 (14 μM (n=4)), -6.41 ± 0.05 (0.39 μM (n=3)), -6.66 ± 0.06 (0.22 μM (n=3)), -4.32 ± 0.02 (48 μM (n=3)) and -6.08 ± 0.08 (0.85 μM (n=4)) respectively (Figure 4.3.12). From these values it can be seen that methacholine is at least 10-fold more potent at the M\textsubscript{2} and M\textsubscript{4} receptors than the M\textsubscript{1} and M\textsubscript{3} receptors. A direct comparison of methacholine concentration-response curves between PTx pre-treated (100 ng ml\textsuperscript{-1}) and untreated CHO-M\textsubscript{1} and -M\textsubscript{3} cells revealed that when the M\textsubscript{1} and M\textsubscript{3} receptor subtypes only had access to the pertussis toxin insensitive pool of G proteins methacholine appeared more potent (Students t-test P < 0.05) (Figure 4.3.13). Figure 4.3.13 also shows the change in the hill slope for methacholine-stimulated [\textsuperscript{35}S]-GTP\textgamma{}S binding in the presence of PTx compared to the absence of the toxin. The log M EC\textsubscript{50} values for methacholine at the M\textsubscript{1} receptor were -4.76 ± 0.10 (18 μM) and -5.41 ± 0.06 (3.9 μM) in the absence and presence of pertussis toxin respectively (n=3). The M\textsubscript{3} receptor produced EC\textsubscript{50} values for methacholine of -4.56 ± 0.19 (33 μM) and -4.69 ± 0.07 (21 μM) log M in the absence and presence of pertussis toxin respectively (n=3).

Analysis of the muscarinic acetylcholine receptor ‘partial’ agonists arecoline and pilocarpine at the level of the [\textsuperscript{35}S]-GTP\textgamma{}S binding response revealed that arecoline acted as a ‘full’ agonist at the M\textsubscript{2} and M\textsubscript{4} receptor subtypes, but behaved as a ‘partial’ agonist at the M\textsubscript{1} and M\textsubscript{3} receptor subtypes. Arecoline displayed E\textsubscript{max} values of 113 ± 11, 129 ± 9 and 135 ± 7 % (n=3) when expressed as a percentage of 1 mM methacholine response in the M\textsubscript{2} and M\textsubscript{4} expressing cell lines CHO-M\textsubscript{2}, CHO-SLM\textsubscript{2} and CHO-M\textsubscript{4} respectively (Figure 4.3.14). Yet in the M\textsubscript{1} and M\textsubscript{3} receptor expressing cell lines arecoline elicited E\textsubscript{max} values of 55 ± 2 and 58 ± 4 % (n=3) when expressed as a percentage of 1 mM methacholine-stimulated [\textsuperscript{35}S]-GTP\textgamma{}S binding (Figure 4.3.14). However, another reported ‘partial’ agonist pilocarpine acted as partial agonist at all the receptor subtypes, revealing E\textsubscript{max} values of 36 ± 5, 57 ± 3, 59 ± 3, 48 ± 6 % and 25 ± 2 % (n=3) expressed as a percentage of 1 mM methacholine-stimulated [\textsuperscript{35}S]-GTP\textgamma{}S binding for the CHO-M\textsubscript{1}, CHO-M\textsubscript{2}, CHO-SLM\textsubscript{2}, CHO-M\textsubscript{3} and CHO-M\textsubscript{4}.
and CHO-M4 cell lines respectively (Figure 4.3.13). These findings are in agreement with previously published data (Lazareno et al., 1993).

Concentration-response analysis of both arecoline and pilocarpine was carried out for the M2 and M4 receptor subtypes in the CHO-M2, CHO-SLM2, and CHO-M4 cell lines. Also, arecoline concentration-response analysis at the level of [35S]-GTPγS binding was carried out for the M3 receptor in the CHO-M3 cell line. Full concentration-response analyses were not carried out for pilocarpine at the M1 and M3 muscarinic receptor subtypes, or arecoline at the M3 subtype, because the maximal responses were too small to allow accurate assessment of concentration-dependent effects.

A maximal concentration of arecoline (3 mM) elicited a small increase over basal [35S]-GTPγS binding of 18 ± 1% from the M3 receptor subtype expressed in CHO-M3 cells (Table 4.3.2 (n=4)) and a 27 ± 1% increase over basal [35S]-GTPγS binding at the M1 muscarinic receptor subtype (n=4). Arecoline (100 μM) elicited maximal responses in the CHO-M2, CHO-SLM2 and CHO-M4 cell lines of 110 ± 11, 142 ± 9, and 232 ± 11% increases over basal [35S]-GTPγS binding respectively (n=3) (Figure 4.3.15). Arecoline displayed partial agonist effects at the M1 receptor as discussed above, yet although the maximal response to arecoline was truncated it still displayed a high potency reflected in the EC50 values obtained. The log M EC50 values for arecoline at the M1, M2 and M4 receptor subtypes expressed in CHO-M1, CHO-M2, CHO-SLM2 and CHO-M4 cells are -5.52 ± 0.11 (3.1 nM (n=4)), -5.82 ± 0.13 (1.6 nM (n=3)), -6.06 ± 0.08 (0.9 nM (n=3)) and -5.90 ± 0.11 (1.3 nM (n=3)) log M respectively (Figure 4.3.15). From these values, and those above, it can be seen that arecoline is more potent at the M2 and M4 receptor subtypes than methacholine.

Pilocarpine at a concentration of 3 mM elicited 17 ± 2 and 15 ± 2 percentage increases over basal [35S]-GTPγS binding, for the M1 and M3 receptor subtypes respectively (Table 4.3.2 (n=4)). This level of maximal stimulation for these receptor subtypes was insufficient to allow full characterisation. Pilocarpine (100 μM) produced maximal responses (expressed as percentage increases over basal [35S]-GTPγS binding) of 56 ± 3, 65 ± 4 and 44 ± 3 in the CHO-M2, CHO-SLM2 and CHO-M4 cell lines respectively.
Full concentration-response analyses were performed producing log M EC$_{50}$ values for pilocarpine of -6.01 ± 0.03 (0.81 nM (n=3)), -6.10 ± 0.03 (0.84 nM (n=3)) and -5.97 ± 0.06 (1.16 nM (n=4)) in the CHO-M$_2$, CHO-SLM$_2$ and CHO-M$_4$ cell lines respectively (Figure 4.3.16). All the results obtained for the muscarinic receptor agonists are in general agreement with those reported by Lazareno et al. (1993a).

For comparison to the EC$_{50}$ values obtained for methacholine-stimulated [$^{35}$S]-GTP$_{Y}$S binding, methacholine binding affinities were obtained under the same conditions utilised for the [$^{35}$S]-GTP$_{Y}$S binding assay. Hence binding profile experiments were performed in the presence of 70 pM GTP$_{Y}$S, 10 µM GDP and 100 mM NaCl (Figure 4.3.17 and Table 4.3.3) and apparent K$_a$ values were obtained (Leff and Dougall, 1993). In Figure 4.3.17 it can be seen that 70 pM GTP$_{Y}$S and 10 µM GDP can elicit changes in both the binding affinity and Hill slope values obtained at all of the receptor subtypes in response to methacholine (Table 4.3.3). The presence of the guanine nucleotides elicits increases in the K$_a$ values as the guanine nucleotides uncouple the G protein from the receptor causing the receptor to shift to a lower affinity state. When the values obtained by methacholine-stimulated [$^{35}$S]-GTP$_{Y}$S binding and methacholine-induced [$^3$H]-NMS displacement at the muscarinic receptor subtypes are compared it can be seen that methacholine appears more potent when evaluated at the level of G protein activation (Table 4.3.4).

Identification of atropine-inhibited basal [$^{35}$S]-GTP$_{Y}$S binding in CHO-cell membranes expressing recombinant muscarinic acetylcholine receptors.

Atropine was shown in the cell lines expressing adenylyl cyclase-linked muscarinic acetylcholine receptor subtypes to mediate a decrease in basal [$^{35}$S]-GTP$_{Y}$S binding. The direct comparison of methacholine-stimulated [$^{35}$S]-GTP$_{Y}$S binding and atropine-induced inhibition of basal [$^{35}$S]-GTP$_{Y}$S binding is shown in Figure 4.3.18. In the CHO-M$_2$, CHO-SLM$_2$ and CHO-M$_4$ cell membranes 1mM methacholine-stimulated 196 ± 5, 268 ± 9 and 224 ± 11 % increases of basal [$^{35}$S]-GTP$_{Y}$S binding whereas atropine-mediated 9.4 ± 1.8, 22.7 ± 0.5 and 15.6 ± 1.6 % decreases in basal [$^{35}$S]-GTP$_{Y}$S binding. The levels of methacholine-stimulated increases in basal [$^{35}$S]-GTP$_{Y}$S
binding can be seen to correlate to the receptor density measured in the CHO-M2, 
CHO-SLM2 and CHO-M4 cell lines (see Chapter 3, Section 3.3 ‘Results’).

To characterise the reduction in basal $[^{35}\text{S}]-\text{GTP}\gamma\text{S}$ binding further, concentration-
response analyses were performed (Figure 4.3.19). IC$_{50}$ values for the atropine-
mediated decrease in basal $[^{35}\text{S}]-\text{GTP}\gamma\text{S}$ binding were 1.1 nM (n=4), 1.6 nM (n=4) and 
2.9 nM (n=4) in the M$_2$ and M$_4$ receptor expressing cell lines CHO-M$_2$, CHO-SLM$_2$
and CHO-M$_4$ respectively. These values correlate closely to previously reported
values for atropine binding affinity estimates (Michel and Whiting 1988; Buckley et
al., 1989; Lazareno and Roberts, 1989; Lazareno et al., 1990). In an attempt to
maximise the reduction in basal $[^{35}\text{S}]-\text{GTP}\gamma\text{S}$ binding the Na$^+$ concentration in the
assay was reduced from 100 mM to 10 mM, and the GDP concentration was altered
from $1 \times 10^{-7}$ to $1 \times 10^{-5}$ M (Figure 4.3.20). Altering either the Na$^+$ or GDP present in
the assay failed to increase the atropine-mediated change in basal $[^{35}\text{S}]-\text{GTP}\gamma\text{S}$
binding observed in any of the cell lines under investigation. In the CHO-M$_2$ cell
membranes (GDP, 10 μM) atropine-mediated a 9.8 ± 1.1 and 12.1 ± 0.5 % decrease in
basal $[^{35}\text{S}]-\text{GTP}\gamma\text{S}$ binding in the presence of 10 and 100 mM NaCl respectively.
There was no statistically significant difference in the values obtained in high and low
sodium (Paired Student’s t-test, P > 0.05). CHO-SLM$_2$ cell membranes (GDP, 10 μM)
showed atropine-mediated decreases of 14.3 ± 1.1 and 21.6 ± 1.2 % in basal $[^{35}\text{S}]-
\text{GTP}\gamma\text{S}$ binding in the presence of 10 and 100 mM NaCl. Atropine-mediated decreases
in basal $[^{35}\text{S}]-\text{GTP}\gamma\text{S}$ binding in CHO-M$_4$ cell membranes of 10.4 ± 1.7 and 15.0 ± 1.6
% in the presence of 10 and 100 mM Na$^+$. Altering the Na$^+$ and GDP concentrations
present in the assay failed to increase the atropine-inhibition of basal $[^{35}\text{S}]-\text{GTP}\gamma\text{S}$
binding observed in any of the 3 cell lines under investigation.
Figure 4.3.1.

Time-course of methacholine-stimulated $[^{35}\text{S}]$-GTP$\gamma$S binding in CHO-cell membranes.

Time-courses of $[^{35}\text{S}]$-GTP$\gamma$S binding to $M_1$-$M_4$ cell membranes. CHO-cell membranes (100 µg) were incubated in the presence of GDP (10 µM) and $[^{35}\text{S}]$-GTP$\gamma$S (70 pM) for the time indicated. Data are expressed as cpm bound above basal for all time-points observed and are shown as means ± s.e.mean of values obtained from 3 separate experiments, performed in duplicate.
Effect of methacholine upon $[^{35}\text{S}]$-GTP$\gamma$S binding in CHO-K1 untransfected cell membranes.

Time-course of $[^{35}\text{S}]$-GTP$\gamma$S binding to untransfected CHO-K1 cell membranes. CHO-K1 (100 μg) cell membranes were incubated in the presence of GDP (10 μM) and $[^{35}\text{S}]$-GTP$\gamma$S (70 pM) for the time indicated. Data are expressed as cpm bound for all time-points and are shown as means ± s.e.mean of values obtained from 3 separate experiments, performed in duplicate.
Figure 4.3.3.a.

Effect of GDP upon 1 mM methacholine-stimulated $[^{35}\text{S}]$-GTPγS binding in CHO-M₁ cell membranes.

[Graph showing effect of GDP on basal and stimulated $[^{35}\text{S}]$-GTPγS binding.]

Figure 4.3.3.b.

Effect of GDP at increasing concentrations on basal and methacholine-stimulated $[^{35}\text{S}]$-GTPγS binding in CHO-M₁ cell membranes.

[Graph showing effect of GDP on basal and stimulated $[^{35}\text{S}]$-GTPγS binding.]

CHO-cell membranes (100μg) were incubated in the presence and absence of 1 mM methacholine at 30°C, and the change in basal and stimulated $[^{35}\text{S}]$-GTPγS binding was observed after 30 min. Data are expressed as the mean percentage increases in basal $[^{35}\text{S}]$-GTPγS binding ± s.e.mean of 4 individual experiments (a), and the mean cpm bound ± s.e.mean of 4 individual experiments (b).
Figure 4.3.4.a.
Effect of GDP upon methacholine-stimulated $[^{35}\text{S}]-\text{GTP}\gamma\text{S}$ binding in CHO-M$_2$ cell membranes.

![Graph showing effect of GDP on methacholine-stimulated $[^{35}\text{S}]-\text{GTP}\gamma\text{S}$ binding in CHO-M$_2$ cell membranes.](image1)

Figure 4.3.4.b.
Effect of GDP at increasing concentrations on basal and methacholine-stimulated $[^{35}\text{S}]-\text{GTP}\gamma\text{S}$ binding in CHO-M$_2$ cell membranes.

![Graph showing effect of GDP on basal and methacholine-stimulated $[^{35}\text{S}]-\text{GTP}\gamma\text{S}$ binding in CHO-M$_2$ cell membranes.](image2)

CHO-M$_2$ membranes (100 μg) were incubated in the presence or absence of 1 mM methacholine. The change in $[^{35}\text{S}]-\text{GTP}\gamma\text{S}$ binding after exposure to varying GDP concentrations (as indicated) was observed after 30 min incubations. The data are expressed as the mean percentage increases in basal $[^{35}\text{S}]-\text{GTP}\gamma\text{S}$ binding ± s.e.mean of 3 individual experiments (a), and the mean ± s.e.mean cpm bound (n=3) (b).
Figure 4.3.5.a.
Effect of GDP upon methacholine-stimulated [$^{35}$S]GTPyS binding in CHO-SLM$_2$ cell membranes.

![Graph showing the effect of GDP on methacholine-stimulated $[^{35}\text{S}]$GTPyS binding.](image)

Figure 4.3.5.b.
Effect of GDP at increasing concentrations on basal and methacholine-stimulated [$^{35}$S]GTPyS binding in CHO-SLM$_2$ cell membranes.

![Graph showing the effect of GDP on basal and methacholine-stimulated $[^{35}\text{S}]$GTPyS binding.](image)

CHO-SLM$_2$ cell membranes were incubated with varying concentrations of GDP in the presence or absence of 1 mM methacholine. The change in basal and methacholine-stimulated [$^{35}$S]GTPyS binding was observed after 30 min incubations. The data are expressed as either the mean percentage increases in basal [$^{35}$S]GTPyS binding ± s.e.mean of (n=3) (a), or the mean cpm bound ± s.e.mean (n=3) (b).
Figure 4.3.6.a.
Effect of GDP upon methacholine-stimulated $[^{35}\text{S}]-\text{GTP}\gamma\text{S}$ binding in CHO-M$_3$ cell membranes.

![Graph showing effect of GDP upon methacholine-stimulated $[^{35}\text{S}]-\text{GTP}\gamma\text{S}$ binding in CHO-M$_3$ cell membranes.](image)

Figure 4.3.6.b.
Effect of GDP at increasing concentrations on basal and methacholine-stimulated $[^{35}\text{S}]-\text{GTP}\gamma\text{S}$ binding in CHO-M$_3$ cell membranes.

CHO-cell membranes (100µg) were incubated in the presence and absence of 1 mM methacholine at 30°C, and the change in basal and stimulated $[^{35}\text{S}]-\text{GTP}\gamma\text{S}$ binding was observed after 30 min. Data are expressed as the mean percentage increases in basal $[^{35}\text{S}]-\text{GTP}\gamma\text{S}$ binding ± s.e.mean of 4 individual experiments (a), and the mean cpm bound ± s.e.mean of 4 individual experiments (b).
Figure 4.3.7.a.
Effect of GDP upon methacholine-stimulated \[^{35}\text{S} \]-GTP\(\gamma\)S binding in CHO-M4 cell membranes.

![Graph showing the effect of GDP on \(^{35}\text{S}\)-GTP\(\gamma\)S binding.](image1)

Figure 4.3.7.b.
Effect of GDP at increasing concentrations on basal and methacholine-stimulated \(^{35}\text{S}\)-GTP\(\gamma\)S binding in CHO-M4 cell membranes.

![Graph showing the effect of GDP concentration on basal and stimulated \(^{35}\text{S}\)-GTP\(\gamma\)S binding.](image2)

CHO-M4 cell membranes were incubated in assay buffer in the presence or absence of 1 mM methacholine, the assay buffer also included \(^{35}\text{S}\)-GTP\(\gamma\)S (70 pM) and the GDP concentration indicated (n=3). The change in basal and stimulated \(^{35}\text{S}\)-GTP\(\gamma\)S binding was observed after 30 min incubations at 30°C. The data are expressed as the percentage increases in basal \(^{35}\text{S}\)-GTP\(\gamma\)S binding ± s.e.mean of 3 individual experiments (a) and the mean ± s.e.mean cpm bound (n=3) (b).
Figure 4.3.8.

Effect of pertussis toxin pre-treatment (100 ng ml\(^{-1}\)) on methacholine-stimulated \(^{35}\)S-GTP\(_{\gamma}\)S binding in CHO-cell membranes.

Treated or untreated CHO-cell membranes were incubated for 30 min at 30 °C, in the presence of 70 pM \(^{35}\)S-GTP\(_{\gamma}\)S and 10 μM GDP. Data are expressed as \(^{35}\)S-GTP\(_{\gamma}\)S bound above basal (cpm) and are shown as means ± s.e.mean for ≥ 3 separate experiments, performed in duplicate. * represents P < 0.05 and # represents P > 0.05 for comparisons with basal binding, paired Students's t-test.
Effect of varying concentrations of pertussis toxin upon methacholine-stimulated $[^{35}\text{S}]-\text{GTP} \gamma \text{S}$ binding in CHO-cell membranes.

The effect of pertussis toxin pre-treatment (20-24 h) upon methacholine-stimulated $[^{35}\text{S}]-\text{GTP} \gamma \text{S}$ binding to CHO-cell membranes. CHO-cell membranes (100 μg) were incubated in the presence of GDP (10 μM) and $[^{35}\text{S}]-\text{GTP} \gamma \text{S}$ (70 pM) for 30 min. Data are expressed as cpm bound above basal for all concentrations observed and are shown as means ± s.e.mean of values obtained from 4 separate experiments.
Figure 4.3.10.

The effect of pertussis toxin (100 ng ml⁻¹) upon the time-course of methacholine-stimulated [³⁵S]-GTPγS binding in CHO-M₁ and -M₃ cell membranes.

Time-courses of [³⁵S]-GTPγS binding to pertussis toxin pre-treated and untreated cell membranes. CHO-M₁ and -M₃ cell membranes were incubated in the presence of GDP (10 μM) and [³⁵S]-GTPγS (70 pM) for the times indicated. Data are expressed as cpm bound above basal for all time points observed and are shown as means ± s.e.mean obtained from 3 separate experiments, all experiments were performed in duplicate.
Figure 4.3.11.a.
Non-linear graphical representation of $[^{35}\text{S}]$-GTPγS isotope dilution performed in CHO-M$_2$ cell membranes.

CHO-cell membranes (100 μg) were incubated in the presence of increasing concentrations of unlabelled GTPγS and 70 pM $[^{35}\text{S}]$-GTPγS. The change in $[^{35}\text{S}]$-GTPγS binding was observed after 30 min incubation at 30°C. Data are shown as means ± s.e.mean for a representative experiment performed in duplicate.

Figure 4.3.11.b.
Linear graphical representation of $[^{35}\text{S}]$-GTPγS isotope dilution performed in CHO-M$_2$ cell membranes.
Concentration-dependent stimulation of $[^{35}\text{S}]-\text{GTP}\gamma\text{S}$ binding in CHO-cell membranes by methacholine.

CHO-cell membranes were incubated in the presence GDP (10 μM) for 30 min. Data are expressed as percentage stimulation over basal for all time-points and are shown as means ± s.e.mean for ≥ 3 separate experiments. All experiments were performed in duplicate.
Effect of pertussis toxin (100 ng ml\(^{-1}\)) upon methacholine-stimulated \(^{35}\)S-GTP\(_{\gamma}\)S binding in CHO-M, and -M, cell membranes.

The effect of pertussis toxin pre-treatment (20-24 h) upon methacholine-stimulated \(^{35}\)S-GTP\(_{\gamma}\)S binding to CHO-M, and -M, cell membranes. Untreated and pre-treated CHO-cell membranes were incubated in the presence of GDP (10 \(\mu\)M) and \(^{35}\)S-GTP\(_{\gamma}\)S (70 pM) for 30 min. Data are expressed as cpm bound above basal for all concentrations observed and are shown as means \(\pm\) s.e.mean of values obtained from 3 separate experiments.
Figure 4.3.14.

Effect of different cholinergic agonists on $[^3S]$-GTPγS binding in CHO-cell membranes.

Agonist $E_{max}$ values are expressed relative to the effect of 1 mM methacholine. CHO-cell membranes were incubated for 30 min at 30 °C, in the presence of 70 pM $[^3S]$-GTPγS and 10 µM GDP. Data are expressed as percentage increases in $[^3S]$-GTPγS bound relative to the percentage increase elicited by 1 mM methacholine and are shown as means ± s.e.mean for ≥ 3 separate experiments, performed in duplicate.
Figure 4.3.15.

Concentration-dependent stimulation of $[^{35}S]$-GTP$\gamma$S binding in CHO-cell membranes by arecoline.

CHO-cell membranes (100 µg) were incubated for 30 min in the presence of 70 pM $[^{35}S]$-GTP$\gamma$S and 10 µM GDP (referred to as GXP in the key). Data are expressed as percentage increases in $[^{35}S]$-GTP$\gamma$S bound and are shown as means ± s.e.mean for ≥ 3 separate experiments. All experiments were performed in duplicate.
Figure 4.3.16.

Concentration-dependent stimulation of $[^{35}\text{S}]-\text{GTP}\gamma\text{S}$ binding in CHO-cell membranes by pilocarpine.

CHO-$M_2$, -SLM$_2$ and -$M_4$ cell membranes were incubated for 30 min at 30°C in the presence of 70 pM $[^{35}\text{S}]-\text{GTP}\gamma\text{S}$ and 10 μM GDP. Data are expressed as percentage increases of $[^{35}\text{S}]-\text{GTP}\gamma\text{S}$ bound and is shown as means ± s.e.mean for ≥ 3 separate experiments, performed in duplicate.
Figure 4.3.17.

Effect of guanine nucleotides upon methacholine-induced displacement of [³H]-NMS binding in CHO-cell membranes.

CHO-cell membranes (100 μg) were incubated for 30 min in the presence and absence of 70 pM GTPγS and 10 μM GDP. Data are expressed as percentage inhibition of total [³H]-NMS bound for by methacholine and are shown as means ± s.e.mean for ≥ 3 separate experiments, performed in duplicate.
Effect of 1 mM methacholine $[^{35}S]$-GTPγS binding in CHO-cell membranes.

![Bar graph showing the effect of 1 mM methacholine on $[^{35}S]$-GTPγS binding.]

Effect of 10 μM atropine on $[^{35}S]$-GTPγS binding in CHO-cell membranes.

![Bar graph showing the effect of 10 μM atropine on $[^{35}S]$-GTPγS binding.]

CHO-cell membranes (100 μg) were incubated in assay buffer as described in the Methods section entitled '$[^{35}S]$-GTPγS Radioligand Binding mediated by human muscarinic receptors stably expressed in CHO cells' with the inclusion of 1 mM methacholine or 10 μM atropine (n=4). The change in basal $[^{35}S]$-GTPγS binding was observed after 30 min incubations at 30°C. The data are expressed as the mean percentage increases or decreases in basal $[^{35}S]$-GTPγS binding ± s.e.mean of 4 individual experiments.
Effect of increasing concentrations of atropine upon ‘basal’ [³⁵S]-GTPγS binding in CHO-cell membranes.

Concentration-dependence of atropine-induced reductions in [³⁵S]-GTPγS binding to CHO-cell membranes. CHO-cell membranes (100 µg) were incubated for 30 min in the presence of 70 pM [³⁵S]-GTPγS and 10 µM GDP. Data are expressed as cpm bound for all concentrations and are shown as means ± s.e.mean for ≥ 3 separate experiments, performed in duplicate.
Figure 4.3.20.a.
Effect of GDP upon atropine-inhibited $[^{35}S]$-GTPγS binding in CHO-cell membranes in the presence of 100 mM Na$^+$. 

CHO-cell membranes (100 µg) were incubated in the presence and absence of atropine. The change in $[^{35}S]$-GTPγS binding was observed after 30 min incubation at 30°C. The data are expressed as the mean percentage decreases in basal $[^{35}S]$-GTPγS binding ± s.e.mean of individual experiments (n=4).

Figure 4.3.20.b.
Effect of GDP upon atropine-inhibited $[^{35}S]$-GTPγS binding in CHO-cell membranes in the presence of 10 mM Na$^+$. 

CHO-cell membranes (100 µg) were incubated in the presence and absence of atropine. The change in $[^{35}S]$-GTPγS binding was observed after 30 min incubation at 30°C. The data are expressed as the mean percentage decreases in basal $[^{35}S]$-GTPγS binding ± s.e.mean of individual experiments (n=4).
Table 4.3.1.

GTPγS binding parameters derived from [35S]-GTPγS isotope dilution experiments in CHO-cell membranes.

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>B&lt;sub&gt;max&lt;/sub&gt; (f mol mg&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>K&lt;sub&gt;D&lt;/sub&gt; (nM)</th>
<th>B&lt;sub&gt;max&lt;/sub&gt; (f mol mg&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>K&lt;sub&gt;D&lt;/sub&gt; (nM)</th>
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</thead>
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<tr>
<td>CHO-M&lt;sub&gt;1&lt;/sub&gt;</td>
<td>1860 ± 291</td>
<td>3.58 ± 0.89</td>
<td>1957 ± 302</td>
<td>3.37 ± 0.92</td>
</tr>
<tr>
<td>CHO-M&lt;sub&gt;2&lt;/sub&gt;</td>
<td>1379 ± 32</td>
<td>1.11 ± 0.04</td>
<td>1469 ± 108</td>
<td>1.18 ± 0.08</td>
</tr>
<tr>
<td>CHO-SLM&lt;sub&gt;2&lt;/sub&gt;</td>
<td>1527 ± 164</td>
<td>1.20 ± 0.07</td>
<td>1669 ± 224</td>
<td>1.27 ± 0.03</td>
</tr>
<tr>
<td>CHO-M&lt;sub&gt;3&lt;/sub&gt;</td>
<td>1424 ± 125</td>
<td>4.40 ± 0.53</td>
<td>1540 ± 154</td>
<td>4.85 ± 0.47</td>
</tr>
<tr>
<td>CHO-M&lt;sub&gt;4&lt;/sub&gt;</td>
<td>2248 ± 142</td>
<td>1.26 ± 0.07</td>
<td>2254 ± 157</td>
<td>1.21 ± 0.06</td>
</tr>
</tbody>
</table>

CHO-cell membranes were incubated with 70 pM [35S]-GTPγS and increasing concentrations of unlabelled GTPγS at 30 °C for 30 min. From these experimental data, bound and free GTPγS could be calculated and represented graphically as both a linear and non-linear regression. The comparison of these 2 data sets are shown above. It can be seen that the data from the linear and non-linear regression analyses are comparable, the data quoted will be derived from the non-linear regression.
Table 4.3.2.

Maximal stimulations of [35S]-GTPγS binding elicited by various muscarinic agonists in CHO-cell membranes.

<table>
<thead>
<tr>
<th></th>
<th>Methacholine</th>
<th>Arecoline</th>
<th>Pilocarpine</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHO-M₁</td>
<td>46.8 ± 2.1</td>
<td>26.8 ± 1.1</td>
<td>17.3 ± 2.3</td>
</tr>
<tr>
<td>CHO-M₂</td>
<td>98.0 ± 8.3</td>
<td>110.4 ± 10.5</td>
<td>56.0 ± 2.7</td>
</tr>
<tr>
<td>CHO-SLM₂</td>
<td>110.0 ± 4.9</td>
<td>141.5 ± 9.4</td>
<td>65.3 ± 3.8</td>
</tr>
<tr>
<td>CHO-M₃</td>
<td>30.8 ± 0.8</td>
<td>18.0 ± 1.1</td>
<td>14.6 ± 1.9</td>
</tr>
<tr>
<td>CHO-M₄</td>
<td>171.8 ± 14.4</td>
<td>231.8 ± 10.9</td>
<td>43.6 ± 3.3</td>
</tr>
</tbody>
</table>

The data shown in the Table are derived from [35S]-GTPγS binding experiments. Data are expressed as the mean maximum percentage stimulation over basal ± s.e.mean derived from individual experiments (n≥3).
Table 4.3.3.

The effect of guanine nucleotides on methacholine induced $[^{3}H]$-NMS displacement in CHO-cell membranes.

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Apparent $K_B$ (µM)</th>
<th>Hill slope (nH)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHO-M₁</td>
<td>25.6 ± 7.2</td>
<td>0.76 ± 0.07</td>
</tr>
<tr>
<td>CHO-M₁ + GXP</td>
<td>99.4 ± 12.9</td>
<td>0.98 ± 0.08</td>
</tr>
<tr>
<td>CHO-SLM₂</td>
<td>1.0 ± 0.4</td>
<td>0.48 ± 0.06</td>
</tr>
<tr>
<td>CHO-SLM₂ + GXP</td>
<td>8.2 ± 2.0</td>
<td>0.75 ± 0.04</td>
</tr>
<tr>
<td>CHO-M₃</td>
<td>16.6 ± 2.0</td>
<td>0.79 ± 0.04</td>
</tr>
<tr>
<td>CHO-M₃ + GXP</td>
<td>30.6 ± 4.1</td>
<td>0.90 ± 0.05</td>
</tr>
<tr>
<td>CHO-M₄</td>
<td>2.1 ± 0.3</td>
<td>0.58 ± 0.05</td>
</tr>
<tr>
<td>CHO-M₄ + GXP</td>
<td>12.0 ± 7.2</td>
<td>0.88 ± 0.02</td>
</tr>
</tbody>
</table>

The effect of guanine nucleotides utilised in $[^{35}S]$-GTPγS binding experiments on methacholine-induced $[^{3}H]$-NMS displacement, GXP = GDP 10 µM and 70 pM GTPγS. Apparent $K_B$ values are derived according to Leff and Dougall (1993). Hill slopes were derived using GraphPad Prism 2. Data are expressed as the means ± s.e.mean of values obtained from the individual experiments performed in duplicate (n=3).
Comparison of methacholine binding affinity with functional efficacy at Hm1-Hm4 receptors expressed in CHO-cell membranes.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Apparent $K_B$ (µM) $[^3H]$-NMS binding</th>
<th>$EC_{50}$ (µM) $[^35S]$-GTPγS binding</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHO-M₁</td>
<td>99.4 ± 12.9</td>
<td>13.9 ± 2.0</td>
</tr>
<tr>
<td>CHO-SLM₂</td>
<td>8.2 ± 2.0</td>
<td>0.22 ± 0.03</td>
</tr>
<tr>
<td>CHO-M₃</td>
<td>30.6 ± 4.1</td>
<td>47.6 ± 2.0</td>
</tr>
<tr>
<td>CHO-M₄</td>
<td>12.0 ± 7.2</td>
<td>0.85 ± 0.15</td>
</tr>
</tbody>
</table>

The data displayed in the above Table are derived from methacholine-induced $[^3H]$-NMS displacement binding in the presence of guanine nucleotides used in the GTPγS binding assay buffer and from the methacholine-stimulated $[^35S]$-GTPγS binding. Data are expressed as the means ± s.e.mean derived from individual experiments. $[^3H]$-NMS binding (n=3) and $[^35S]$-GTPγS binding (n=4) refer Table 4.2.1 and Graph 4.1.1.
4.4. Discussion

In this study agonist-stimulated [$^{35}$S]-GTP$\gamma$S binding to G proteins was used as a measure of receptor-mediated activation of heterotrimeric G proteins by different muscarinic acetylcholine receptor subtypes. The nature of the G protein populations to which the muscarinic receptor subtypes may couple has been inferred by their pertussis toxin sensitivity. Agonist-stimulated increases in [$^{35}$S]-GTP$\gamma$S binding were observed in all the transfected CHO-cell clones (CHO-M$_1$, CHO-M$_2$, CHO-SLM$_2$, CHO-M$_3$, and CHO-M$_4$), whereas in untransfected CHO-K1 cells no agonist-stimulated [$^{35}$S]-GTP$\gamma$S binding was seen. This illustrates that the response observed is receptor-mediated and that the CHO-K1 cell line does not posses any endogenous muscarinic receptors.

Methacholine-stimulated increases in [$^{35}$S]-GTP$\gamma$S binding were not observed in CHO-M$_1$, CHO-M$_2$, CHO-SLM$_2$, CHO-M$_3$, and CHO-M$_4$ cell membranes in the absence of GDP. With increasing concentrations of GDP, basal [$^{35}$S]-GTP$\gamma$S binding was dose-dependently inhibited, while the methacholine-stimulated [$^{35}$S]-GTP$\gamma$S binding, whilst still being reduced, was not inhibited to the same degree. Therefore, with increasing concentrations of GDP, methacholine-stimulated [$^{35}$S]-GTP$\gamma$S binding was increased as a percentage of basal binding in CHO-M$_1$, CHO-M$_2$, CHO-SLM$_2$, CHO-M$_3$, and CHO-M$_4$ cell membranes. The requirement for micromolar concentrations of GDP to enhance agonist effects in membrane preparations has been reported in a number of studies using a variety of cell lines expressing different GPCRs (Hilf et al., 1989; Hilf and Jakobs, 1992; Lazareno et al., 1993a, 1993b; Lorenzen et al., 1993; Williams et al., 1997). GDP has been shown to decrease basal [$^{35}$S]-GTP$\gamma$S binding to a greater extent than agonist-stimulated [$^{35}$S]-GTP$\gamma$S, allowing the signal to be maximised. The requirement for exogenous GDP suggests that a proportion of G proteins in the membrane preparation are guanine nucleotide free, because if the level of GDP in the membrane preparation was sufficient to saturate the $\alpha$-subunit binding site exogenous GDP would not be required. This idea is supported by the agonist-displacement studies as a guanine nucleotide shift was observed. It is believed that guanine nucleotide-free G proteins will interact with [$^{35}$S]-GTP$\gamma$S in an
agonist-independent manner. Receptor-stimulation is postulated to increase the rate of GDP dissociation from the $G\alpha$ subunit, thus allowing GTP to interact (Higashijima et al., 1987; Shiozaki and Haga, 1992; Bourne, 1997). Thus, it appears that GDP is required to interact with G proteins to allow their activation after receptor stimulation and this action of GDP is probably a consequence of its competition for the nucleotide binding site on all classes of G proteins. Also exogenous GDP serves to reduce the total binding of $[^{35}S]$-GTPyS. GDPβS has been shown to be an unsatisfactory replacement for GDP in cardiac membranes (Hilf et al., 1989). One possible explanation for the inability of GDPβS to replace GDP may be due to the relative dissociation rates of both of the nucleotides. If GDPβS dissociates slowly, then the ability of $[^{35}S]$-GTPyS to bind may be impaired. Currently there are no studies to substantiate the idea of varying dissociation rates. GDP was shown to be metabolised rapidly at 30°C in HL60 membranes, and required exogenous GDP to observe FMLP-induced $[^{35}S]$-GTPyS binding (Wieland et al., 1992). When similar experiments were carried out at 0°C the metabolism of endogenous GDP was reduced allowing agonist-stimulated $[^{35}S]$-GTPyS binding to be observed in the absence of exogenous GDP (Wieland et al., 1992). It appears that receptor-activation of G proteins requires the GDP liganded form of $G\alpha$ to interact with, allowing receptor-induced exchange of GDP for GTPyS. In membrane-based systems GDP can be rapidly released and degraded resulting in a higher level of nucleotide-free G proteins. This may elicit GTPyS binding to G proteins independently of agonist-activation of receptors. Therefore conditions which maximise agonist-stimulated levels of $[^{35}S]$-GTPyS binding and minimise the basal levels of $[^{35}S]$-GTPyS binding are routinely evaluated and used in studies of this nature.

The effect of pre-treatment with pertussis toxin on agonist-stimulated $[^{35}S]$-GTPyS binding is dependent on the muscarinic receptor subtype expressed. PTx pre-treatment of CHO-M2, CHO-SLM2, and CHO-M4 cells prior to membranes preparation reduced basal $[^{35}S]$-GTPyS binding by around 80%. This suggests that in these cell lines nearly all of the basal $[^{35}S]$-GTPyS binding is to $G_\gamma$-like G proteins. PTx pre-treatment (100 ng ml$^{-1}$) also abolished agonist-mediated effects in the M2 and M4 expressing cell lines, confirming that these muscarinic acetylcholine receptor subtypes transduce their
signal by coupling solely to G\textsubscript{t}-like G proteins (Dell'Acqua et al., 1993; Migeon et al., 1995). This finding is in agreement with previously published data where the complete loss of agonist-mediated effects was observed after stimulation of PTx pre-treated M\textsubscript{2} and M\textsubscript{4} receptor expressing cells (Lazareno et al., 1993a; Burford et al., 1995b). In the CHO-M\textsubscript{1} and CHO-M\textsubscript{3} cell membranes PTx pre-treatment reduces both basal and methacholine-stimulated \(^{35}\text{S}\)-GTP\textsubscript{y}S binding. In contrast to the CHO-M\textsubscript{2}, CHO-SLM\textsubscript{2}, and CHO-M\textsubscript{4} cell membranes, in the CHO-M\textsubscript{1} and CHO-M\textsubscript{3} cell membrane preparations basal \(^{35}\text{S}\)-GTP\textsubscript{y}S binding was reduced by around 50\%, a residual level of basal \(^{35}\text{S}\)-GTP\textsubscript{y}S binding in CHO-M\textsubscript{1} and CHO-M\textsubscript{3} cells has also been observed by Lazareno et al, (1993a) and Burford et al, (1995b). Also in the CHO-M\textsubscript{1} and CHO-M\textsubscript{3} cell membranes a statistically significant stimulation of \(^{35}\text{S}\)-GTP\textsubscript{y}S binding is still observed in the presence of agonist. The residual basal binding and methacholine-stimulated binding in the M\textsubscript{1} and M\textsubscript{3} expressing cells may be due to G\textsubscript{q/11}\textsubscript{α}-mediated \(^{35}\text{S}\)-GTP\textsubscript{y}S binding. The M\textsubscript{1} and M\textsubscript{3} receptor subtypes are known to couple to G\textsubscript{q/11}\textsubscript{α} to activate PLC (Caulfield 1993, Eglen et al., 1996) and therefore interaction with G\textsubscript{q/11}\textsubscript{α} would be expected. So this finding supports the idea that the M\textsubscript{1} and M\textsubscript{3} receptor subtypes interact with both pertussis-toxin-sensitive and -insensitive G proteins (Offermanns et al., 1994a). The differential loss of agonist-stimulated \(^{35}\text{S}\)-GTP\textsubscript{y}S binding after pertussis toxin pre-treatment in the CHO-M\textsubscript{1} (50\%) and CHO-M\textsubscript{3} (24\%) cell membrane preparations may indicate a difference in the G protein coupling efficiency of these receptor subtypes to G\textsubscript{t}-like G proteins.

It has previously been shown that ADP-ribosylation of G proteins by PTx interferes with receptor-G protein interaction and does not interfere with the intrinsic functions of the G protein i.e. GTPase activity and subunit dissociation (Haga et al., 1985; Enomoto and Asakawa, 1986, Huff and Neer, 1986; Katada et al., 1986). The loss of basal \(^{35}\text{S}\)-GTP\textsubscript{y}S binding by PTx pre-treatment in the CHO-cell lines suggests a number of possibilities. The reduction in basal \(^{35}\text{S}\)-GTP\textsubscript{y}S binding may represent a portion of PTx-sensitive G proteins that were coupled to receptors (endogenous and/or recombinant) in the absence of receptor stimulus. This supports the idea that unoccupied receptors are not silent and that G protein activation can be seen in the absence of agonist. Agonist-independent receptor activation has been observed in a
number of systems to date (For reviews see, Lefkowitz et al., 1993; Milligan et al., 1995) and has been termed constitutive activity. A mutant β2-adrenoceptor has been identified that shows constitutive activation of G protein in the absence of agonist (Samama et al., 1993). This study led to an extension of the ternary complex model of receptor activation by the inclusion of an isomerisation step between, at least, two interconvertible receptor states one active (R*) and one inactive (R), with agonist binding affecting the R\(\leftrightarrow\)R* equilibrium, but not causing the active conformation to be assumed (Lefkowitz et al., 1993; Milligan et al., 1995; Leff et al., 1997, 1998). Uncoupling unoccupied receptors from PTx-sensitive G proteins, by PTx pre-treatment, will lead to a reduction in total \[^{35}\text{S}]\text{GTPyS}\) binding. Therefore, it appears reasonable to assume that the loss of binding seen in this study may be due to the loss of agonist-independent G protein activation. This argument is supported by the differing loss of basal \[^{35}\text{S}]\text{GTPyS}\) binding after PTx pre-treatment between the even and odd numbered muscarinic family members. The level of constitutive activity would be expected to be differentially dependent upon the G protein interactions of the specific receptor subtype. The reduction in basal \[^{35}\text{S}]\text{GTPyS}\) binding in CHO-cell clones expressing the M2 and M4 receptor subtypes was more pronounced than the loss of basal binding in the M1 and M3 expressing cells. This supports the idea that the basal \[^{35}\text{S}]\text{GTPyS}\) binding is agonist-independent activation of G\(_r\)-like G proteins. As the M2 and M4 receptor subtypes activate G\(_r\)-like G proteins (Dell’Acqua et al., 1993; Migeon et al., 1995) they may therefore activate a larger proportion of PTx-sensitive G proteins in the absence of agonist compared to the M1 and M3 subtypes which are believed to couple to G\(_{q/11}\) G proteins. However these experiments were performed in the presence of 100 mM NaCl, and Na\(^+\) ions are believed to uncouple unoccupied receptors from G proteins and reduce basal \[^{35}\text{S}]\text{GTPyS}\) binding (Hilf et al., 1989; Gierschik et al., 1989). Therefore the reduction in binding in the presence of PTx seems high.

Quantitation of G proteins activated by methacholine-stimulation in CHO-cell membrane preparations expressing recombinant muscarinic receptors revealed differences between the receptor subtypes. The K\(_D\) values for agonist-stimulated high affinity \[^{35}\text{S}]\text{GTPyS}\) binding, for each of the cell lines, are in agreement with previous
studies of this nature which also produce low nanomolar values (Traynor and Nahorski, 1995; Selley et al., 1996; Breivogel et al., 1998). Although the $K_D$ values are in the expected range, the values obtained revealed differences between the odd and even numbered muscarinic receptor subtypes. The $K_D$ values, reflecting the difference in the ability of each subtype to induce a high affinity GTP binding site in the G proteins, were 3-5 fold lower in the $M_2$ and $M_4$ expressing cells when compared to the $M_1$ and $M_3$ expressing cells. As the results with pertussis toxin illustrated, the $M_2$ and $M_4$ expressing cells interact with one class of G proteins, whereas the $M_1$ and $M_3$ expressing cells can couple to members of more than one G protein class. Therefore the differences in the $K_D$ values may reflect varying affinities of binding within the activated G protein population (Wessling-Resnick and Johnson, 1987). In opposition to this argument the analysis of this data set revealed only single site binding however, as the $K_D$ values for $G_i$ and $G_q$ differ by less than 10 fold the difference may not be easily detected.

The $B_{max}$ values indicating the number of G proteins activated by each subtype in response to methacholine, revealed a ratio of approximately 1:2 (muscarinic receptor binding sites: [$^{35}$S]-GTPγS binding sites) in the $M_2$ and $M_4$ expressing cells. The number of [$^{35}$S]-GTPγS binding sites occupied after agonist stimulation is around twice the number of muscarinic receptor binding sites determined by [$^3$H]-NMS binding in the CHO-M$_2$, CHO-SLM$_2$ and CHO-M$_4$ cell membranes. This ratio is in broad agreement with the ratio found between muscarinic receptors and [$^{35}$S]-GTPγS binding sites in porcine cardiac membranes (Hilf et al., 1989) and $\mu$-opioid receptors and [$^{35}$S]-GTPγS binding sites in SH-SY5Y human neuroblastoma cells (Traynor and Nahorski, 1995). However in the $M_1$ and $M_3$ expressing cell lines, CHO-M$_1$ and CHO-M$_3$, a ratio of less than 1:1 was observed. These findings provide no evidence for amplification of the signal at this stage in the signalling pathway. The formation of an activated-receptor-G protein ternary complex results in the generation of G$\alpha$-[${}^{35}$S]-GTPγS in this assay system, and in theory the activated receptor will be released to activate a second G protein allowing signal amplification to occur with time. However the GTP analogue locks the G$\alpha$ subunit in the activated conformation due to its specific chemical properties, whereby it dissociates slowly and is resistant to the
GTPase action of the $\alpha$ subunit. Then with time it is possible that the G protein population mediating productive collisions would be reduced as unactivated G protein becomes scarce (Taylor, 1993). So these results may be due to a limiting number of G proteins compared to activated receptors, so only a limited number of $G_\alpha$-[35S]-GTP$_\gamma$S can be formed, in these cell lines. Alternatively, the lack of amplification from all the muscarinic receptor subtypes may be a reflection of the relative abundance of $G_{q/11}\alpha$ and $G_l$-like G proteins endogenously expressed in CHO-cells. To advance this idea the G protein expression levels would have to manipulated and further measurements made.

Full and partial muscarinic agonists were evaluated at the level of [35S]-GTP$_\gamma$S binding. When the full agonist, methacholine (1 mM), was assessed the maximal increase in [35S]-GTP$_\gamma$S binding observed at 30 min in the CHO-cell clones did not solely reflect expression level but also the particular muscarinic receptor subtype expressed. CHO-M$_2$, CHO-SLM$_2$ and CHO-M$_4$ cell membranes produced larger increases in agonist-stimulated [35S]-GTP$_\gamma$S binding despite expressing relatively lower levels than the CHO-M$_1$ and CHO-M$_3$ cells. This finding may be due to the experimental conditions utilised. Agonist-stimulated [35S]-GTP$_\gamma$S binding, expressed as a percentage above basal, may not necessarily identify that one particular muscarinic receptor subtype is more efficiently coupled to their respective G proteins. The experimental conditions may have elicited preferential binding of [35S]-GTP$_\gamma$S to certain classes of G proteins. It has been observed that $G_l$-like G proteins bind GTP$_\gamma$S with a greater affinity than $G_s$ and $G_q$ G proteins (Bokoch et al., 1984; Wessling-Resnick and Johnson, 1987; Blank et al., 1991). Therefore, although it appears that the M$_2$ and M$_4$ receptor subtypes couple more efficiently to G proteins than the M$_1$ and M$_3$ subtypes, this may just reflect preferential binding of [35S]-GTP$_\gamma$S to one particular class of G proteins (e.g. $G_l$) over other G protein species (e.g. $G_{q/11}$), which may be involved in coupling to the other muscarinic receptor subtypes. The use of approx. 70 pM [35S]-GTP$_\gamma$S in this series of experiments may cause a selective binding of [35S]-GTP$_\gamma$S with PTx-sensitive G proteins compared to PTx-insensitive G proteins. Utilising a higher concentration of [35S]-GTP$_\gamma$S may increase the labelling.
of lower affinity sites, and hence may alter the proportion of $[^{35}\text{S}]$GTP$\gamma$S bound to $G_{q11}$.

Methacholine is assumed to act as a full agonist at all the muscarinic receptor subtypes in this study and the EC$_{50}$ values obtained for methacholine were in agreement with those reported by Lazareno et al. (1993a). When EC$_{50}$ values for methacholine-stimulated $[^{35}\text{S}]$GTP$\gamma$S binding were compared in pertussis toxin pre-treated and untreated cells, the potency of methacholine was greater in the PTx pre-treated cells than in the control cells. This is also in agreement with the potency changes reported by Lazareno et al. (1993a). As the $M_1$ and $M_3$ receptor subtypes are known to couple to PLC via $G_{q11}\alpha$, it would be expected that when activating their preferred G protein class that a higher affinity interaction would occur and hence the potency would increase. When comparing EC$_{50}$ values for cAMP accumulation and Ca$^{2+}$ release in CHO-$M_1$ and -$M_3$ cells the potency of carbachol was reported to be markedly reduced when assessed at the level of cAMP (Burford et al., 1995a). Therefore, potency changes can be seen for a single agonist when coupling to different G proteins.

The maximal effects of arecoline appeared to be dependent upon the muscarinic receptor subtype expressed, with the $M_3$ and $M_4$ expressing cells displaying $E_{\text{max}}$ values comparable to methacholine. In contrast the $M_1$ and $M_3$ expressing cells produced $E_{\text{max}}$ values relative to methacholine that indicated partial agonist activity. Based on maximal functional effects, Stoll and Muller (1991) predicted that arecoline and pilocarpine are both partial agonists when compared to methacholine in a study of short term receptor down-regulation in whole mouse brain. Yet the high concentration of GDP utilised in this study, 10 $\mu$M, may be affecting the efficacy of arecoline as it has been shown that efficacy differences can be magnified by increasing GDP (Pauwels et al., 1997; Selley et al., 1997). However, many other studies have shown that arecoline, when acting at AC-linked muscarinic receptors, acts as a high efficacy agonist comparable to methacholine or carbachol (Olianas and Onali, 1991; Quist, 1991; Olianas and Onali, 1996). Other comprehensive agonist studies have shown an efficacy change in arecoline from high to low when comparing AC-linked and PLC-
linked muscarinic receptor subtypes (Freedman et al., 1988; Spencer et al., 1988; Baumgold and White, 1989). Pilocarpine however gave rise to $E_{\text{max}}$ values which indicated that this compound acts as a partial agonist at all the muscarinic receptor subtypes investigated. Pilocarpine has been directly compared to high efficacy agonists like carbachol and methacholine and in these studies pilocarpine was shown to be a low efficacy agonist at all the muscarinic subtypes studied (Freedman et al., 1988; Baumgold and White, 1989; Olianas and Onali, 1991; Olianas and Onali, 1996). The $EC_{50}$ values obtained for arecoline and pilocarpine are in agreement with those reported by Lazareno et al. (1993a).

As well as maximising the agonist signal in $[^{35}\text{S}]-\text{GTP}\gamma\text{S}$ binding studies GDP also has been shown to have pronounced effects on agonist efficacy and potency in a number of systems. In a study of $\mu$-opioid receptor-stimulated $[^{35}\text{S}]-\text{GTP}\gamma\text{S}$ binding in rat thalamus, SK-N-SH and mMOR-CHO cell lines, efficacy differences were magnified by increasing GDP indicative of the fact that the activity state of G proteins can affect agonist efficacy (Selley et al., 1997). Following this study, Breivogel et al. (1998) showed that cannabinoid receptor agonist efficacy for stimulating $[^{35}\text{S}]-\text{GTP}\gamma\text{S}$ binding to rat cerebellar membranes correlated with agonist-induced decreases in GDP affinity, again illustrating that agonist efficacy is determined by the activity state of G proteins. Studies of the 5-HT$_{1A}$ receptor in conjunction with 5-HT$_{1A}$ and 5-HT$_{1B/D}$ receptor agonists (Pauwels et al., 1997; Pauwels et al., 1998) showed that apparent efficacy of agonists could be altered by G protein activation state and the type of G proteins involved in mediating the response. The addition of exogenous GDP will affect the activity state of G proteins and therefore all $[^{35}\text{S}]-\text{GTP}\gamma\text{S}$ binding studies are subject to experimental bias. In our study all values obtained are relative to the experimental conditions utilised. Therefore the experimental conditions must be quoted in any discussion of overall efficacy of muscarinic agonists.

Agonist-stimulated $[^{35}\text{S}]-\text{GTP}\gamma\text{S}$ binding was compared to agonist binding under the same experimental conditions. Affinity estimates for methacholine in membranes from CHO-cell clones were determined from their abilities to compete with $[^{3}\text{H}]-\text{NMS}$ for binding to the receptors in the presence and absence of 10 $\mu$M GDP and 70 pM
GTPγS. For the M₁, M₂, M₃ and M₄ receptor subtypes the inclusion of guanine
nucleotides lead to a rightward shift and a significantly greater Hill slope. This finding
suggests firstly the absence of a significant level of endogenous GDP and secondly
that GDP and GTP together potently reduce agonist-binding. The effects of GDP and
GTP on agonist-dissociation experiments were as expected (Graeser and Neubig,
1992). In the present study the M₂ and M₄ expressing cell membranes produce larger
guanine nucleotide shifts than the M₁ and M₃ expressing cells, a finding in agreement
with those of Burford et al. (1995b). Also large GTP-shifts in agonist binding have
been seen in studies using cardiac membranes to investigate the M₂ receptor subtype
(Matesic et al., 1989), whereas similar studies involving the PLC-linked receptors
generally show smaller GTP-shifts (Richards et al., 1991; Wess et al., 1990a). The
cause of these observed differences are not defined, but may relate to PLCβ1 acting as
a GAP on Gq₁₁α (Burstein et al., 1992b) increasing the rate of GTP hydrolysis. It has
been suggested that in CHO-cell membrane preparations PLC activity is impaired
(Burford et al., 1995b), and that this lack of PLC activity and, hence reduced
GTP/GDP exchange may result in minimal high affinity agonist binding. Another
explanation could be that there are significant differences in the levels of different
classes of G proteins endogenously expressed in CHO-cells. Alternatively, these
differences in guanine nucleotide-modification of agonist-binding may reflect
differences in the coupling of the AC-linked and PLC-linked muscarinic receptors
with specific G proteins.

A comparison of the affinity of methacholine, and methacholine-stimulated [³⁵S]-
GTPγS binding, shows that the M₃ receptor G protein-coupling is weak in comparison
to the other muscarinic receptor subtypes evaluated. When the M₁ and M₃ muscarinic
receptors are compared at the level of Ins(1,4,5)P₃ accumulation the M₁ receptor
subtype appears more potent then the M₃ subtype (Burford et al., 1995a). So there
appears to be a difference in the relative coupling efficiency of these two PLC-linked
muscarinic receptor subtypes.

Human acetylcholine muscarinic receptor subtypes were also assessed at the level of
G protein activation for inverse agonist activity in CHO-M₂, CHO-SLM₂ and CHO-
M₄ cell lines. [³⁵S]-GTPγS binding was utilised to allow assessment of this phenomenon at the level of guanine nucleotide exchange as this event is so pivotal in the extended ternary complex model (Milligan et al., 1995). Atropine-mediated reductions in basal [³⁵S]-GTPγS binding were observed in all the cell lines. 10-20% of control [³⁵S]-GTPγS binding was inhibited by a maximal concentration of atropine, and full concentration-analyses revealed similar IC₅₀ values for all three cell membrane preparations. It is extremely unlikely that the inhibitory effect of atropine on [³⁵S]-GTPγS binding is due to contamination of the preparation by endogenous acetylcholine. CHO cells do not produce acetylcholine, and there is no other apparent route for acetylcholine to get into the assay. Also the finding that atropine can act as an inverse agonist at muscarinic receptors is in agreement with previously reported data by Hilf and Jakobs (1992) where atropine-inhibition of [³⁵S]-GTPγS binding in cardiac membranes was observed, with atropine mediating a decrease of around 20%. Jakubik et al. (1995, 1996) reported atropine-mediated increases in cAMP in both M₂ and M₄ expressing CHO-cells and M₁ expressing rat cardiomyocytes and atropine-inhibition of inositol phosphate production in M₁ and M₄ expressing CHO-cells. Also studies using a G protein overexpression system by Burstein et al. (1995, 1997) showed that atropine could act as an inverse agonist at all the PLC-coupled muscarinic receptor subtypes.

The inhibition of [³⁵S]-GTPγS binding by atropine in cardiac membranes was found to be modulated by both NaCl and GDP (Hilf and Jakobs, 1992b). The inhibition was maximal in the absence of NaCl, and was reduced in a concentration-dependent manner until finally abolished by 100 mM NaCl. Maximal inhibition by atropine was observed in the presence of 0.3 μM GDP and then progressively decreased as the GDP concentration was increased further. These results are not in accord with our observations, as a reduction in NaCl from 100 mM to 10 mM produced a small reduction in the level of inhibition mediated by a maximal concentration of atropine. Also reducing the concentration of GDP mediated a slight reduction in the level of atropine-inhibition observed. For an inverse agonist to reduce control levels of agonist-independent activity it must preferentially bind to the inactive receptor species altering the position of the equilibrium between R and R*, and in turn this reduces the
level of R*. Na' and GDP both affect the equilibrium between R* and R*G by uncoupling the receptor-G protein complex. Therefore, they would be expected to reduce the level of constitutive activity measured at the level of G protein activation thus making it harder to observe any inverse agonist activity. This may reflect the finding that these cell preparations had a high level of constitutive activity initially, as perhaps indicated by the effects of pertussis toxin, and that altering the receptor equilibrium with GDP and NaCl would not have a large effect.

Similar IC_{50} values for atropine-inhibition of [^{35}S]-GTP\gamma S binding were observed in all three CHO-cell membrane preparations, these values reflect the reported affinity constants for atropine (Caulfield, 1993). The maximal levels of atropine-mediated inhibition of [^{35}S]-GTP\gamma S binding reflected the receptor densities observed in the CHO-M_2 and CHO-SLM_2 cell lines. Together these findings suggest that there is no receptor reserve for the inverse agonist atropine. Using pindobind, a \beta_2\text{-adrenoceptor alkylation agent, in conjunction with the overexpression of the \beta_2\text{-adrenoceptor in myocardial tissue the }EC_{50} \text{ value for the inverse agonist was seen to decrease, suggesting a receptor reserve (Bond et al., 1995). Therefore at higher levels of }M_2 \text{ and }M_4 \text{ receptor expression it may become possible to detect a receptor reserve.}

Expression of homogeneous populations of muscarinic receptor subtypes in single cell lines has allowed the study of the interactions of these receptors with G proteins in a similar cell background. In turn, this has allowed the efficiency of coupling of different muscarinic receptor subtypes to begin to be dissected. However, the use of membrane-based systems always elicits a number of concerns. For example, does the loss of microenvironments disrupt 'normal' receptor-G protein coupling as it is known that most signal transduction machinery is concentrated in specific subcellular domains (Nomura et al., 1997). Also the relatively poor efficiency of coupling of PI-linked muscarinic receptors to G proteins seen in this assay system suggests the possibility that the coupling of }M_1 \text{ and }M_3 \text{ receptors in membranes may be 'weakened'. Agonist-stimulated PLC activation has been reported in membrane preparations of cells (Gutowski et al., 1991) but further studies to investigate muscarinic receptor-PLC coupling in membrane preparations of CHO-cells are
required. Therefore further studies in a permeabilised cell system may allow receptor-G protein coupling to be studied in a more intact preparation and also allow fuller investigation of the $M_1$ and $M_3$ receptor-G protein coupling (Chapter Five).
Chapter Five.

Assessment of $[^{35}\text{S}]-\text{GTP}\gamma\text{S}$ Binding, Ins(1,4,5)P$_3$ Generation and $^{45}\text{Ca}^{2+}$ Release in Permeabilised CHO-cells Expressing Recombinant Human Acetylcholine Muscarinic Receptors.
5.0. Introduction.

The use of agonist-stimulated activation of G proteins in cell free preparations has been a widely used approach to assess stimulus-response relationships at the level of G protein activation. This has been accomplished by assaying either receptor-stimulated high-affinity GTPase activity, or binding of metabolically stable GTP analogues in preparations of cell membranes (Wieland and Jakobs 1994; Gierschik et al., 1994), as reviewed in the last Chapter. Although this provides information on early signal transduction events the use of membranes inevitably leads to a loss of membrane architecture, which may have an important role in the organisation of signal transduction.

Recently G protein α subunits have been identified as caveolin-interacting proteins (Chang et al., 1994; Li et al., 1995; Song et al., 1996; Nomura et al., 1997). G protein α subunits are quite highly concentrated in preparations of caveolae-enriched membranes purified from many diverse sources. Caveolin interacts directly with multiple G protein α subunits, including Gs, Go and G-like G proteins, and can functionally suppress their basal activity by inhibiting GTP/GDP exchange (Li et al., 1995). The interaction with caveolin seems to be located to a specific N-terminal region and appears dependent on the activity state of the G protein α subunit. It has also been shown by immunogold labelling studies that many other G protein signalling related molecules reside within plasma membrane caveolae, including agonist-activated G protein-coupled receptors (Raposo et al., 1987; 1989; Raposo and Benedetti, 1994) and down stream effectors such as Ins(1,4,5)P₃ receptors and the plasma membrane Ca²⁺-ATPase (Schnitzer et al., 1995; Fujimoto et al., 1995). So the use of membrane-based systems to assess G protein activation always elicits a number of concerns. For example, as it is known that most signal transduction machinery is concentrated in specific subcellular domains, does the loss of microenvironments disrupt 'normal' receptor-G protein coupling. Further limitations of such approaches in membranes are that subsequent downstream signalling events are often absent in such preparations. Yet in more intact cellular preparations, on the other hand, G protein activation is usually determined more indirectly by measurement of effector...
activities such as adenylyl cyclase activity and phosphoinositidase C activity by monitoring cAMP or inositol phosphate accumulations. However, these distal signalling events only give very limited information about the primary interaction of receptors with G proteins.

The development of a single experimental system to permit the quantitative analysis of many stages of receptor signalling would allow a more direct comparison with subsequent signalling events. $[^{35}S]$-GTPγS binding utilised as a quantitative indicator of the activation of G proteins is routinely performed using cell membranes, yet it also has been monitored in a more intact cellular system in a study of myeloid differentiated human leukaemia (HL60) cells. HL60 cells permeabilised with digitonin demonstrated $[^{35}S]$-GTPγS binding induced by three distinct chemoattractant receptors (Wieland et al., 1995). This study illustrated that $[^{35}S]$-GTPγS binding can be evaluated in a more intact cellular system, and perhaps this type of methodology can overcome some of the disadvantages associated with membrane filtration-based studies.

The signalling pathways of muscarinic acetylcholine receptors have been studied at the level of receptor-G protein activation and second messenger generation, but usually in vastly different experimental preparations. Therefore, further studies in a permeabilised cell system may allow receptor-G protein coupling to be studied in a more intact preparation. Here, data are presented using a permeabilised cell preparation which allows assessment not only of agonist-stimulated $[^{35}S]$-GTPγS binding, but also allows measurement of $M_1/M_3$ receptor signalling via phosphoinositide hydrolysis leading to inositol 1,4,5-trisphosphate generation and ultimately calcium mobilisation.

5.1 Methods.

For details on the methodology used throughout this Chapter see Chapter Two 'Experimental Methodology' with reference to the sections entitled 'Permeabilised Cell Preparation', '$[^3H]$-Adenine Release from Permeabilised Cells', 'Mobilisation
of Intracellular Ca$^{2+}$ Stores in Permeabilised CHO-cells after Muscarinic Receptor Activation’, ‘$[^{35}S]$-GTP$\gamma$S Radioligand Binding in Permeabilised CHO-cells after Muscarinic Receptor Stimulation’, ‘Methacholine-Stimulated Inositol 1,4,5-Trisphosphate Generation in Permeabilised CHO-cells’, and ‘Protein Determinations’.

5.2. Data Analysis.

$[^{35}S]$-GTP$\gamma$S radioligand binding, inositol trisphosphate and calcium release data were analysed using a least-sum-of-squares non-iterative curve fitting programme, computer software Prism 2 (concentration-response curve (variable slope)) to determine EC$_{50}$ and Hill coefficient (nH) values. Prism 2 was supplied by GraphPAD software Inc., San Diego, CA, U.S.A. All data are shown as means ± s.e.mean for ‘n’ separate experiments.

5.3. Results.

Evaluation of permeabilising agents.

Initially different detergents, commonly used to permeabilise cells to allow assessment of intracellular signalling, and the duration of their exposure were compared in CHO-M$_3$ cells at the level of $[^{3}H]$-adenine metabolite release. The cells were pre-labelled with $[^{3}H]$-adenine so that this nucleotide would become incorporated into the intracellular milieu and when the cells were exposed to various detergents the $[^{3}H]$-adenine or its metabolites would be released providing a rough indicator of permeabilisation. Digitonin (500 $\mu$g ml$^{-1}$), $\beta$-escin (50 $\mu$g ml$^{-1}$) and saponin (100 $\mu$g ml$^{-1}$) were compared at 2 and 5 min. Digitonin (500 $\mu$g ml$^{-1}$) caused the release of a maximal level of $[^{3}H]$-adenine after 2 min, where as $\beta$-escin and saponin caused a maximal release at 5 min (Figure 5.3.1). At 5 min all the detergents assessed elicited the release of 30-40% of the total $[^{3}H]$-adenine incorporated (data not shown), this appeared to be due to incorporation of the radiolabelled nucleotide into nucleic acids. The effect of the different detergents on receptor-generated signalling
was assessed by the ability of methacholine to induce mobilisation of intracellular Ca\textsuperscript{2+} stores in either β-escin (50 µg ml\textsuperscript{-1}), digitonin (500 µg ml\textsuperscript{-1}) or saponin (100 µg ml\textsuperscript{-1}) permeabilised CHO-cell suspensions. Digitonin (500 µg ml\textsuperscript{-1}) and saponin (100 µg ml\textsuperscript{-1}) permeabilised CHO-M\textsubscript{3} cells did not produce a measurable response to methacholine at the level of intracellular Ca\textsuperscript{2+} mobilisation. Yet the response to Ins(1,4,5)P\textsubscript{3} remained intact producing \(EC_{50}\) values of 80 nM and 84 nM for Ins(1,4,5)P\textsubscript{3}-stimulated \(^{45}\)Ca\textsuperscript{2+} release from digitonin and saponin permeabilised CHO-M\textsubscript{3} cells respectively (Figure 5.3.2). This indicates that the effects of digitonin (500 µg ml\textsuperscript{-1}) or saponin (100 µg ml\textsuperscript{-1}) are at the level of the cell membrane and not at the level of the intracellular Ca\textsuperscript{2+} stores. However, β-escin (50 µg ml\textsuperscript{-1}) allowed methacholine as well as Ins(1,4,5)P\textsubscript{3} to be evaluated at the level of Ca\textsuperscript{2+} mobilisation, methacholine and Ins(1,4,5)P\textsubscript{3} produced \(EC_{50}\) values of 290 nM and 75 nM respectively for \(^{45}\)Ca\textsuperscript{2+} release in β-escin (50 µg ml\textsuperscript{-1}) permeabilised CHO-M\textsubscript{3} cells (Figure 5.3.11). Therefore, all further permeabilised cell experiments were conducted on CHO-cell suspensions exposed to β-escin (50 µg ml\textsuperscript{-1}) for 5 min.

**Effect of time and GDP concentration on methacholine-stimulated \(^{35}\)S-GTPγS binding in permeabilised CHO-cells expressing recombinant muscarinic receptors.**

The use of β-escin (50 µg ml\textsuperscript{-1}) permeabilised CHO-cell suspensions to measure the specific binding of \(^{35}\)S-GTPγS to activated G proteins was assessed. Initial time-course experiments illustrated that an elevation over basal of \(^{35}\)S-GTPγS binding upon stimulation with 1 mM methacholine could be obtained in a permeabilised cell system (in the presence of 10 µM GDP and 120 mM KCl), with maximal stimulations occurring at the longest time point evaluated (60 min) (Figure 5.3.3). At 60 min the CHO-M\textsubscript{1}, CHO-SLM\textsubscript{2}, CHO-M\textsubscript{3} and CHO-M\textsubscript{4} cell lines produced maximal methacholine-stimulated \(^{35}\)S-GTPγS binding expressed as \(^{35}\)S-GTPγS bound above basal (fmol mg\textsuperscript{-1} protein) of; 79 ± 4, 93 ± 6, 68 ± 9 and 87 ± 9 (n=3), representing 28-38% increases over basal values of 278 ± 19, 259 ± 13, 236 ± 49, and 231 ± 18 (Figure 5.3.3). All further experiments were therefore incubated for 60 min. The time-course was not evaluated beyond 60 min due to the finite nature of the ATP-
regeneration system in the cytosol-like buffer (CLB) utilised in this set of experiments. Methacholine-stimulated $[^{35}S]$-GTP$_\gamma$S binding was also evaluated in non-transfected CHO-cells, these cells produced no response to methacholine at all time points up to 60 min (data not shown).

The GDP dependence of $[^{35}S]$-GTP$_\gamma$S binding in permeabilised CHO-cells was investigated by assessing the level of methacholine-stimulated $[^{35}S]$-GTP$_\gamma$S binding after 60 min in the absence or presence of GDP (1 and 10 µM). Upon stimulation with a maximal concentration of methacholine (1 mM), in the presence of varying concentrations of GDP, increases in $[^{35}S]$-GTP$_\gamma$S binding above basal were obtained at all concentrations of GDP tested in all four cell lines investigated (Figure 5.3.4 and Table 5.3.1). From these data it can be seen that decreasing the concentration of GDP, from 10 µM to 0 increased total agonist-stimulated $[^{35}S]$-GTP$_\gamma$S binding in all cell lines but it also dramatically increased the variability. So in all the $[^{35}S]$-GTP$_\gamma$S binding assays the inclusion of GDP (10 µM) was utilised to minimise variability.

The methacholine-mediated stimulation of $[^{35}S]$-GTP$_\gamma$S binding to β-escin (50 µg ml$^{-1}$) permeabilised CHO-cell suspensions was completely blocked by atropine in all four cell lines (Figure 5.3.5). Using binding conditions of 60 min incubations at 30°C with the inclusion of 10 µM GDP the β-escin (50 µg ml$^{-1}$) permeabilised CHO-M$_1$, CHO-SLM$_2$, CHO-M$_3$ and CHO-M$_4$ cells produced maximal methacholine-stimulated $[^{35}S]$-GTP$_\gamma$S binding values of 32 %, 29%, 19% and 25% over basal respectively (n=3). In parallel incubations of methacholine (1 mM) and atropine (10 µM) at 30°C for 60 min completely prevented these increases (Figure 5.3.5). These data illustrate that the $[^{35}S]$-GTP$_\gamma$S binding responses observed in permeabilised cell suspensions are receptor-mediated.

Assays were routinely performed at 30°C with 200-300 µg of protein (β-escin 50 µg ml$^{-1}$ permeabilised CHO-cell suspension) for 60 min to afford maximal stimulation of $[^{35}S]$-GTP$_\gamma$S binding over basal levels in the presence of 1 mM methacholine. The extent of receptor-stimulated $[^{35}S]$-GTP$_\gamma$S binding (maximally around 1.4 fold) was essentially independent of the cell-line utilised.
Pharmacological characterisation of agonist-stimulated [35S]-GTPγS binding in β-escin permeabilised CHO-cells expressing recombinant muscarinic acetylcholine receptors.

Using the characterised assay conditions comparisons of the muscarinic acetylcholine receptor 'full' agonist methacholine and the muscarinic receptor 'partial' agonists arecoline and pilocarpine were performed in all four cell lines used in this series of experiments. 1 mM methacholine elicited increases over basal [35S]-GTPγS binding of 74 ± 20, 74 ± 2, 54 ± 12 and 81 ± 11 expressed as [35S]-GTPγS bound over basal fmol mg⁻¹ protein in the CHO-M₁, CHO-SLM₂, CHO-M₃ and CHO-M₄ permeabilised cell suspensions. Comparative values for arecoline-stimulated (1 mM) [35S]-GTPγS binding in the CHO-M₁, CHO-SLM₂, CHO-M₃ and CHO-M₄ permeabilised cell suspensions were 44 ± 16, 74 ± 5, 33 ± 13 and 69 ± 9 (Figure 5.3.6). Thus, arecoline displayed $E_{max}$ values expressed as a percentage of 1 mM methacholine response of 100 ± 7 % and 86 ± 10 % in CHO-SLM₂ and CHO-M₄ respectively (n=3), whilst in the M₁ and M₃ receptor expressing cell lines $E_{max}$ values were only 60 ± 20 % and 41 ± 7 % of the methacholine-stimulated [35S]-GTPγS binding (Figure 5.3.6). So it can be seen that when arecoline is evaluated at the level of [35S]-GTPγS binding in permeabilised CHO-cell suspensions it acts as a partial agonist at the M₁ and M₃ receptor subtypes, whereas at the M₂ and M₄ receptor subtypes it appears to function as a full agonist. This is in agreement with the data reported in Chapter Four when arecoline was assessed at the level of [35S]-GTPγS binding in cell membranes it was seen that arecoline showed full agonist activity at the M₂ and M₄ receptor subtypes.

Pilocarpine displayed partial agonist activity at all the muscarinic receptor subtypes producing $E_{max}$ values of 31 ± 3 %, 59 ± 15 %, 33 ± 5 % and 68 ± 4 % when expressed as a percentage of 1 mM methacholine-stimulated [35S]-GTPγS binding at the M₁, M₂, M₃ and M₄ subtypes respectively (Figure 5.3.6). Again these data are in agreement with those reported in CHO-cell membranes in Chapter Four.

Full concentration-response analyses of methacholine were performed in the permeabilised cell preparation for the M₁, M₂, M₃ and M₄ expressing CHO-cells. The
EC<sub>50</sub> values for methacholine at the M<sub>1</sub>, M<sub>2</sub>, M<sub>3</sub> and M<sub>4</sub> receptor subtypes expressed in CHO-M<sub>1</sub>, CHO-SLM<sub>2</sub>, CHO-M<sub>3</sub> and CHO-M<sub>4</sub> are -5.17 ± 0.07 (6.9 μM), -5.73 ± 0.09 (2.0 μM), -5.31 ± 0.14 (5.6 μM) and -5.78 ± 0.09 (1.2 μM) expressed as log M values respectively (Figure 5.3.7). The Hill coefficient values from the methacholine concentration-response curves were 0.98 ± 0.07, 0.82 ± 0.09, 1.02 ± 0.06 and 1.16 ± 0.21 (n=4) for the CHO-M<sub>1</sub>, CHO-SLM<sub>2</sub>, CHO-M<sub>3</sub> and CHO-M<sub>4</sub> cell lines respectively. From these former values it can be seen that methacholine appears 3-4 fold more potent at the M<sub>2</sub> and M<sub>4</sub> receptors, compared to the M<sub>1</sub> and M<sub>3</sub> receptors when evaluated at this level in permeabilised cell suspensions.

**Effect of pertussis toxin pre-treatment on methacholine-stimulated [³⁵S]-GTPγS binding in β-escin permeabilised CHO-cells expressing recombinant muscarinic receptors.**

Pertussis toxin (PTx) pre-treatment (100 ng ml<sup>−1</sup> 18-20 h, for detail reference the Experimental Methodology section entitled 'Cell Culture Techniques') resulted in a 30-45 % decrease in the basal [³⁵S]-GTPγS binding and a 50-60 % decrease in methacholine-stimulated [³⁵S]-GTPγS binding in the permeabilised CHO-M<sub>1</sub>, CHO-SLM<sub>2</sub>, CHO-M<sub>3</sub> and CHO-M<sub>4</sub> cell suspensions. The methacholine-stimulated [³⁵S]-GTPγS binding in absence and presence of PTx in the CHO-M<sub>1</sub>, CHO-SLM<sub>2</sub>, CHO-M<sub>3</sub> and CHO-M<sub>4</sub> cell lines are shown in Table 5.3.2. After pertussis toxin pre-treatment agonist-stimulated [³⁵S]-GTPγS binding was no longer statistically significantly increased compared to basal levels in the CHO-M<sub>1</sub>, CHO-SLM<sub>2</sub>, CHO-M<sub>3</sub> and CHO-M<sub>4</sub> cell suspensions (P > 0.05, paired Student’s t-test). The pertussis toxin concentration of 100 ng ml<sup>−1</sup> utilised is maximally effective as increasing the concentration to 300 ng ml<sup>−1</sup> produces no further decrease in either the basal or agonist-stimulated [³⁵S]-GTPγS binding (Data not shown). Therefore the [³⁵S]-GTPγS binding responses reported in the CHO-M<sub>1</sub>, CHO-SLM<sub>2</sub>, CHO-M<sub>3</sub> and CHO-M<sub>4</sub> permeabilised cell suspensions appear to be mediated predominantly by pertussis toxin-sensitive G proteins (>85%).
Effect of atropine on $[^{35}\text{S}]$-GTP$\gamma$S binding in $\beta$-escin permeabilised CHO-cells expressing recombinant muscarinic receptors.

Atropine has been shown to inhibit methacholine-stimulated $[^{35}\text{S}]$-GTP$\gamma$S binding in $\beta$-escin (50 $\mu$g ml$^{-1}$) permeabilised CHO-cell suspensions (data described above). Yet in CHO-cell membranes atropine mediated an inhibition of basal $[^{35}\text{S}]$-GTP$\gamma$S binding (see Chapter Four). The effect of atropine on basal $[^{35}\text{S}]$-GTP$\gamma$S binding in permeabilised CHO-cell suspensions was evaluated at the $M_1$, $M_2$, $M_3$ and $M_4$ receptor subtypes. Atropine had no effect on basal $[^{35}\text{S}]$-GTP$\gamma$S binding under standard assay conditions of 60 min incubations at 30°C with the inclusion of 10 $\mu$M GDP. To alter the G protein population mediating the basal level of $[^{35}\text{S}]$-GTP$\gamma$S binding in permeabilised cell suspensions the ability of atropine to affect the level of $[^{35}\text{S}]$-GTP$\gamma$S binding was assessed in pertussis toxin (100 ng ml$^{-1}$) pre-treated and untreated cells in the absence or presence of GDP (1 or 10 $\mu$M). Atropine showed no significant effects upon basal $[^{35}\text{S}]$-GTP$\gamma$S binding under any of the conditions evaluated (Table 5.3.3).

Evaluation of inositol 1,4,5-trisphosphate generation and intracellular $\text{Ca}^{2+}$ mobilisation in $\beta$-escin permeabilised CHO-$M_1$ and -$M_3$ cells.

Having evaluated a permeabilised cell system to allow the investigation of G protein activation, by using $[^{35}\text{S}]$-GTP$\gamma$S binding as a quantitative parameter, the examination of distal signalling events was undertaken in the same cell preparation. In this single cell preparation inositol 1,4,5-trisphosphate generation and intracellular $\text{Ca}^{2+}$ mobilisation were evaluated after agonist-stimulation of the $M_1$ and $M_3$ receptor subtypes.

Time-course analysis of inositol 1,4,5-trisphosphate generation in permeabilised CHO-$M_1$ and -$M_3$ cells showed that Ins(1,4,5)$\text{P}_3$ reached a maximal level after 5 min incubation at 37°C with 1, 10 or 100 $\mu$M methacholine (Figures 5.3.8 and 5.3.9). $M_1$ and $M_3$ receptor stimulation produced increases of 93.4 ± 6.0 and 55.7 ± 1.4 Ins(1,4,5)$\text{P}_3$ pmol mg$^{-1}$ protein with 100 $\mu$M methacholine, respectively. Further
experiments demonstrated no significant differences in the time profiles of
Ins(1,4,5)P₃ generation between control and PTx pre-treated cells (data not shown).
Ins(1,4,5)P₃ generation assays were therefore routinely performed at 37°C with 200-
300 µg of protein for 5 min.

Inositol 1,4,5-trisphosphate accumulation in permeabilised cell suspensions was
partially pertussis toxin-sensitive. Treatment with PTx (100 ng ml⁻¹, 18-24 h) reduced
both basal and methacholine-stimulated accumulations. Pertussis toxin decreased the
basal value by 44.3 ± 10.7 % over 4 experiments in the CHO-M₁ cell suspensions and
50.1 ± 13.6 % over 3 experiments in the CHO-M₃ cell suspensions. Methacholine-
stimulated Ins(1,4,5)P₃ accumulations decreased by 23.5 ± 12.6 % and 33.7 ± 9.8 %
when levels in pertussis toxin pre-treated cells were compared to untreated CHO-M₁
and CHO-M₃ cell suspensions.

Full concentration-response analyses to methacholine at the level of Ins(1,4,5)P₃
generation in untreated and pertussis toxin pre-treated (100 ng ml⁻¹) CHO-M₁ cell
suspensions produced log (M) EC₅₀ values of -5.75 ± 0.16 (2.1 µM) and -5.57 ± 0.11
(3.0 µM) (Figure 5.3.10). Further concentration-response analysis to methacholine at
the level of Ins(1,4,5)P₃ generation in untreated and pertussis toxin pre-treated CHO-
M₃ cell suspensions produced log (M) EC₅₀ values of -5.23 ± 0.28 (7.0 µM) and -5.07
± 0.12 (9.2 µM) respectively (Figure 5.3.10). The M₁ and M₃ receptor subtypes are
reportedly linked to phosphoinositidase C via pertussis toxin-insensitive G proteins
(Caulfield, 1993) yet in this cellular preparation there is evidence that they mediate
signalling by coupling to both pertussis toxin-sensitive and -insensitive G proteins.
This is reflected in both the Eₘₐₓ values and the EC₅₀ values for the M₁ and M₃ cells. The Eₘₐₓ
values for both the cell lines show a decrease in the presence of PTx, perhaps
indicating a Gβγ component in the activation of PLC. Also the EC₅₀ values for
methacholine-stimulated Ins(1,4,5,)P₃ accumulation (Table 5.3.4) show a small but
consistent decrease in both the CHO-M₁ and- M₃ PTx pre-treated cells, this may
further illustrate a Gβγ component involved in the activation of PLC.
Methacholine-stimulated $^{45}\text{Ca}^{2+}$-release in permeabilised cells gave rise to a similar maximal release of $^{45}\text{Ca}^{2+}$ in CHO-M₁ and -M₃ cell suspensions (84 ± 4; 87 ± 2% of ionomycin-releasable pool, respectively (Figure 5.3.11)). Further experiments demonstrated no significant differences in the maximal levels of $^{45}\text{Ca}^{2+}$-released in pertussis toxin pre-treated (100 ng ml⁻¹) cells (Figure 5.3.11). Full concentration-response analyses of methacholine-stimulated $^{45}\text{Ca}^{2+}$-release in pertussis toxin pre-treated and untreated CHO-M₁ cell suspensions produced log (M) EC₅₀ values of -6.26 ± 0.20 (352 nM) and -6.24 ± 0.24 (425 nM) (n=4). In CHO-M₃ cell suspensions, further concentration-response analyses at the level of methacholine-stimulated $^{45}\text{Ca}^{2+}$-release were performed in pertussis toxin pre-treated and untreated cells producing log (M) EC₅₀ values of -6.57 ± 0.01 (272 nM) and -6.48 ± 0.02 (330 nM) respectively (n=4). No significant effects of pertussis toxin pre-treatment were seen at the level of $^{45}\text{Ca}^{2+}$-release in either the CHO-M₁ or CHO-M₃ cells. Comparison of Ins(1,4,5)P₃ and $^{45}\text{Ca}^{2+}$-release concentration-effect relationships revealed lower EC₅₀ values for intracellular $^{45}\text{Ca}^{2+}$-mobilisation indicating signal amplification (see Table 5.3.4).
Figure 5.3.1.


CHO-M$_3$ cell suspensions were incubated in the presence or absence of a given detergent for the time indicated. (See the Methods section entitled '[$^3$H]-Adenine Release from Permeabilised CHO-cells'). Data are expressed as the initial [$^3$H]-adenine released for all time-points and are shown as means ± s.e.mean for 3 separate experiments carried out in duplicate.
Figure 5.3.2.

The effect of detergents on receptor-mediated signalling.

The effect of saponin (100 \( \mu \text{g ml}^{-1} \)) on \(^{45}\text{Ca}^{2+}\) release in CHO-M3 cells.

![Graph showing the effect of saponin on \(^{45}\text{Ca}^{2+}\) release](image)

The effect of digitonin (500 \( \mu \text{g ml}^{-1} \)) on \(^{45}\text{Ca}^{2+}\) release in CHO-M3 cells.

![Graph showing the effect of digitonin on \(^{45}\text{Ca}^{2+}\) release](image)

CHO-cells were incubated in CLB as described in Methods section ‘Mobilisation of intracellular \(\text{Ca}^{2+}\) stores in permeabilised CHO-cells after muscarinic receptor activation’ with the inclusion of methacholine (filled symbols) or Ins(1,4,5)P3 (open symbols) \((n = 3)\). The maximal calcium release was defined with ionomycin \((20 \mu \text{M})\), data are shown as a percentage of the total ionomycin releasable calcium pool. All experiments were performed in duplicate.
Figure 5.3.3.
Time-course of 1 mM methacholine-stimulated $^{[35]S}$-GTPγS binding in β-escin permeabilised CHO-cells.

Time-course of $M_1$-M$_4$ receptor-mediated $^{[35]S}$-GTPγS binding in permeabilised cells. CHO-cells permeabilised with β-escin (50 μg ml$^{-1}$) were incubated in the presence of 10 μM GDP and 1.2 nM $^{[35]S}$-GTPγS for the times indicated. Data are expressed as fmol $^{[35]S}$-GTPγS bound mg$^{-1}$ protein above basal for all time-points and are shown as means ± s.e.mean for 3 separate experiments carried out in duplicate.
The effect of GDP upon 1 mM methacholine-stimulated [35S]-GTPγS binding in β-escin permeabilised CHO-cells.

The effect of GDP upon $M_1$-$M_4$ receptor-mediated [35S]-GTPγS binding in permeabilised CHO-cells. β-escin (50 μg ml$^{-1}$) permeabilised CHO-cells were incubated in the absence or presence of GDP (1 or 10 μM) for 60 min. Data are expressed as fmol [35S]-GTPγS bound mg$^{-1}$ protein above basal and are shown as means ± s.e.mean for ≥ 3 separate experiments carried out in duplicate.
The effect of atropine on methacholine-stimulated $[^{35}\text{S}]-\text{GTPyS}$ binding in $\beta$-escin permeabilised CHO-cells. $\beta$-escin (50 $\mu$g ml$^{-1}$) permeabilised CHO-cells were incubated in the presence or absence of 1 mM methacholine for 60 min, with the inclusion of 10 $\mu$M atropine in antagonist-treated reactions. Data are expressed as fmol $[^{35}\text{S}]-\text{GTPyS}$ bound mg$^{-1}$ protein and are shown as means ± s.e.mean for 3 separate experiments carried out in duplicate.
Effect of various muscarinic agonists upon [³⁵S]-GTPγS binding in β-escin permeabilised CHO-cells.

Effect of methacholine (1 mM), arecoline (1 mM) and pilocarpine (1 mM) upon [³⁵S]-GTPγS binding in permeabilised CHO-cells. CHO-M₁-M₄ cells were incubated in the presence of GDP 10 μM and 1.2 nM [³⁵S]-GTPγS for 60 min. Data are expressed as fmol [³⁵S]-GTPγS bound mg⁻¹ protein above basal for all time-points and are shown as means ± s.e.mean for 3 separate experiments carried out in duplicate.
Figure 5.3.7.

Methacholine-stimulated $[^{35}\text{S}]-\text{GTP}\gamma\text{S}$ binding in $\beta$-escin permeabilised CHO-cells

Concentration-dependence of methacholine-induced $[^{35}\text{S}]-\text{GTP}\gamma\text{S}$ binding in $\beta$-escin permeabilised cells. CHO-cells were incubated in the presence of GDP (10 $\mu$M) and 1.2 nM $[^{35}\text{S}]-\text{GTP}\gamma\text{S}$ for 60 min. Data are expressed as fmol $[^{35}\text{S}]-\text{GTP}\gamma\text{S}$ bound mg$^{-1}$ protein and are shown as means ± s.e.mean for 4 separate experiments performed in duplicate.
Figure 5.3.8.
The effect of time on inositol 1,4,5-trisphosphate generation in permeabilised CHO-M_{i} cells stimulated with methacholine.

CHO-cells were incubated in the presence of methacholine (1, 10 or 100 \( \mu \)M) for the times indicated as described in Methods section 'Methacholine-stimulated inositol 1,4,5-trisphosphate generation in permeabilised CHO-cells'. The data shown are representative of 3 further experiments. Further experiments demonstrated no significant differences in the patterns of IP_{3} generation between control and PTx-treated cells. All experiments were performed in triplicate.
CHO-cells were incubated in the presence of methacholine (1, 10 or 100 μM) for the times indicated as described in Methods section ‘Methacholine-stimulated inositol 1,4,5-trisphosphate generation in permeabilised CHO-cells’. The data shown are representative of 3 experiments. Further experiments demonstrated no significant differences in the patterns of IP₃ generation between control and PTx-treated cells, experiments were performed in triplicate.
Effect of pertussis toxin pre-treatment (100 ng ml\(^{-1}\) 20-24 h) upon CHO-M\(_1\) and -M\(_3\) mediated Ins(1,4,5)P\(_3\) generation in β-escin permeabilised cells.

Effect of methacholine at increasing concentrations on Ins(1,4,5)P\(_3\) generation in PTx pre-treated and control permeabilised CHO-cell suspensions. Data are expressed as pmol Ins(1,4,5)P\(_3\) generated mg\(^{-1}\) protein for all concentrations observed and are shown as means ± s.e.mean for 4 separate experiments performed in duplicate.
Figure 5.3.11.

Effect of pertussis toxin pre-treatment (100 ng ml⁻¹, 20-24 h) upon methacholine-mediated ⁴⁵Ca²⁺ release in β-escin permeabilised CHO-M₁ and -M₃ cells.

Concentration-dependence of methacholine-induced ⁴⁵Ca²⁺ release in permeabilised CHO-cells. CHO-M₁ (filled symbols) and -M₃ (open symbols) cells were challenged with increasing concentrations of methacholine for 1 min. Data are expressed as a percentage of the total ⁴⁵Ca²⁺ released by 20 μM ionomycin and are shown as means ± s.e.mean for 4 separate experiments performed in duplicate.
Table 5.3.1.
Effect of GDP upon $[^{35}S]$-GTPγS binding in β-escin (50 μg ml$^{-1}$) permeabilised CHO-cells.

<table>
<thead>
<tr>
<th></th>
<th>Basal</th>
<th>1mM methacholine</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHO-M$_1$ [GDP]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>1234 ± 174</td>
<td>1413 ± 237</td>
<td>3</td>
</tr>
<tr>
<td>1</td>
<td>699 ± 71</td>
<td>860 ± 69</td>
<td>4</td>
</tr>
<tr>
<td>10</td>
<td>272 ± 15</td>
<td>347 ± 12*</td>
<td>4</td>
</tr>
<tr>
<td>CHO-SLM$_2$ [GDP]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>1458 ± 343</td>
<td>1645 ± 406</td>
<td>3</td>
</tr>
<tr>
<td>1</td>
<td>794 ± 168</td>
<td>949 ± 112</td>
<td>3</td>
</tr>
<tr>
<td>10</td>
<td>242 ± 13</td>
<td>344 ± 10*</td>
<td>3</td>
</tr>
<tr>
<td>CHO-M$_3$ [GDP]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>1244 ± 134</td>
<td>1429 ± 214</td>
<td>3</td>
</tr>
<tr>
<td>1</td>
<td>730 ± 88</td>
<td>881 ± 94</td>
<td>4</td>
</tr>
<tr>
<td>10</td>
<td>268 ± 22</td>
<td>354 ± 14*</td>
<td>4</td>
</tr>
<tr>
<td>CHO-M$_4$ [GDP]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>1219 ± 643</td>
<td>2050 ± 148</td>
<td>3</td>
</tr>
<tr>
<td>1</td>
<td>1067 ± 226</td>
<td>1314 ± 289</td>
<td>3</td>
</tr>
<tr>
<td>10</td>
<td>299 ± 57</td>
<td>396 ± 71*</td>
<td>3</td>
</tr>
</tbody>
</table>

Effects of GDP upon basal and methacholine-stimulated $[^{35}S]$-GTPγS binding in β-escin permeabilised cells. CHO-cells were incubated in the presence or absence of 1 mM methacholine with the indicated concentrations of GDP for 60 min. Data are shown as fmol $[^{35}S]$-GTPγS bound mg$^{-1}$ protein. All values are expressed as means ± s.e.mean where ‘n’ represents the number of individual experiments performed. Statistical significance * P < 0.05; paired Student’s t-test.
Table 5.3.2.
The effects of pertussis toxin upon basal and 1 mM methacholine-stimulated $^{[35]S}$-GTPγS binding in β-escin permeabilised cells.

<table>
<thead>
<tr>
<th></th>
<th>Basal</th>
<th>1mM methacholine</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHO-M$_1$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- PTx</td>
<td>227 ± 17</td>
<td>318 ± 12 *</td>
<td>3</td>
</tr>
<tr>
<td>+ PTx</td>
<td>146 ± 15</td>
<td>157 ± 18</td>
<td>3</td>
</tr>
<tr>
<td>CHO-SLM$_2$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- PTx</td>
<td>193 ± 10</td>
<td>286 ± 3 *</td>
<td>3</td>
</tr>
<tr>
<td>+ PTx</td>
<td>114 ± 2</td>
<td>127 ± 14</td>
<td>3</td>
</tr>
<tr>
<td>CHO-M$_3$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- PTx</td>
<td>237 ± 10</td>
<td>339 ± 25 *</td>
<td>3</td>
</tr>
<tr>
<td>+ PTx</td>
<td>161 ± 36</td>
<td>172 ± 38</td>
<td>3</td>
</tr>
<tr>
<td>CHO-M$_4$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- PTx</td>
<td>217 ± 42</td>
<td>293 ± 60 *</td>
<td>3</td>
</tr>
<tr>
<td>+ PTx</td>
<td>118 ± 12</td>
<td>124 ± 14</td>
<td>3</td>
</tr>
</tbody>
</table>

CHO-cells were exposed to pertussis toxin for 20-24 h prior to cell preparation (see Methods section entitled 'Permeabilised Cell Preparation').

Pertussis toxin pre-treated (+PTx; 100 ng ml$^{-1}$) and untreated (-PTx) permeabilised CHO-cells were incubated with $^{[35]S}$-GTPγS (1.2 nM), GDP (10 μM) in the presence and absence of 1 mM methacholine for 60 min. All data are shown as $^{[35]S}$-GTPγS bound fmol mg$^{-1}$ protein, means ± s.e.mean of values obtained from separate experiments (n = number of individual experiments). Statistical significance * P < 0.05; paired Student’s t-test.
Table 5.3.3.

Effect of pertussis toxin and GDP on atropine inhibition of $[^{35}\text{S}]$-GTPγS binding.

<table>
<thead>
<tr>
<th></th>
<th>Control Basal</th>
<th>Control + Atr</th>
<th>+PTx Basal</th>
<th>+PTx + Atr</th>
</tr>
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<tbody>
<tr>
<td>CHO-Mₙ</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>[GDP] μM</td>
<td>0</td>
<td>997 ± 61</td>
<td>975 ± 71</td>
<td>728 ± 25</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>758 ± 10</td>
<td>729 ± 22</td>
<td>489 ± 60</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>226 ± 7</td>
<td>226 ± 4</td>
<td>122 ± 10</td>
</tr>
<tr>
<td>CHO-SLM₂</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>[GDP] μM</td>
<td>0</td>
<td>1099 ± 85</td>
<td>1126 ± 120</td>
<td>741 ± 58</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>396 ± 10</td>
<td>462 ± 61</td>
<td>435 ± 55</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>177 ± 23</td>
<td>185 ± 14</td>
<td>109 ± 10</td>
</tr>
<tr>
<td>CHO-M₃</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>[GDP] μM</td>
<td>0</td>
<td>1079 ± 42</td>
<td>1066 ± 42</td>
<td>595 ± 66</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>745 ± 25</td>
<td>759 ± 59</td>
<td>411 ± 58</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>213 ± 13</td>
<td>217 ± 22</td>
<td>106 ± 3</td>
</tr>
<tr>
<td>CHO-M₄</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>[GDP] μM</td>
<td>0</td>
<td>1063 ± 84</td>
<td>1083 ± 82</td>
<td>637 ± 57</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>790 ± 34</td>
<td>764 ± 81</td>
<td>424 ± 23</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>237 ± 21</td>
<td>238 ± 13</td>
<td>118 ± 9</td>
</tr>
</tbody>
</table>

Effect of GDP and PTx on basal and atropine-influenced $[^{35}\text{S}]$-GTPγS binding in β-escin permeabilised cells. CHO-cells were incubated in the presence or absence of 10 μM atropine with the indicated concentrations of GDP for 60 min. Data are shown as fmol $[^{35}\text{S}]$-GTPγS bound mg⁻¹ protein. All values are expressed as means ± s.e.mean of values obtained from individual experiments (n=3).
Table 5.3.4.

The effects of pertussis toxin upon the responses elicited by methacholine in CHO-M₁ and CHO-M₃ cells permeabilised with β-escin.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>[³⁵S]-GTPγS binding</th>
<th>Ins(1,4,5)P₃ accumulation</th>
<th>⁴⁵Ca²⁺ release</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHO-M₁</td>
<td>5.31 ± 0.14 (n=4)</td>
<td>5.75 ± 0.16 (n=4)</td>
<td>6.26 ± 0.20 (n=4)</td>
</tr>
<tr>
<td>CHO-M₃</td>
<td>5.17 ± 0.07 (n=4)</td>
<td>5.23 ± 0.18 (n=3)</td>
<td>6.57 ± 0.01 (n=4)</td>
</tr>
<tr>
<td>CHO-M₁ + PTx</td>
<td>N/A</td>
<td>5.57 ± 0.11 (n=4)</td>
<td>6.24 ± 0.24 (n=4)</td>
</tr>
<tr>
<td>CHO-M₃ + PTx</td>
<td>N/A</td>
<td>5.07 ± 0.12 (n=3)</td>
<td>6.48 ± 0.02 (n=4)</td>
</tr>
</tbody>
</table>

Comparison of different responses in pertussis toxin pre-treated and untreated CHO-M₁ and CHO-M₃ cells. Data are expressed as the mean-log molar EC₅₀ values ± s.e.mean from individual experiments, the number of experiments are shown in parentheses.
5.4. Discussion.

In this series of experiments agonist-stimulated $[^{35}S]$-GTPyS binding to G proteins was used as a measure of receptor-mediated activation of heterotrimeric G proteins by individual muscarinic acetylcholine receptor subtypes in permeabilised cells. This allowed the G protein activation from each receptor subtype to be assessed in a more intact cellular preparation, allowing the responses to both ‘full’ and ‘partial’ agonists to be documented.

No methacholine-stimulated $[^{35}S]$-GTPyS binding was seen in untransfected CHO-K1 cells but agonist-stimulated $[^{35}S]$-GTPyS binding was observed in all the transfected CHO-cell clones evaluated (CHO-M$_1$, CHO-SLM$_2$, CHO-M$_3$, and CHO-M$_4$), illustrating that the responses observed in the transfected cells are receptor-mediated. It also confirms that the CHO-K1 (parental) cell line does not possess endogenous muscarinic receptors. Agonist-stimulated $[^{35}S]$-GTPyS binding was completely inhibited when atropine was included at 10 µM, further illustrating that the observed responses were muscarinic receptor-mediated.

A number of surfactants well known to permeabilise the plasma membrane by complexing with cholesterol were assessed. Detergent evaluation was carried out on two levels, firstly the ability to produce holes in the cell surface allowing flux of intracellular components out of the cell, and secondly the ability to allow signal transduction from the extracellular surface to occur. Digitonin, saponin and β-escin are all cholesterol-complexing glycosides, and these were all assessed at the level of $[^{3}H]$-adenine metabolite release. The detergents all showed the ability to permeabilise the CHO-cells after 2 and 5 min exposures, at the concentrations utilised. However, when these were evaluated at the level of $^{45}$Ca$^{2+}$ release they showed differential abilities to allow the signal from the cell surface to be transduced to the intracellular machinery, with only β-escin permeabilised cells producing a measurable $^{45}$Ca$^{2+}$ response to methacholine. The lack of a $^{45}$Ca$^{2+}$ response to methacholine in CHO-cells permeabilised with saponin or digitonin may be due to major disruption of the cell membrane prohibiting receptor signalling due to the loss of membrane architecture.
and hence receptor configuration, or alternatively it may be due to a loss of intracellular membrane structure.

Time-course data for [35S]-GTPγS binding in CHO-M₁, CHO-SLM₂, CHO-M₃ and CHO-M₄ cell suspensions produced more equivalent time profiles than those observed in the membrane filtration-based assay system and the membrane based-immunoprecipitation assays (see Chapters Four and Six respectively), for all the four receptor subtypes evaluated. It can be seen from those data that the M₂ and M₄ receptor subtypes appear to elicit a slightly faster and larger response than the M₁ and M₃ receptor subtypes. This trend was seen in the membrane filtration-based assay system, yet in the permeabilised cell system these differences are not as pronounced. One factor that may explain these observed differences is that the permeabilised cell suspensions may require more careful equilibration of all the assay components. In one of the original studies of muscarinic receptor-mediated [35S]-GTPγS binding, a preincubation step was utilised to ensure that the tested muscarinic agents were at equilibrium throughout the labelling period (Lazareno et al., 1993b). The inclusion of a preincubation step in the permeabilised cell suspensions may have revealed more pronounced differences between the muscarinic receptor subtypes. With the inclusion of this step there would not be the equilibration to overcome which would be expected to be equal in these cells as the entire cell transfects are CHO-cells permeabilised with the same agent for the same duration. Also the pertussis toxin data from this series of experiments shows that the [35S]-GTPγS binding was almost solely mediated by Gᵢ-like G proteins. The pertussis toxin data show that we cannot fully evaluate muscarinic receptor-mediated activation of G₉₁α in a permeabilised CHO-cell preparation, so we are evaluating the interaction of the receptor subtypes with a different population of G proteins from the membrane based assay system. To investigate fully the M₁, M₂, M₃ and M₄ receptor subtype interactions with Gᵢ-like G proteins a Gᵢ₁₁α-specific [35S]-GTPγS binding time-course would be required.

One major experimental change in this series of experiments, compared to the membrane filtration-based experiments, was that KCl replaced NaCl in this set of experiments. Cytosol-like buffer (CLB), the composition of which reflects the
intracellular milieu, appeared adequate to allow the measurement of receptor-stimulated $[^{35}\text{S}]-\text{GTPyS}$ binding in permeabilised cells, and it has been shown that FMLP-mediated $[^{35}\text{S}]-\text{GTPyS}$ binding in permeabilised HL-60 cells elicits identical results with either 150 mM NaCl or 150 mM KCl (Wieland et al., 1995). So $[^{35}\text{S}]-\text{GTPyS}$ binding in permeabilised cells appears to require an isotonic ionic strength, but the monovalent cation appears unimportant. However, the monovalent cation had a pronounced affect upon carbachol-stimulated hydrolysis of GTP in cardiac membranes, where a potency order $\text{Na}^+ > \text{Li}^+ > \text{K}^+$ has been reported (Hilf and Jakobs, 1989). This may indicate that in the permeabilised CHO-cell system, although KCl could be utilised, the results may have been enhanced by the use of NaCl in a buffer more reflective the extracellular medium. This, however was not assessed due to the requirement for a buffer reflective of the intracellular medium to perform Ins(1,4,5)P$_3$ generation, or $^{45}\text{Ca}^{2+}$ release assays.

In contrast to $[^{35}\text{S}]-\text{GTPyS}$ binding in cellular membranes, $[^{35}\text{S}]-\text{GTPyS}$ binding could be assessed in a permeabilised cell system in the absence of exogenous GDP. The presence of endogenous guanine nucleotides in assays with permeabilised cells may explain the receptor-stimulated $[^{35}\text{S}]-\text{GTPyS}$ binding observed in the absence of exogenous GDP. This finding directly contrasts with the findings based in cell membranes and presented in Chapter Four, where receptor-stimulated $[^{35}\text{S}]-\text{GTPyS}$ binding was not seen in the absence of exogenous GDP. The lack of receptor-mediated $[^{35}\text{S}]-\text{GTPyS}$ binding in the absence of GDP in the membrane-filtration based assay is probably due to the loss of endogenous guanine nucleotides caused by extensive washing steps in the preparation of cell membranes.

Differential effects of the muscarinic receptor agonists, methacholine, arecoline and pilocarpine, were seen at the muscarinic receptor subtypes when assessed by $[^{35}\text{S}]-\text{GTPyS}$ binding in permeabilised CHO-cells. Again, as seen in Chapter Four, the maximal effects of arecoline appeared to be dependent upon the muscarinic receptor subtype expressed. The $M_2$ and $M_4$ expressing cells displayed $E_{\text{max}}$ values for arecoline comparable to methacholine, whereas, at the $M_1$ and $M_3$ receptor subtypes arecoline displayed partial agonist activity when compared directly to the methacholine-
mediated response. Receptor subtype-dependent variations in the efficacy of arecoline have been reported in many other studies. Olianas and Onali, (1991), Quist, (1991) and Olianas and Onali, (1996) have shown that arecoline acting at AC-linked muscarinic receptors is a high efficacy agonist comparable to methacholine or carbachol. Further detailed agonist studies have shown an efficacy change in arecoline from high to low when comparing AC-linked and PLC-linked muscarinic receptor subtypes (Freedman et al., 1988; Spencer et al., 1988; Baumgold and White, 1989). Pilocarpine however gave rise to $E_{\text{max}}$ values which indicated that this compound acts as a partial agonist at all the muscarinic receptor subtypes investigated. Matesic et al, (1991) directly illustrated the partiality of pilocarpine agonism at the level of G protein activation, reporting that reduced amounts of total receptor-G protein complexes were observed when pilocarpine was compared to carbachol. In many other studies pilocarpine has been directly compared to high efficacy agonists like carbachol and methacholine and was shown to be a low efficacy agonist at all the muscarinic subtypes studied (Freedman et al., 1988; Baumgold and White, 1989; Olianas and Onali, 1991; 1996).

Full concentration-analysis at the $M_2$ and $M_4$ receptors produced $EC_{50}$ values for methacholine that correlated closely with the $EC_{50}$ values generated in membrane filtration-based values. The $M_1$ and $M_3$ receptor subtypes, however, produced $EC_{50}$ values for methacholine which were significantly different from those generated in the membrane filtration-based assay. As discussed above the G protein population activated in these two assay designs appears to diverge, therefore it is not surprising that the $EC_{50}$ values are not in close agreement. The fold stimulations over basal for all four receptor subtypes were small (~1.4 fold maximum) (Akam et al., 1998), but this compares favourably with data obtained in digitonin-permeabilised adherent human embryonic kidney cells expressing the $M_3$ muscarinic receptor where a 1.6 fold stimulation over basal $[^{35}\text{S}]$-GTP$\gamma$S binding was observed (Wieland et al., 1995).

Methacholine-stimulated $[^{35}\text{S}]$-GTP$\gamma$S binding in $\beta$-escin permeabilised cells was essentially abolished by pertussis toxin pre-treatment, indicating that in this assay system all receptor subtypes couple predominately to $G_r$-like G proteins. The

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experimental conditions may have elicited preferential binding of \([^{35}S]-\text{GTP}\gamma S\) to certain classes of G proteins. It has been reported that G\(_i\)-like G proteins bind GTP\(\gamma\)S with a greater affinity than G\(_s\) and G\(_q\) G proteins (Bokoch et al., 1984; Wessling-Resnick and Johnson, 1987; Blank et al., 1991). Thus, it is possible that higher concentrations of \([^{35}S]-\text{GTP}\gamma S\) would have facilitated the evaluation of a pertussis-toxin insensitive component of \([^{35}S]-\text{GTP}\gamma S\) binding.

Methacholine-stimulated Ins(1,4,5)P\(_3\) accumulation in CHO-M\(_i\) and CHO-M\(_3\) cells was maintained in permeabilised cells suspended in cytosol-like buffer. This suggests that permeabilisation of CHO-M\(_i\) and CHO-M\(_3\) cells in a cytosol-like buffer, where Ca\(^{2+}\) is buffered to approx. 100 nM, did not preclude receptor-mediated activation of PLC. The time-course of Ins(1,4,5)P\(_3\) generation in permeabilised cells is different to that observed in intact cells, which is characterised by both a peak and plateau phase (Tobin et al., 1992; Wojcikiewicz et al., 1994; Wojcikiewicz et al., 1995; Burford et al., 1995a). The lack of defined peak and plateau phases of Ins(1,4,5)P\(_3\) generation in permeabilised cells may be due to the deregulation of the desensitisation machinery under these experimental conditions (Wojcikiewicz et al., 1994). The maximal response in permeabilised CHO-cells was less than that measured by Burford et al., (1995a) in intact cells. The reduced maximal response may be due to a reduction in the intrinsic activity of PLC under these experimental conditions (particularly the alteration in Ca\(^{2+}\) concentration), or an increased metabolism of Ins(1,4,5)P\(_3\). The permeabilised CHO-M\(_i\) cells produced a maximal Ins(1,4,5)P\(_3\) accumulation which was around twice that observed in CHO-M\(_3\) cells. This reflects the findings of Buck and Fraser (1990), where carbachol stimulation of M\(_i\) muscarinic receptors in CHO cells, produced a maximal total inositol phosphate response which was twice that observed in M\(_3\) expressing cells, when expressed at similar expression levels. This suggests that M\(_i\) receptors couple more efficiently to PLC activation than M\(_3\) receptors.

The EC\(_{50}\) values obtained for methacholine-stimulated Ins(1,4,5)P\(_3\) generation are in agreement with those reported by Burford et al., (1995a). Additionally in this study the EC\(_{50}\) values illustrate that the M\(_i\) receptor appears slightly more efficacious when
activating PLC compared to the M3 mediated response. Other studies have revealed this trend, with Peralta et al. (1988) reporting a 4-16 fold lower EC\textsubscript{50} value for carbachol acting at the M1 subtype when compared directly to the M3 subtype. For rat muscarinic receptors expressed in CHO-cells, the order of potency with respect to PI turnover was M1 > M3 (Gurwitz et al., 1994). Again the order of potency with respect to PI turnover was found to be M1 > M3 in A9L cells expressing human muscarinic receptors (Richards and van Giersbergen, 1995). The discussion of differences in the coupling of the M1 and M3 receptor subtypes is continued in Chapter Six.

Pertussis toxin pre-treatment had a small, yet reproducible, effect upon CHO-M1 and -M3 mediated Ins(1,4,5)P\textsubscript{3} generation in β-escin permeabilised cells. The EC\textsubscript{50} values for methacholine-stimulated Ins(1,4,5)P\textsubscript{3} accumulation showed a small, but consistent increase in EC\textsubscript{50} value for both the CHO-M1 and -M3 in the presence of pertussis toxin. This finding may illustrate a G\textsubscript{\textbeta\gamma} component involved in the activation of PLC after M1 and M3 receptor stimulation. Many G protein-coupled receptors stimulate the hydrolysis of phosphatidylinositol 4,5-bisphosphate by phospholipase C\textbeta through G protein subunits ultimately to release calcium from the endoplasmic reticulum (Clapham, 1995). Both G\textsubscript{\textalpha q} and G\textsubscript{11\textalpha} subunits have been shown to mediate PLC\textbeta activity (Blank et al., 1991), and it has also been illustrated in a variety of studies, that G\textbeta\gamma can stimulate PLC\textbeta activity (Blank et al., 1992; Boyer et al., 1992; Camps et al., 1992a; Camps et al., 1992b; Katz et al., 1992; Blank et al., 1993; Park et al., 1993; Herrlich et al., 1997; Jiang et al., 1997; Tomura et al., 1997). Pertussis toxin blocks some, but not the majority, of muscarinic receptor-mediated PLC\textbeta activation in permeabilised CHO-cells, hence both pertussis toxin-sensitive and -insensitive G proteins may be involved. As no pertussis toxin-sensitive G\textalpha subunits have been shown to activate PLC\textbeta to date, the PTx-sensitive activation is ultimately by G\textbeta\gamma. G\textbeta\gamma has been shown to play a dominant role over G\textalpha in the M3-mediated activation of PLC\textbeta in Xenopus oocytes (Stehno-Bittel et al., 1995). G\textalpha-GDP and a βARK fragment were utilised as G\textbeta\gamma binding agents, these served to inhibit acetylcholine-induced PLC\textbeta stimulation, whereas direct injection of G\textbeta\gamma subunits induced intracellular calcium release. Stehno-Bittel et al. (1995) concluded in their study that receptor coupling specificity of the G\textsubscript{\textalpha q}/G\textbeta\gamma heterotrimer was determined by G\textsubscript{\textalpha q}, but
activating PLC compared to the M3 mediated response. Other studies have revealed this trend, with Peralta et al. (1988) reporting a 4-16 fold lower EC50 value for carbachol acting at the M1 subtype when compared directly to the M3 subtype. For rat muscarinic receptors expressed in CHO-cells, the order of potency with respect to PI turnover was M1 > M3 (Gurwitz et al., 1994). Again the order of potency with respect to PI turnover was found to be M1 > M3 in A9L cells expressing human muscarinic receptors (Richards and van Giersbergen, 1995). The discussion of differences in the coupling of the M1 and M3 receptor subtypes is continued in Chapter Six.

Pertussis toxin pre-treatment had a small, yet reproducible, effect upon CHO-M1 and -M3 mediated Ins(1,4,5)P3 generation in β-escin permeabilised cells. The EC50 values for methacholine-stimulated Ins(1,4,5)P3 accumulation showed a small, but consistent increase in EC50 value for both the CHO-M1 and -M3 in the presence of pertussis toxin. This finding may illustrate a Gβγ component involved in the activation of PLC after M1 and M3 receptor stimulation. Many G protein-coupled receptors stimulate the hydrolysis of phosphatidylinositol 4,5-bisphosphate by phospholipase Cβ through G protein subunits ultimately to release calcium from the endoplasmic reticulum (Clapham, 1995). Both Gqα and G11α subunits have been shown to mediate PLCβ activity (Blank et al., 1991), and it has also been illustrated in a variety of studies, that Gβγ can stimulate PLCβ activity (Blank et al., 1992; Boyer et al., 1992; Camps et al., 1992a; Camps et al., 1992b; Katz et al., 1992; Blank et al., 1993; Park et al., 1993; Herrlich et al., 1997; Jiang et al., 1997; Tomura et al., 1997). Pertussis toxin blocks some, but not the majority, of muscarinic receptor-mediated PLCβ activation in permeabilised CHO-cells, hence both pertussis toxin-sensitive and -insensitive G proteins may be involved. As no pertussis toxin-sensitive Gα subunits have been shown to activate PLCβ to date, the PTx-sensitive activation is ultimately by Gβγ. Gβγ has been shown to play a dominant role over Gα in the M3-mediated activation of PLCβ in Xenopus oocytes (Stehno-Bittel et al., 1995). Gα-GDP and a βARK fragment were utilised as Gβγ binding agents, these served to inhibit acetylcholine-induced PLCβ stimulation, whereas direct injection of Gβγ subunits induced intracellular calcium release. Stehno-Bittel et al. (1995) concluded in their study that receptor coupling specificity of the Gqα/Gβγ heterotrimer was determined by Gqα, but
that Gβγ was the predominant signalling molecule activating oocyte PLCβ. Our study supports the idea that muscarinic receptors involve Gβγ in the activation of PLCβ, but the predominant activator in permeabilised CHO-cells appears to be Gq11α. PLCβ1, PLCβ2 and PLCβ3 have all been shown to translocate to the plasma membrane after stimulation of muscarinic receptors with methacholine in SH-SY5Y cells (L. Wheldon personal communication). As the different isoforms of PLCβ have differing sensitivities to both Ga and Gβγ, with activated Gq11α subunits stimulating PLCβ isoforms with the rank order of potency PLCβ1 > PLCβ3 > PLCβ2 and Gβγ sensitivity being shown to be PLCβ3 > PLCβ2 > PLCβ1 (Rhee and Bae, 1997), it would seem unnecessary to recruit isoforms other than PLC-β1 if all the activation were mediated by Gq11α. Further evidence to support a role for Gβγ after muscarinic stimulation is shown in Chapter Six, where both the M1 and M3 muscarinic receptor subtypes activate a statistically significant population of Gq-like G proteins in response to methacholine. These findings make it highly probable that Gβγ will play a role in the activation of PLCβ after muscarinic M1 or M3 receptor stimulation.

Methacholine-stimulated 45Ca2+ release in CHO-M1 and CHO-M3 cells was also maintained in permeabilised cells suspended in cytosol-like buffer. This suggests that permeabilisation of CHO-M1 and CHO-M3 cells with β-escin in a cytosol-like buffer, where Ca2+ is buffered to approx. 100 nM, did not preclude receptor-mediated Ca2+ mobilisation. It has been demonstrated previously that permeabilised SH-SY5Y cells, which express mainly M1 receptors, can stimulate Ins(1,4,5)P3 generation in response to muscarinic agents and this can subsequently release 45Ca2+ from intracellular stores (Wojcikiewicz et al., 1990a; 1990b; Safrany and Nahorski, 1994; Burford et al., 1995a). This study shows that CHO-M1 and CHO-M3 cells can be permeabilised by β-escin and the uptake of 45Ca2+ into intracellular stores stimulated in the presence of ATP. 90% of loaded 45Ca2+ could subsequently be released by ionomycin (20 μM). The remaining 10% may represent the high affinity binding of 45Ca2+ to calcium binding proteins within the cell and/or a proportion of 45Ca2+ from the buffer which has become trapped in the cell pellet during centrifugation.
Methacholine-stimulated $^{45}\text{Ca}^{2+}$ release in CHO-M$_1$ and CHO-M$_3$ cells reached a similar maximal level in both the clones. The potency of methacholine appeared almost equal for the M$_1$ and M$_3$ receptor subtypes, and the EC$_{50}$ values were in close agreement with those reported by Burford et al. (1995a). As discussed above and in Chapters Four and Six, the M$_1$ receptor subtype appears slightly more potent when compared to the M$_3$ receptor subtype, although any observation made at this level must be regarded as highly subjective due to the lack of statistical support. Pre-treatment with pertussis toxin had no significant effect upon CHO-M$_1$ and -M$_3$ mediated $^{45}\text{Ca}^{2+}$ release in β-escin permeabilised cells, although small rightward shifts were observed in both the CHO-M$_1$ and -M$_3$ cells. The insensitivity to pertussis toxin, at the level of $\text{Ca}^{2+}$ release, is surprising considering the pertussis toxin sensitivity of Ins(1,4,5)P$_3$ generation. This finding may seem to argue against the idea of a G$\beta\gamma$ component in M$_1$ and M$_3$ signal generation, but the amplification between Ins(1,4,5)P$_3$ generation and $\text{Ca}^{2+}$ release from stores may mask the effect of pertussis toxin if the G$\beta\gamma$ activation of PLC$\beta$ is secondary to that mediated by $G_{q/11}\alpha$. The lack of an observable pertussis toxin effect may suggest that any possible effects of toxin pre-treatment on muscarinic receptor agonist-stimulation would be at a level prior to the Ins(1,4,5)P$_3$ receptor. Alternatively these results may suggest that M$_1$ and M$_3$ receptor-stimulation elicits a non-Ins(1,4,5,)P$_3$ mediated $\text{Ca}^{2+}$ signal.

The apparent potency differences between methacholine-stimulated Ins(1,4,5)P$_3$ generation and $^{45}\text{Ca}^{2+}$ release may be due to an amplification step in the pathway between Ins(1,4,5)P$_3$ generation and $\text{Ca}^{2+}$ release from stores. Alternatively, the potency differences may reflect a difference in the protein concentrations used in the two different assay systems. Perhaps in order to compare these two signal transduction events, Ins(1,4,5)P$_3$ generation and $\text{Ca}^{2+}$ release, in a more comparable manner measurements of cytosolic calcium using fluorescent indicators should be performed in intact cells and compared to the Ins(1,4,5)P$_3$ generation also performed in an intact cell system. Such experiments were not performed in this study as the main aim was to evaluate the many levels of cell signalling in a single cell preparation. Another major point for discussion from this data-set is that basal levels of Ins(1,4,5)P$_3$ (calculated from Ins(1,4,5)P$_3$ mass accumulation studies) are thought
to be 1-3 µM in many intact cells (Challiss et al., 1990; Shears, 1991). Yet 3 µM Ins(1,4,5)P$_3$ causes a near-maximal release of calcium in permeabilised CHO-cells in this study. This may indicate that Ins(1,4,5)P$_3$ is compartmentalised at sites in the cell where it does not come into contact with Ins(1,4,5)P$_3$ receptors gating Ca$^{2+}$ flux from stores. Alternatively, permeabilisation of CHO-cells results in an increased sensitivity of Ins(1,4,5)P$_3$ receptors to Ins(1,4,5)P$_3$, by removing an inhibitory constraint.

Atropine inhibited agonist-stimulated $[^{35}\text{S}]$-GTP$_\gamma$S binding in β-escin permeabilised cells at all muscarinic acetylcholine receptor subtypes, when included in the reaction at a concentration of 10 µM. However, when atropine was incubated alone it did not mediate a reduction in the basal level of $[^{35}\text{S}]$-GTP$_\gamma$S binding to a population of either G$\alpha$ and G$_{q/11}$$\alpha$ or G$_{q/11}$$\alpha$ alone, at any concentration of GDP studied. These studies were performed at a range of GDP concentrations as the inhibition of $[^{35}\text{S}]$-GTP$_\gamma$S binding by atropine in cardiac membranes was found to be modulated by both GDP and NaCl (Hilf and Jakobs, 1992). This is in contrast to the results outlined in Chapter Four where it was observed, by a reduction in basal $[^{35}\text{S}]$-GTP$_\gamma$S binding, that atropine acts as an inverse agonist at the M$_2$ and M$_4$ receptor subtypes. One explanation may be that the experimental conditions utilised biased the assay towards the evaluation of agonist behaviour. This assay system may be insensitive to the small reductions in basal $[^{35}\text{S}]$-GTP$_\gamma$S binding mediated by atropine due to the high level of KCl present. The inhibition mediated by atropine in cardiac membranes was found to be modulated by both GDP and NaCl (Hilf and Jakobs, 1992), and was maximal in the absence of NaCl. The inhibition of basal $[^{35}\text{S}]$-GTP$_\gamma$S binding was reduced in a concentration-dependent manner by increasing the Na$^+$ and GDP concentrations. A reduction in agonist-independent activity mediated by an inverse agonist involves preferential binding to the inactive receptor species (R), altering the position of the equilibrium between inactive (R) and active (R*), in turn reducing the level of R*. Na$^+$ and GDP both influence the equilibrium between R and R* by increasing the level of R, so they would be expected to minimise the level of constitutive activity. Thus, a high level of KCl may make it harder to observe inverse agonist activity; thus in this assay system, with the inclusion of 120 mM KCl, it perhaps was not surprising that we are unable to observe inverse properties of atropine. An alternative
explanation is that atropine is a neutral antagonist. Yet this is unlikely as atropine has been widely reported to act as an inverse agonist (Burstein et al., 1995; 1997; Jakubik et al., 1995; Jakubik et al., 1996). This evidence would suggest that the inverse agonist properties of atropine cannot be evaluated in a permeabilised[^3S]-GTPγS binding assay system.

In conclusion the data presented in this series of experiments indicate that, with a suitable permeabilising agent, permeabilised cells may be used to analyse receptor stimulated G protein activation and subsequent effector regulation.
Chapter Six.

Differential Coupling of Recombinant $M_1$, $M_2$, $M_3$ and $M_4$ Acetylcholine Muscarinic Receptors to Subsets of Guanine Nucleotide Binding Proteins in Chinese Hamster Ovary Cells.
6.1. Introduction.

As discussed in previous Chapters agonist-stimulated $[^{35}\text{S}]-\text{GTPyS}$ binding has been used extensively to assess both the potency and efficacy of a number of compounds which act at various GPCRs (Lorenzen et al., 1993; Gardner et al., 1996; Stanton and Beer, 1996; Akam et al., 1996, 1997, 1998; Williams et al., 1997). Muscarinic receptor family G protein coupling has been assessed in this way, both in this study and by Lazareno et al. (1993a, 1993b) and Burford et al. (1995b). Though, with all studies of this nature the activation measured only reflects overall G protein activation following receptor-stimulation.

Membrane $[^{35}\text{S}]-\text{GTPyS}$ binding methodology has allowed the dissection of G protein activation down to the pertussis toxin-sensitive/-insensitive level, yet multiple subtypes of $G_\alpha$ and $G_\delta$ are pertussis-toxin sensitive and this only allows an inference to receptor coupling partners. Also, it is becoming increasingly evident that single GPCRs can productively couple to many different G protein subtypes to elicit a plethora of responses (Schmidt et al., 1996). Activation of effector molecules by $\beta\gamma$-subunits is also being observed more frequently (Birnbaumer, 1992, Clapham and Neer, 1993; Sternweis, 1994 and references therein). So it is becoming increasingly important to be able to dissect the initial G protein activation.

Specific G protein activation can be assessed using radiolabelled GTP analogue binding and antisera specific to different G$\alpha$ subunits. Immunoprecipitation of specific G protein $\alpha$ subunits in response to stimuli has allowed the assessment of the first stages in a complex signalling cascade for a variety of receptor types including the dopamine receptor family members, 5-HT$_{1A}$ receptor, NKR-P1 receptor, $\mu$-opioid receptor, LPA receptor, thrombin receptors and the substance P receptor (Friedman et al., 1993; Raymond et al., 1993; Wang et al., 1995; Al-Aoukaty et al., 1997; Burford et al., 1998; Fukushima et al., 1998; Ogino et al., 1998; Roush et al., 1998).

The even numbered muscarinic receptor subtypes mediate the inhibition of adenylyl cyclase and it has been shown that $G_i$, $G_{12}$ and $G_{13}$ can all inhibit cAMP
accumulation in mammalian cells (Wong et al., 1992). As the M₂ and M₄ receptor subtypes both couple to adenylyl cyclase they may mediate this effect by interaction with one, or more, of many G protein subtypes. So although there appeared to be no significant agonist-stimulated [³⁵S]-GTPγS binding after pertussis toxin pre-treatment mediated by the M₂ and M₄ receptor subtypes, the total [³⁵S]-GTPγS binding may still be mediated by more than one Gα subunit. In studies of M₂ muscarinic receptors, derived from heart and cerebellum, coupling to multiple G protein α subunits has been shown by purification of the muscarinic receptor-G protein complexes using an immunoprecipitation protocol followed by western blotting (Matesic et al., 1989, 1991). Also, in a comparison of the M₂ and M₄ receptor subtypes using a luciferase reporter gene under the transcriptional control of a cAMP response element, both the M₂ and M₄ receptor subtypes have been shown to couple to multiple pertussis toxin-sensitive G protein α subunits (Migeon et al., 1995).

In the case of the odd numbered family members, the M₁ and M₃ receptor subtypes have been shown to mediate productive interactions with members of the Gq/11 and Gi classes of G proteins both in this study and studies by Lazareno et al. (1993a, 1993b) and Burford et al. (1995b). In a study by Offermanns et al. (1994) subtype-specific immunoprecipitation of G protein α subunits bound to a photoaffinity GTP label was used to study muscarinic receptor activation in HEK cell membranes. In this study the M₁ and M₃ receptor subtypes were shown to interact with members of both the Gq/11 and Gi classes of G proteins. So it can be seen that individual muscarinic receptor subtypes have the potential to interact productively with multiple G proteins. In this series of experiments a direct comparison of the M₁, M₂, M₃ and M₄ muscarinic receptor subtypes at the level of specific G protein activation was performed.

The aim of this series of experiments was to further our understanding of muscarinic receptor subtype-specific G protein coupling by utilising immunoprecipitation of [³⁵S]-GTPγS-bound Gα-subunits after muscarinic receptor activation. In previous Chapters it was established that homogeneous populations of human muscarinic acetylcholine receptor subtypes were expressed in Chinese hamster ovary cells displaying similar G protein profiles. Also it was established using pertussis toxin that
the odd numbered family members mediate the total $[^{35}S]$-GTPγS binding seen after receptor-stimulation by interaction with more than one class of G protein whereas the even family members elicit the activation of only one class of G proteins.

With the receptor subtypes $M_1$, $M_2$, $M_3$ and $M_4$ expressed in the same cellular background the individual receptor-G protein coupling could be evaluated with $G\alpha$ specific antisera. This will allow any differences in receptor-G protein coupling to be attributed only to the receptor subtype expressed and not to cell type-specific variations. Also in these cells the receptor densities have been matched for the $M_2/M_4$ subtypes and $M_1/M_3$ receptor subtypes to minimise differences that may arise due to variations in receptor expression. Hopefully this will enable the dissection of individual coupling events, allowing the traditional coupling of the odd and even family members to be evaluated to see if they interact with limited or expanded G protein populations. In addition to this, it has been documented that all the subtypes under investigation show some degree of pertussis toxin-sensitive coupling and this methodology will enable the identification of the particular G protein subtypes mediating this effect. In addition to assessing qualitative differences in the receptor-G protein coupling, this methodology can also be utilised to evaluate quantitative differences such as potency and efficacy differences at the level of specific G protein activation.


For details on the methodology used throughout this Chapter see Chapter Two ‘Experimental Methodology’ with reference to the sections entitled ‘$G$ Protein Western Blotting’, ‘Immunoprecipitation of $[^{35}S]$-GTPγS-bound Specific $G\alpha$ Subunits in Response to Muscarinic Receptor Activation’, ‘Muscarinic Agonist-Stimulated Inhibition of cAMP Accumulation’ and ‘Cyclic AMP Determination’.
6.2. Data Analysis.

\[^{35}\text{S}]\text{-GTPyS}\) radioligand binding data and cAMP accumulation data were analysed using a least-sum-of-squares non-iterative curve fitting programme, computer software Prism 2 (dose-response curve (variable slope)) to determine EC\(_{50}\) and Hill coefficient (nH) values. Prism 2 was supplied by GraphPAD software Inc., San Diego, CA, U.S.A. All data are shown as means ± s.e.mean for ‘n’ separate experiments.

6.3. Results.

Identification of different Go proteins present in CHO transfects.

Initial western blot experiments were performed upon cellular membranes prepared from CHO cells transfected with the human muscarinic acetylcholine receptors m1-m4. These experiments examined the presence of different Go proteins and gave a crude quantitative indicator of protein levels. Across all the transfected cells no large differences in the levels of any specific Go protein were seen, and cells expressed all Go proteins tested for including G\(_{\gamma\delta}\) (refer to Figures 3.3.2.-3.3.6). As all the Go proteins tested for were present it meant that we could begin to test for stimulation of a wide range of G proteins after muscarinic acetylcholine receptor occupation.

Effect of incubation time and GDP concentration on methacholine-stimulated \[^{35}\text{S}]\text{-GTPyS}\) binding to G proteins in CHO-cell membranes expressing recombinant muscarinic receptors.

The muscarinic acetylcholine receptors have been widely studied and are thought to transduce their signals via the G\(_{\gamma\delta1}\) class of G proteins for the odd numbered family members (m1, m3 and m5), with the even numbered family members (m2 and m4) interacting with the G\(_{i}\)-like family of G proteins (Caulfield, 1993). Therefore initial time-courses carried out for the M\(_2\) and M\(_4\) receptor subtypes were against G\(_{\gamma\delta1}\) (antiserum that cross reacts with G\(_{\gamma\delta1}\), G\(_{\beta\gamma}\) and G\(_{\gamma\delta}\)), and for the M\(_1\) and M\(_3\) receptor subtypes antiserum against G\(_{\gamma\delta1}\) was utilised. Studies in both tissues (Hilf et al.,
1989) and cell lines (Lazareno et al., 1993a) have shown that optimal responses for receptor-mediated $[^{35}S]$-GTPγS binding are strongly dependent upon GDP concentration. Offermanns et al. (1994) reported a difference between the muscarinic acetylcholine receptors in their absolute requirement for GDP when measuring the activation of G proteins with a photoaffinity probe after carbachol stimulation. Therefore, these initial experiments were carried out in the presence of 100 mM NaCl and in the absence or presence of GDP at either 1 or 10 μM.

Methacholine-stimulated $[^{35}S]$-GTPγS binding to $G_{11,3}\alpha$ was observed in the $M_2$ (Figure 6.3.1) and $M_4$ muscarinic acetylcholine receptor (Figure 6.3.2) expressing CHO-cells under all conditions, even in the absence of GDP. The activation of $G_{11,3}\alpha$ is a rapid event with both the $M_2$ and $M_4$ muscarinic receptor subtypes activating a statistically significant population of $G_{11,3}\alpha$ at the earliest time-point studied (1 min) in the absence and presence of GDP (1 and 10 μM). The kinetics of activation of this family of G proteins by the $M_2$ and $M_4$ muscarinic receptor subtypes appear to be similar in the absence or presence of GDP (1 or 10 μM) (All data are shown in Tables 6.3.2 and 6.3.4 (n≥4)). 10 μM GDP was utilised in further experiments as this allowed the results obtained to be directly compared to the membrane-filtration based data.

At 1, 2 and 5 min (10 μM GDP) the $M_2$ receptor caused increases of 6089 ± 815, 9510 ± 1142 and 6129 ± 1697 cpm above basal respectively. For the $M_4$ receptor subtype in the presence of 10 μM GDP similar stimulations were observed at these time-points (3924 ± 261, 5408 ± 873 and 5238 ± 419 cpm above basal). The maximal stimulation occurred in the $M_2$ and $M_4$ expressing cells at 2 min, with the $[^{35}S]$-GTPγS bound over basal reducing at 5 min in the $M_2$ expressing cells. All stimulations were statistically significant when assessed by Student’s t-test for paired observations P<0.05. No response was observed in untransfected CHO-cells with the $G_{11,3}\alpha$ antiserum (data not shown).

Methacholine-stimulated $[^{35}S]$-GTPγS binding to $G_{q/11}\alpha$ was seen in both $M_1$ and $M_3$ expressing cells in the presence and absence of GDP with the highest level of stimulation being observed in the absence of GDP (Figures 6.3.3 and 6.3.4). The
methacholine-stimulated \[^{35}\text{S}\]-GTP\gamma S binding to G\(_{q/1}\alpha\) via both the M\(_1\) and M\(_3\) receptor subtypes was statistically significant at all the time-points studied in the absence and presence of GDP (1 and 10 \(\mu\)M), for details see Tables 6.3.1 and 6.3.3. 1 \(\mu\)M GDP was utilised in further experiments as this allowed any variations in the levels of endogenous guanine nucleotides to be equalised. The activation of the M\(_1\) subtype when directly compared to the M\(_3\) muscarinic acetylcholine receptor subtype in the presence of 1 \(\mu\)M GDP, shows a somewhat different activation profile. At 2 min (GDP, 1 \(\mu\)M) the M\(_1\) muscarinic receptor activation caused a much greater increase in G\(_{q/1}\alpha\)-\[^{35}\text{S}\]-GTP\gamma S binding compared to M\(_3\) muscarinic receptor (M\(_1\), 37359 \(\pm\) 3208; M\(_3\), 12527 \(\pm\) 2318 cpm above basal respectively). The activation of G\(_{q/1}\alpha\) also appeared to occur more rapidly when the M\(_1\) muscarinic receptor subtype was activated with a maximal concentration of methacholine (1 mM), with the M\(_3\) muscarinic receptor activation of G\(_{q/1}\alpha\) being slower (response at 1 min, GDP 1 \(\mu\)M: M\(_1\), 36383 \(\pm\) 1625; M\(_3\), 6746 \(\pm\) 883 cpm above basal (n=3)). No response was observed in untransfected CHO-cells using the G\(_{q/1}\alpha\) antiserum (data not shown).

Optimal assay conditions for the four subtypes were defined as 2 min incubations at 30°C in the presence of 1 \(\mu\)M GDP for the M\(_1\) and M\(_3\) receptor subtypes, or 10 \(\mu\)M GDP for the M\(_2\) and M\(_4\) receptor subtypes. All stimulations were statistically significant when compared to basal values and assessed by Student’s t test for paired observations (P < 0.05).

**Effect of atropine on specific G\(\alpha\) \[^{35}\text{S}\]-GTP\gamma S binding in CHO-cell membranes expressing recombinant muscarinic receptors.**

Atropine blockade of agonist-stimulated G\(\alpha\) \[^{35}\text{S}\]-GTP\gamma S binding (Figures 6.3.5 and 6.3.6) was obtained for all muscarinic acetylcholine receptor subtypes when atropine was included at 10 \(\mu\)M. Atropine at this concentration reduced the methacholine-stimulated \[^{35}\text{S}\]-GTP\gamma S binding to basal levels. Figure 6.3.5 shows atropine-mediated inhibition of G\(_{11,3}\alpha\) \[^{35}\text{S}\]-GTP\gamma S binding after M\(_2\) and M\(_4\) receptor stimulation. Only the methacholine-stimulated values attained statistical significance when compared to basal levels (Student’s t-test; P < 0.05).
Atropine inhibition of methacholine-stimulated $G_{q11}\alpha^{[35S]}$-GTPγS binding was also demonstrated in the cell membranes expressing the $M_1$ and $M_3$ receptor subtypes (Figure 6.3.6). Basal levels of 2980 ± 470 and 1194 ± 125 cpm were seen for $G_{q11}\alpha^{[35S]}$-GTPγS binding mediated by the $M_1$ and $M_3$ subtypes (n=3), with methacholine-stimulated values of 37406 ± 2207 and 13725 ± 1645 respectively (n=3). Coincubation with atropine (10 μM) completely prevented the methacholine-stimulated increase. Thus, only the methacholine-stimulated values attained statistical significance when compared to basal levels (Student’s t-test; P < 0.05).

**Full and partial agonist effects on G protein activation in CHO-cell membranes expressing recombinant muscarinic acetylcholine receptors.**

The next set of experiments was carried out to allow assessment of the ability of individual muscarinic receptor subtypes to interact with a limited or expanded $G\alpha$ population. The muscarinic acetylcholine receptor ‘full’ agonist methacholine was compared to the ‘partial’ agonist pilocarpine in their abilities to activate the $G$ proteins identified as being present in these cells after activation of specific muscarinic receptor subtypes. As documented in previous Chapters methacholine displays ‘full’ agonist activity at all the muscarinic receptor subtypes investigated and pilocarpine displays ‘partial’ agonist activity at all the muscarinic receptor subtypes. This allowed the assessment of both a full and partial agonist at the level of specific $G\alpha$ activation. So in addition to testing the ability of pilocarpine to activate $G_{q11}\alpha$ after $M_1$ and $M_3$ stimulation and $G_{i1,3}\alpha$ after $M_2$ and $M_4$ activation, we tested the abilities of both methacholine and pilocarpine to activate a range of $G\alpha$ subunits by using antisera directed against $G_{q11}\alpha$, $G_{i1,3}\alpha$, $G_{i1/2}\alpha$, $G_{i3/6}\alpha$, $G_{i}\alpha$ and $G_{o}\alpha$.

Methacholine (1 mM) elicits the activation of a significant population of $G\alpha$ proteins, using the general antiserum against $G_{i1,3}\alpha$, after $M_2$ and $M_4$ receptor stimulation (8068 ± 1271 and 9265 ± 890 cpm above basal (n=4) Figure 6.3.7). $G_{i}\alpha$ activation was further dissected with more specific antisera to $G_{i1/2}\alpha$, and $G_{i3/6}\alpha$ and activation of these two groups of $G\alpha$ proteins could also be seen upon stimulation of the adenylyl cyclase inhibiting muscarinic receptor subtypes with maximal methacholine (1 mM).
The M₂ and M₄ receptor subtypes produced significant activation within both populations ([³⁵S]-GTPγS bound above basal M₂; 4017 ± 384, 5158 ± 366 and M₄; 2269 ± 275, 6641 ± 678 cpm for G₁₁α and G₁₃α₀α respectively (n=4) Figure 6.3.7).

Maximal methacholine (1 mM) appears to have no statistically significant effect via the M₂ and M₄ receptor subtypes upon G₉¹α, G₆α or G₄α after 2 min incubation in the presence of 10 µM GDP (n=4). From these observations we can postulate that the activations seen when using antisera directed against G₁₃α₀α is predominantly from the activation of G₁₃α.

Maximal pilocarpine (1 mM) however showed an impaired ability to activate the members of the Gα family after stimulation by M₂ and M₄ receptor subtypes, with the activation of G₁₁α decreasing to 5177 ± 579 and 2474 ± 1095 cpm above basal respectively (Figure 6.3.8). These values correspond to 64 % and 27 % of the [³⁵S]-GTPγS binding produced by 1 mM methacholine after stimulation of M₂ and M₄ receptor subtypes. The larger reduction seen with pilocarpine for the M₄ receptor subtype was verified by a complete loss of stimulation of G₁₁α with the signal from pilocarpine apparently being transduced by the G₁₃α₀α isoforms solely (1688 ± 220 cpm above basal (n=4)). The remaining stimulation of the G₁₃α₀α isoform represents approximately 25 % of the activation elicited by the ‘full’ agonist methacholine. However, the activation of both G₁₁α and G₁₃α₀α can still be observed after M₂ muscarinic receptor stimulation amounting to values corresponding to 23 % and 45 % of the [³⁵S]-GTPγS binding produced in response to 1 mM methacholine (Figure 6.3.8).

The activation profile for G proteins coupled to M₁ and M₃ muscarinic receptor subtypes after maximal methacholine (1 mM) stimulation also showed interesting differences between the subtypes. Under optimal conditions the M₁ receptor subtype was able to activate all of the Gα proteins assessed, whereas the M₃ receptor subtype showed a more limited activation repertoire (Figure 6.3.9). Thus, the M₃ muscarinic receptor subtype, despite ultimately causing the stimulation of the same effector system, only causes the populations of G₉¹α, G₁₁α and G₁₃α₀α to increase binding of
[\textsuperscript{35}S]-GTP\gamma S significantly. From these data it can be seen that the M\textsubscript{1} receptor subtype is more efficient at activating the G protein population especially the G\textsubscript{q11}\alpha isoform (Figure 6.3.9).

When the partial agonist pilocarpine was used the M\textsubscript{1} muscarinic receptor subtype was only able significantly to activate G\textsubscript{q11}\alpha, G\textsubscript{i1,3}\alpha and G\textsubscript{i3,6}\alpha [\textsuperscript{35}S]-GTP\gamma S binding, producing stimulations of 5202 ± 81, 565 ± 90 and 290 ± 29 cpm above basal respectively (n=3) (Figure 6.3.10). Pilocarpine had a reduced ability, relative to methacholine, to activate the M\textsubscript{1} linked G proteins, with a 75 % reduction in the activation of G\textsubscript{q11}\alpha, a 83 % reduction in the activation of G\textsubscript{i1,3}\alpha and an 87 % reduction in the activation of G\textsubscript{i3,6}\alpha. So, it can be seen that the M\textsubscript{1} activation profiles for methacholine and pilocarpine are markedly different. The ability of pilocarpine to activate G\textsubscript{q11}\alpha, G\textsubscript{i1,3}\alpha and G\textsubscript{i3,6}\alpha after M\textsubscript{1} activation was dramatically different to that observed with the M\textsubscript{1} subtype. The activation of G\textsubscript{q11}\alpha (493 ± 135 cpm above basal (n=3)) was just statistically significant (p=0.047), with this number representing just 23 % of the activation seen with the ‘full’ agonist methacholine. In contrast, the activation of G\textsubscript{i1,3}\alpha and G\textsubscript{i3,6}\alpha was still large (2692 ± 766 and 1654 ± 336 cpm above basal value respectively). The level of [\textsuperscript{35}S]-GTP\gamma S binding to G\textsubscript{i1,3}\alpha caused by pilocarpine-stimulation of the M\textsubscript{1} receptor subtype remained at a level comparable to that elicited by methacholine. So it appears that pilocarpine and methacholine activate different populations of G\textsubscript{i}-like G proteins after M\textsubscript{1} muscarinic receptor stimulation and both the ‘full’ and ‘partial’ agonists can robustly stimulate G\textsubscript{i}-like G proteins.

**Pharmacological characterisation of agonist-stimulated G\alpha specific [\textsuperscript{35}S]-GTP\gamma S binding in CHO-cell membranes expressing recombinant muscarinic acetylcholine receptors.**

As was seen in the two previous Chapters, methacholine activated total G\alpha populations in a concentration-dependent manner. Here, the immunoprecipitation methodology allows assessment of the concentration dependence of [\textsuperscript{35}S]-GTP\gamma S binding to individual G\alpha subunits.
The ability of the M2 and M4 receptor subtypes to activate the Gi family can be seen to be concentration-dependent. Figure 6.3.11 shows concentration-response curves for the activation of G\textsubscript{i3/0} by the M2 and M4 muscarinic receptor subtypes. Methacholine displays varying potency at the M2 and M4 receptor subtypes when assessed at the level of \[^{35}\text{S}]-\text{GTP\gammaS} binding to G\textsubscript{i3/0}. The 'full' agonist appears to be 12-fold more potent in causing activation of G\textsubscript{i3/0} through the M2 muscarinic receptor compared to the M4 muscarinic receptor, with the M2 and M4 receptor subtypes displaying EC\textsubscript{50} values of 1.8 \textmu M and 21 \textmu M (log EC\textsubscript{50} (M): -5.79 ± 0.15 and -4.71 ± 0.11 (n=3) respectively).

After the initial assessment of G\textsubscript{q1/1} protein activation by the PLC\beta activating muscarinic acetylcholine receptor subtypes it appears that the M1 muscarinic receptor subtype is both more rapid and much larger in its activation of G\textsubscript{q1/1} compared to the M3 muscarinic receptor subtype. The effect of methacholine on the activation of G\textsubscript{q1/1} through these two subtypes is clearly concentration dependent (Figure 6.3.12), and again clear differences between the M1 and M3 muscarinic receptor subtypes were shown to exist. The M1 receptor subtype mediated a 4-5 fold greater increase in \[^{35}\text{S}]-\text{GTP\gammaS} binding than the M3 receptor. Also, methacholine displayed a 5 fold higher potency acting at the M1 muscarinic receptor subtype when compared directly through these experiments with the M3 subtype. Thus, EC\textsubscript{50} values of 4.5 \textmu M and 23.8 \textmu M (log EC\textsubscript{50} (M) -5.37 ± 0.13 and -4.63 ± 0.05) were obtained respectively for M1- and M3-stimulation respectively (n=3).  

**Pharmacological characterisation of agonist-stimulated inhibition of cAMP accumulation in CHO-SLM\textsubscript{2} and -M4 cells expressing recombinant muscarinic acetylcholine receptors.**

To assess further the differential activation of the G\textsubscript{i}-like G proteins in response to pilocarpine, both methacholine and pilocarpine were evaluated at the level of cAMP accumulation in M2- and M4-expressing cells. Concentration-response relationships were performed employing 10 min incubations at 37°C of intact CHO-SLM\textsubscript{2} and CHO-M4 cells in suspension. Both methacholine and pilocarpine caused inhibition of
forskolin-stimulated cAMP (Figures 6.3.13 and 6.3.14). Methacholine produced a log
M IC\textsubscript{50} value of -6.87 ± 0.05 (136 nM) in the CHO-SLM\textsubscript{2} cells, no number could be
evaluated for the CHO-M\textsubscript{4} cells as this response appeared to be biphasic, with
increasing cAMP accumulation seen at methacholine concentration greater than 1 μM.
Pilocarpine produced log M IC\textsubscript{50} values of -5.52 ± 0.04 (3.0 μM) and -5.57 ± 0.05 (2.7
μM) for the M\textsubscript{2} and M\textsubscript{4} receptor subtypes respectively. Thus, when pilocarpine was
assessed at this level, no significant differences were seen between the M\textsubscript{2} and M\textsubscript{4}
muscarinic receptor subtypes.
Effect of GDP concentration on methacholine-stimulated $G_{i,3,\alpha}$ specific $[^{35}S]$-GTP$\gamma$S binding in CHO-SLM$_2$ cell membranes.

CHO-SLM$_2$ (75 $\mu$g) cell membranes were incubated in the absence or presence of GDP (1 or 10 $\mu$M) for the times indicated. Data are expressed as cpm bound above basal for all time-points and are shown as means ± s.e.mean for 4 separate experiments performed in duplicate.
Effect of GDP concentration on methacholine-stimulated $G_{i,3}\alpha$ specific $[^{35}S] \text{-GTP}\gamma\text{S}$ binding in CHO-M$_4$ cell membranes.

Time-course of M$_4$ receptor-mediated $[^{35}S] \text{-GTP} \gamma \text{S}$ binding to $G_{i,3}\alpha$ in the presence of varying concentrations of GDP. CHO-M$_4$ (75 µg) cell membranes were incubated in the absence or presence of GDP (1 or 10 µM) for the times indicated. Data are expressed as cpm bound above basal for all time-points and are shown as means ± s.e.mean for 5 separate experiments carried out in duplicate.
Figure 6.3.3.

Effect of GDP concentration on methacholine-stimulated $G_{q11} \alpha$ specific [$^{35}$S]-GTP$\gamma$S binding in CHO-M$_1$ cell membranes.

Time-course of $M_1$ receptor-mediated [$^{35}$S]-GTP$\gamma$S binding to $G_{q11} \alpha$ in the presence of 0, 1 or 10 $\mu$M GDP. CHO-M$_1$ (75 $\mu$g) cell membranes were incubated in the presence of 1 nM [$^{35}$S]-GTP$\gamma$S for the times indicated. Data are expressed as cpm bound above basal for all time-points and are shown as means ± s.e.mean for 4 separate experiments carried out in duplicate.
Figure 6.3.4.

Effect of GDP concentration on methacholine-stimulated G$_{q/13}$ specific [$^{35}$S]-GTP$\gamma$S binding in CHO-M$_3$ cell membranes.

CHO-M$_3$ (75 µg) cell membranes were incubated in the absence or presence of GDP (1 or 10 µM) for the times indicated. Data are expressed as cpm bound above basal for all time-points observed and are shown as means ± s.e.mean for 4 separate experiments performed in duplicate.
Figure 6.3.5
Effect of atropine on methacholine-stimulated G_{i,3}α specific $[^{35}\text{S}]-\text{GTP}_y\text{S}$ binding in CHO-cell membranes.

![Graph](image1)

Figure 6.3.6
Effect of atropine on methacholine-stimulated G_{q11}α specific $[^{35}\text{S}]-\text{GTP}_y\text{S}$ binding in CHO-cell membranes.

![Graph](image2)

CHO-cell membranes (75 μg) were incubated in assay buffer with the inclusion of 1 mM methacholine (MCh), 10μM atropine (Atr) or both 1 mM MCh and 10 μM Atr (n=3). The binding defined with the specific antisera was abolished with the inclusion of atropine illustrating that the methacholine-stimulated $[^{35}\text{S}]-\text{GTP}_y\text{S}$ binding is receptor-dependent.
Figure 6.3.7.

Effect of methacholine on Gα specific [35S]-GTPγS binding in CHO-SLM₂ and -M₄ cell membranes.

CHO-cell membranes (75 μg) were incubated in the presence of 1 nM [35S]-GTPγS and 10 μM GDP in the presence and absence of 1 mM methacholine. Immunoprecipitates from various specific G protein antisera (x axis) were counted for radioactivity. Data are expressed as c.p.m bound above basal and are shown as means ± s.e.mean for 4 separate experiments. * Statistical significance defined as P < 0.05, for increases over basal [35S]-GTPγS binding, paired Student's t-test.
Figure 6.3.8.

Effect of pilocarpine on Gα specific [35S]-GTPγS binding in CHO-SLM2 and -M4 cell membranes.

Pilocarpine-stimulated [35S]-GTPγS binding (in cpm) following immunoprecipitation with a specific G protein antiserum (x axis). Data are represented as means ± s.e.mean for 4 separate experiments. * Significantly greater (P < 0.05) when compared to basal binding, paired Student's t-test.
Effect of methacholine on G\(\alpha\) specific \(^{35}\)S-GTP\(\gamma\)S binding in CHO-M\(_1\) and -M\(_3\) cell membranes.

Methacholine-stimulated \(^{35}\)S-GTP\(\gamma\)S binding (in cpm) following immunoprecipitation with G protein antisera (x axis). Immunoprecipitates were counted for radioactivity, here data are expressed as means ± s.e.means for 3 separate experiments. * Significantly greater (\(P < 0.05\)) when compared to basal binding (paired Student’s t-test).
Figure 6.3.10.

Effect of pilocarpine on Gα specific [35S]-GTPγS binding in CHO-M₁ and -M₃ cell membranes.

CHO-cell membranes (75 μg) were incubated in the presence of 1 nM [35S]-GTPγS and 1 μM GDP in the presence and absence of 1 mM pilocarpine and then immunoprecipitation was carried out using various specific G protein antisera (x axis). Data are expressed as cpm bound above basal for the antisera utilised, and are shown as means ± s.e.mean for 3 separate experiments. * Significantly greater (P < 0.05) when compared to basal binding, paired Student’s t-test.
Concentration-dependent effects of methacholine on G\textsubscript{13\alpha} specific \[^{35}\text{S}]-\text{GTPyS} binding in CHO-SLM\textsubscript{2} and -M\textsubscript{4} cell membranes.

Membranes (75 µg) were incubated for 2 min in the presence of 1 nM \[^{35}\text{S}]-\text{GTPyS} and 10 µM GDP and then immunoprecipitation was carried out using a G\textsubscript{13\alpha}-specific antiserum. Data are expressed as cpm specifically bound for all concentrations used and are shown as means ± s.e.mean for 4 separate experiments.
Concentration-dependent effects of methacholine on $G_{q11}\alpha$ specific $[^{35}S]$-GTP$\gamma$S binding in CHO-M$_1$ and -M$_3$ cell membranes.

Effect of methacholine at increasing concentrations on binding of $[^{35}S]$-GTP$\gamma$S to $G_{q11}\alpha$ subunits in CHO-cell membranes. Incubations were carried out as detailed in Methods section X. Immunoprecipitation was carried out using a $G_{q11}\alpha$ specific antiserum, and the immunoprecipitates were counted for radioactivity. Data are expressed as cpm bound above basal for all time-points and are shown as means ± s.e.mean for 3 separate experiments.
Figure 6.3.13.

Concentration-dependent effects of methacholine on forskolin-stimulated cAMP generation in CHO-SLM₂ and -M₄ cells.

CHO-SLM₂ and -M₄ cells were incubated in the presence of 10 μM forskolin and increasing concentrations of methacholine. [cAMP] was determined after 10 min incubations at 37°C. Data are shown as means ± s.e.mean for 4 experiments performed in duplicate.
Concentration-dependent effects of pilocarpine on forskolin-stimulated cAMP generation in CHO-SLM₂ and -M₄ cells.

CHO-SLM₂ and -M₄ cells were incubated in the presence of 10 μM forskolin and increasing concentrations of pilocarpine. [cAMP] was determined after 10 min incubations at 37°C. Data are shown as means ± s.e.mean for 4 experiments performed in duplicate.
Effects of guanosine-5'-diphosphate (GDP) and time on methacholine-stimulated \(^{35}\text{S}\)-GTP\(\gamma\)S binding to membrane preparations of CHO-M\(_1\) cells. Membranes were incubated with \(^{35}\text{S}\)-GTP\(\gamma\)S in the presence and absence of 1 mM methacholine and the indicated concentrations of GDP in assay buffer for the time indicated. The data are shown as the means ± s.e.mean for 4 separate experiments performed in duplicate. All cpm above basal increases were significantly greater P < 0.05 (paired Student's t-test).

Table 6.3.1. Time-course immunoprecipitation data for CHO-M\(_1\) cells.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Basal (cpm)</th>
<th>Methacholine (cpm)</th>
<th>Increases above basal (cpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 GDP</td>
<td>1 GDP</td>
<td>10 GDP</td>
</tr>
<tr>
<td>0.25</td>
<td>2255 ± 601</td>
<td>914 ± 299</td>
<td>97 ± 75</td>
</tr>
<tr>
<td>1</td>
<td>5142 ± 1406</td>
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<td>5447 ± 493</td>
<td>3153 ± 608</td>
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</tr>
<tr>
<td>5</td>
<td>7241 ± 300</td>
<td>3677 ± 306</td>
<td>1569 ± 153</td>
</tr>
</tbody>
</table>

Effects of guanosine-5'-diphosphate (GDP) and time on methacholine-stimulated \(^{35}\text{S}\)-GTP\(\gamma\)S binding to membrane preparations of CHO-M\(_1\) cells. Membranes were incubated with \(^{35}\text{S}\)-GTP\(\gamma\)S in the presence and absence of 1 mM methacholine and the indicated concentrations of GDP in assay buffer for the time indicated. The data are shown as the means ± s.e.mean for 4 separate experiments performed in duplicate. All cpm above basal increases were significantly greater P < 0.05 (paired Student's t-test).
Table 6.3.2. Time-course immunoprecipitation data for CHO-SLM\textsubscript{2} cells.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Basal (cpm)</th>
<th>MCh (cpm)</th>
<th>Increases above basal (cpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 GDP</td>
<td>1 GDP</td>
<td>10 GDP</td>
</tr>
<tr>
<td>0.25</td>
<td>571 ± 221</td>
<td>776 ± 51</td>
<td>7.6 ± 6.6</td>
</tr>
<tr>
<td>1</td>
<td>9387 ± 1124</td>
<td>8091 ± 256</td>
<td>2722 ± 182</td>
</tr>
<tr>
<td>2</td>
<td>16651 ± 2709</td>
<td>21301 ± 1363</td>
<td>8661 ± 1208</td>
</tr>
<tr>
<td>5</td>
<td>28826 ± 233</td>
<td>36547 ± 1421</td>
<td>19429 ± 1251</td>
</tr>
</tbody>
</table>

Effects of guanosine-5'-diphosphate (GDP) and time on methacholine-stimulated \[^{35}\text{S}]\text{-GTP\gamma S} binding to membrane preparations of CHO-SLM\textsubscript{2} cells, following immunoprecipitation with a G\textsubscript{n,3}\alpha specific antiserum. Membranes were incubated with \[^{35}\text{S}]\text{-GTP\gamma S} in the presence and absence of 1 mM methacholine and the indicated concentrations of GDP in assay buffer for varying durations. The data are shown as the means ± s.e.mean for 4 separate experiments performed in duplicate. All increases above basal were statistically significant (P < 0.05, paired Student's t-test).
Table 6.3.3. Time-course immunoprecipitation data for CHO-M₃ cells.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Basal (cpm)</th>
<th>MCh (cpm)</th>
<th>Increases above basal (cpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 GDP</td>
<td>1 GDP</td>
<td>10 GDP</td>
</tr>
<tr>
<td>0.25</td>
<td>308 ± 62</td>
<td>360 ± 68</td>
<td>252 ± 78</td>
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<tr>
<td></td>
<td>1490 ± 26</td>
<td>829 ± 82</td>
<td>1315 ± 357</td>
</tr>
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<td>1198 ± 66</td>
<td>1188 ± 183</td>
</tr>
<tr>
<td>2</td>
<td>3319 ± 254</td>
<td>2107 ± 281</td>
<td>1301 ± 85</td>
</tr>
</tbody>
</table>

Effects of guanosine-5’-diphosphate (GDP) and time on methacholine-stimulated [³⁵S]-GTPγS binding to membrane preparations of CHO-M₃ cells following immunoprecipitation with a Gᵣ₈₁α specific antiserum. Membranes were incubated with 1 nM [³⁵S]-GTPγS in the presence and absence of 1 mM methacholine and varying concentrations of GDP in assay buffer for the time indicated. The data are shown as the means ± s.e.mean for 4 separate experiments performed in duplicate. All increases achieved statistical significance (P < 0.05) when analysed using paired Student’s t-test.
Table 6.3.4. Time-course immunoprecipitation data for CHO-M4 cells.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Basal (cpm)</th>
<th>Methacholine (cpm)</th>
<th>Increases above basal (cpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 GDP</td>
<td>1 GDP</td>
<td>10 GDP</td>
</tr>
<tr>
<td>0.25</td>
<td>1126 ± 245</td>
<td>830 ± 130</td>
<td>266 ± 119</td>
</tr>
<tr>
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<td>8339 ± 932</td>
<td>8605 ± 951</td>
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<td>16660 ± 1286</td>
<td>14680 ± 899</td>
<td>7132 ± 557</td>
</tr>
<tr>
<td>5</td>
<td>26344 ± 755</td>
<td>26309 ± 1019</td>
<td>14375 ± 1192</td>
</tr>
</tbody>
</table>

Effects of guanosine-5'-diphosphate (GDP) and time on methacholine stimulated [35S]-GTPyS binding to membrane preparations of CHO-M4 cells. Membranes were incubated with [35S]-GTPyS in the presence and absence of 1 mM methacholine and the indicated concentrations of GDP in assay buffer for the time shown followed by immunoprecipitation with a Gi3,α specific antiserum. The data is shown as the means ± s.e.mean for 5 separate experiments performed in duplicate. All cpm above basal increases were significantly greater P < 0.05 (paired Student’s t-test).
6.4. Discussion.

In this study agonist-stimulated [\(^{35}\)S]-GTP\(\gamma\)S binding to G\(\alpha\) proteins and subsequent immunoprecipitation by G\(\alpha\) antisera was used as a measure of receptor-mediated activation of specific heterotrimeric G proteins by individual muscarinic acetylcholine receptor subtypes. In the CHO-cells used the densities of muscarinic receptor subtypes present were equalised as far as possible between the M\(_2/M_4\) subtypes and M\(_1/M_3\) receptor subtypes to minimise differences in G protein coupling that may arise due to variations in receptor expression. This allowed the specific G\(\alpha\) protein activation profiles of each receptor subtype, in response to 'full' and 'partial' agonists, to be documented and any differences between the AC-coupled or the PLC-coupled subtypes to be assessed.

Agonist-stimulated [\(^{35}\)S]-GTP\(\gamma\)S binding was observed in all the transfected CHO-cell clones (CHO-M\(_1\), CHO-SLM\(_2\), CHO-M\(_3\) and CHO-M\(_4\)), whereas in untransfected CHO-K\(_1\) cells, no methacholine-stimulated [\(^{35}\)S]-GTP\(\gamma\)S binding was seen with antisera directed against G\(_{11,3}\)\(\alpha\) or G\(_{q,11}\)\(\alpha\). This illustrates that the responses observed in the transfected cells are muscarinic receptor-mediated and that the CHO-K\(_1\) (parental) cell line does not possess endogenous muscarinic receptors. A complete blockade of agonist-stimulated G\(\alpha\)-[\(^{35}\)S]-GTP\(\gamma\)S binding was obtained for all muscarinic acetylcholine receptor subtypes when atropine (10 \(\mu\)M) was included, further illustrating that the observed responses were muscarinic receptor-mediated.

Methacholine-stimulated [\(^{35}\)S]-GTP\(\gamma\)S binding was observed in CHO-M\(_1\), CHO-SLM\(_2\), CHO-M\(_3\) and CHO-M\(_4\) cell membranes in the absence and presence of GDP. The activation observed in the absence of GDP is in direct contrast to the findings obtained from the membrane-filtration based assays. It has been documented in filtration-based studies that the differential effects of GDP on stimulation of [\(^{35}\)S]-GTP\(\gamma\)S binding is most likely due to the involvement of different classes of G proteins. Traynor and Nahorski (1995) proposed that [\(^{35}\)S]-GTP\(\gamma\)S binding mediated by receptors negatively-coupled to adenylyl cyclase is GDP-dependent. In other studies it can be seen that agonist-dependent binding of [\(^{35}\)S]-GTP\(\gamma\)S and other
hydrolysis-resistant GTP analogues to G-like G proteins can be more readily measured in the presence of GDP (Florio and Sternweis, 1989; Wieland et al., 1992). Alternatively G proteins of the G₄, G₅, and G₁₂ classes can be effectively activated in the absence of GDP (Offermanns et al., 1994a, 1994b). However, in immunoprecipitation-based [³⁵S]-GTPγS binding studies there does not seem to be an absolute requirement for the inclusion of GDP, even for receptors which couple preferentially to the inhibition of adenylyl cyclase. In a study of the activation of heterotrimeric G proteins by NKR-P1 receptors in natural killer cells, no GDP was included and functional coupling of G₁₃α, G₄α, G₈/₁₁α and G₄α was observed (Al-Aoukaty et al., 1997). Also, in studies in striatal membranes stimulated with dopamine, increases in [³⁵S]-GTPγS binding to G₁α, Gᵢα and G₄α were observed even in the absence of GDP (Wang et al., 1995). So it can be seen that even in the absence of GDP [³⁵S]-GTPγS binding to specific Gα subunits from three G protein classes can be observed. Muscarinic receptor-mediated [³⁵S]-GTPγS binding to specific Gα subunits has also been observed in the absence of GDP. Murthy and Makhlouf (1997) reported acetylcholine-stimulated binding of [³⁵S]-GTPγS to G₈/₁₁α and G₁₃α in smooth muscle membranes, mediated by the M₂ and M₃ muscarinic receptor subtypes in the absence of GDP. Also Friedman et al, (1996) reported carbachol-mediated [³⁵S]-GTPγS binding to Gᵢα and G₆α in the absence of GDP after muscarinic receptor activation in rabbit cortical membranes.

Time-courses were performed using the G₁₃,α antiserum after methacholine-stimulation of the M₂ and M₄ receptors. These two receptor subtypes displayed equivalent time-profiles for G-like G protein activation, suggesting equivalence at the kinetic level of G protein coupling. The finding that the M₂ and M₄ receptor subtypes display similar time profiles is in agreement with the time-course data from the membrane-filtration assays (see Figure 4.3.1). Rapid and equal activation of G-like G proteins after the stimulation of M₂ and M₄ muscarinic receptors has been documented in a number of studies using a luciferase reporter gene under the transcriptional control of a cAMP response element after receptor stimulation in mammalian cells (Migeon and Nathanson, 1994; Migeon et al., 1995). Also in insect Sf9 cells a rapid
activation of G-like G proteins has been documented after M_2 muscarinic receptor stimulation (Parker et al., 1991).

Time-courses, using the G_q/1α antiserum, were performed on CHO-M_1 and -M_3 cell membranes after methacholine-stimulation. The [³⁵S]-GTPγS binding to G_q/1α mediated by the M_1 receptor subtype appears to occur more rapidly when compared directly to the M_3 receptor-mediated G_q/1α- [³⁵S]-GTPγS binding. Also in this set of experiments it has been observed that [³⁵S]-GTPγS binding to G_q/1α through the M_1 receptor subtype is larger when compared to the M_3 receptor subtype. The finding that the M_1 and M_3 receptor subtypes display different speeds and magnitudes of [³⁵S]-GTPγS binding, reflecting G protein activation, is also in agreement with the time-course data from membrane-filtration assays (see Figure 4.3.1 and Figure 4.3.10). Also the larger activation of G_q/1α by the M_1 receptor subtype compared to the M_3 receptor appear to be reflected in the larger accumulation of Ins(1,4,5)P_3 after methacholine stimulation of this subtype (Figures 5.3.8, 5.3.9, and 5.3.10). However, functional differences of the M_1 and M_3 receptor subtypes have not been widely reported, with most studies concentrating on functional correlations. The observation that the M_1 receptor subtype displays a more rapid and larger activation of G_q/1α is supported by a study of immediate-early gene activation by the M_1 and M_3 receptor subtypes. In this study the formation of inositol polyphosphates in NG108-15 cells expressing the M_1 and M_3 receptor subtypes at equivalent receptor levels was directly compared (Tohda et al., 1994). The M_1 receptor subtype produced Ins(1,4,5)P_3 more rapidly and to a much greater extent than the M_3 subtype, indicating a larger and more rapid activation of PLC by G_q/1α. Also in this study the M_1 subtype was seen to induce c-fos earlier, and to a greater extent, than the M_3 subtype, yet this may reflect different modes of induction rather than a difference at the level of G_q/1α activation (Tohda et al., 1994).

The M_2 and M_4 receptor subtypes activated G_{iβγ3}α, G_{iβγ6}α and G_{iβγ6}α in response to methacholine, but showed no statistically significant activation of G_q/1α, G_α or G_α. The activation of all three isoforms of G_α has been reported for the M_2 muscarinic receptor subtype (Migeon et al., 1995). Also in CHO-cells, recombinant M_2
muscarinic receptors have been found to couple to $G_{12}\alpha$ and $G_{13}\alpha$ (Dell'Acqua et al., 1993), and in HEK 293 cells the $M_2$ subtype has been shown to mediate the activation of $G_{11}\alpha$, $G_{12}\alpha$ and $G_{13}\alpha$ (Offermanns et al., 1994a). However, in studies by Matesic et al. (1989, 1991) based in heart and cerebellum the $M_2$ subtype has been shown to mediate interactions with $G_{11}\alpha$, $G_{13}\alpha$ and $G_\alpha\alpha$. The activation of $G_\alpha\alpha$ was not seen in this study after $M_2$ receptor stimulation. This may be due to a lower affinity of the antiserum for its substrate resulting in a low immunoprecipitation efficiency, which may in turn preclude the measurement of low level $G_\alpha\alpha$ activation. The lack of statistically significant activation of $G_{4/11}\alpha$, $G_\alpha\alpha$ or $G_\alpha\alpha$ by the $M_2$ or $M_4$ receptor subtypes may reflect the assay conditions utilised and activation may be seen if the GDP concentration was lowered.

Due to the later discovery and pharmacological evaluation of the $M_4$ receptor subtype, there are fewer studies on the $G$ protein coupling of this receptor subtype. However, studies using both chick and human recombinant $M_4$ receptors have shown that this receptor subtype preferentially couples to $G_{12}\alpha$ and $G_\alpha\alpha$, with a limited interaction with $G_{11}\alpha$ and $G_{13}\alpha$ (Migeon and Nathanson, 1994; Migeon et al., 1995). These findings are in agreement with our data for the qualitative coupling of the $M_4$ receptor subtype. Yet, these studies examined the effectiveness of various $G$ proteins to reconstitute the coupling of receptor subtypes to the inhibition of AC. As all the $G_\alpha$ isoforms have been shown to be equipotent in mediating the inhibition of AC (Wong et al., 1992), one may conclude that the $M_4$ receptor subtype preferentially interacts with $G_{12}\alpha$. This quantitative finding can not be corroborated by our study due to potential differences in the immunoprecipitation efficiency of different antisera. In our analysis of the $M_2$ and $M_4$ muscarinic receptor subtypes at the level of cAMP accumulation we observed, in conjunction with an inhibitory response, a stimulatory response to $M_4$ receptor activation at higher methacholine concentrations. A number of recent studies have shown that the $M_4$ receptor subtype is capable of both inhibiting and stimulating adenylyl cyclase, with the stimulatory response dependent upon receptor number and agonist concentration (Jones et al., 1991; Dittman et al., 1994; Migeon and Nathanson, 1994). Only the study by Dittman et al. (1994) proposed that the stimulation of adenylyl cyclase is due to $G_\alpha\alpha$ coupling rather than being mediated
by either $\beta\gamma$ complexes or increases in intracellular calcium. The activation of $G_{i\alpha}$ by the $M_4$ receptor subtype seen in our study approaches statistical significance ($P = 0.055$), yet to fully evaluate this phenomenon further experimentation would be required. Activation of $G_{o\alpha}$ was not seen in this study after $M_4$ receptor stimulation, yet the activation of $G_{o\alpha}$ and $G_{o\alpha}$ has been reported after $M_4$ receptor-stimulation by Migeon et al, (1995). Again as discussed above this may be due to a low affinity of the antiserum for its substrate resulting in a reduced immunoprecipitation efficiency and/or low levels of $G_{o\alpha}$ expression in CHO-cells, which may in turn preclude the measurement of low level $G_{o\alpha}$ activation.

$M_2$ and $M_4$ muscarinic receptor stimulation can also mediate phosphoinositide hydrolysis, detected as total inositol phosphate accumulation (Ashkenazi et al., 1987, 1989; Peralta et al., 1988; Caulfield, 1993). However, these findings in conjunction with this study suggest that the activation of PLC is not being mediated by $G_{q11,\alpha}$, but the stimulation of PLC by the $M_2$ and $M_4$ receptor subtypes is probably being mediated by $\beta\gamma$ subunits from $G_i$-like G proteins (Ashkenazi et al., 1989; Camps et al., 1992; Katz et al., 1992; Lee et al., 1993; Wu et al., 1993), or even activated $G_{i\alpha}$ subunits (Hunt et al., 1994). The discussion above illustrates that the $M_2$ and $M_4$ muscarinic receptor subtypes functionally couple to a single class of G protein in membranes derived from a single cell type.

The partial agonist action of pilocarpine seen both in membranes and permeabilised cell suspensions, is again seen for all four receptor subtypes when evaluated at the level of $G_{o\alpha}$ specific $[^{35}\text{S}]-\text{GTP}_{\gamma}\text{S}$ binding. This is in agreement with other studies involving the comparison of various 'full' agonists and pilocarpine (Freedman et al., 1988; Baumgold and White, 1989; Olianas and Onali, 1991, 1996). A study by Matesic et al, (1991) directly illustrated the partiality of pilocarpine at the level of G protein activation by purifying muscarinic receptor-G protein complexes using an immunoprecipitation protocol followed by western blotting. In this study by Matesic et al, (1991), reduced amounts of total receptor-G protein complexes were reported when pilocarpine was compared to carbachol. In the work reported here, a maximally effective concentration of pilocarpine displayed an impaired ability to activate the
members of the G_2 family after stimulation by the M_2 and M_4 subtypes when compared to methacholine, with the activation of G_{i_2} falling most after the stimulation of the M_4 receptor subtype. The larger reduction seen with pilocarpine against the M_4 subtype is verified by a loss of G_{i_2} activation, with the signal from pilocarpine apparently being transduced solely by the G_{i_2} isoforms. However, the activation of both G_{i_2} and G_{i_3} remains intact after M_2 receptor stimulation. These findings, taken together with the methacholine data, suggest that agonists acting at different receptor subtypes may be capable of inducing relatively selective coupling of the occupied receptor to available G-proteins. Selective activation of inhibitory G-protein α-subunits by partial agonists of the 5-HT_1A receptor have been observed using G protein 4-azidoanilido-[α-32P]-GTP binding immunoprecipitation-based studies (Gettys et al., 1994), suggesting that this may be a more universal G protein-coupled receptor phenomenon.

The M_1 subtype activated G_{q/11}, G_{i_1}, G_{i_2}, G_{i_3}, G_{i_4}, G_{q}, and G_{s} in response to the ‘full’ agonist methacholine whereas the M_3 receptor subtype activated only G_{q/11}, G_{i_3}, and G_{i_3} to a statistically significant level. The activation of G_1-like G proteins by the M_1 and M_3 receptor subtypes can be seen after pertussis toxin pre-treatment in the membrane-filtration based studies discussed in Chapter Four, and also in Chapter Five with the pertussis toxin-sensitivity of Ins(1,4,5)P_3 generation. The finding that the M_1 and M_3 receptor subtypes interact with pertussis toxin-sensitive G proteins is also supported by other [35S]-GTPγS binding studies (Lazareno et al., 1993; Burford et al., 1995b). Also Offermanns et al. (1994) showed that both M_1 and M_3 receptors could activate G_{i_1} and G_{i_3}, as well as illustrating the expected activation of G_{q/11} by using [α-32P]-GTP-azidoanilide and G protein α subunit-specific immunoprecipitation. The observed activation of G_{s} by the M_1 receptor subtype reported in this study is in agreement with a number of studies where a robust activation of adenylyl cyclase is observed after M_1 receptor stimulation (Peralta et al., 1988; Burford et al., 1995a; Burford and Nahorski, 1996). Our current studies illustrate that the M_1 and M_3 muscarinic receptor subtypes can couple to multiple classes of G protein in membranes derived from a single cell type.
The 'partial' agonist pilocarpine stimulated \[^{35}\text{S}]\text{-GTPyS} binding to G_{q11}\alpha, G_{i13}\alpha and G_{i36}\alpha after activation of the M₁ muscarinic receptor subtype. The M₃ receptor subtype also showed activation of these three sets of G protein \(\alpha\) subunits in response to pilocarpine. However, the stimulation of G_{q11}\alpha was only 23% of that elicited by methacholine and only just attained statistical significance, yet the activation of G_{i13}\alpha in response to pilocarpine was approximately equivalent to that seen with methacholine. Pilocarpine has been reported as more efficacious in stimulating phosphoinositide hydrolysis linked to the M₁ receptor subtype, when compared directly to the M₃ receptor subtype in CHO-cells (Wang and El-Fakahany, 1993) and in CHO and A9L cells (Richards and van Giersbergen, 1995). In fact, in the A9L cells pilocarpine failed to elicit a measurable concentration-response when acting through the M₃ receptor subtype. Both of these studies may reflect the very different levels of G_{q11}\alpha activation after pilocarpine stimulation of the M₁ and M₃ receptor subtypes that we report here. Selective activation of inhibitory G protein \(\alpha\)-subunits by pilocarpine through the M₁ receptor compared to the M₃ receptor subtype may be further evidence for agonist trafficking occurring at muscarinic receptor subtypes (see Kenakin, 1995a, 1995b).

The 'full' agonist methacholine appears more potent when activating G_{i36}\alpha through the M₂ compared to the M₄ muscarinic receptor. The M₂ and M₄ receptor subtypes produced EC_{50} values of approximately 1.8 and 21 \(\mu\)M respectively. The EC_{50} values reported for these two subtypes in membrane-filtration based assays were around 1 \(\mu\)M, these observed EC_{50} differences may reflect the activation of different groups of Go proteins. The weaker interaction of the M₄ receptor subtype with G_{i36}\alpha, compared to the M₂ receptor, is in agreement with the findings of Migeon et al., (1995), where the M₂ subtype was seen to mediate robust interactions with G_{i36}\alpha, whereas the M₄ subtype interacted more weakly.

The effect of methacholine on the activation of G_{q11}\alpha through the M₁ and M₃ subtypes is clearly concentration dependent, but again major differences between the M₁ and M₃ muscarinic receptor subtypes exist. The M₁ muscarinic receptor subtype displays a higher potency for the activation of G_{q11}\alpha when compared directly with the M₃.
subtype. This result reflects the findings discussed in Chapter Four where methacholine appears more potent at the M₁ receptor subtype compared to the M₃ muscarinic receptor subtype. The EC₅₀ values obtained at the level of [³⁵S]-GTPγS binding in the presence of pertussis toxin in the filtration-based experiments correlate well with those evaluated by [³⁵S]-GTPγS-G_{q11}α binding in the immunoprecipitation-based assays. At the M₁ receptor subtype EC₅₀ values of 3.9 and 4.5 μM were obtained for methacholine in filtration- and immunoprecipitation-based [³⁵S]-GTPγS binding assays respectively. At the M₃ muscarinic receptor subtype EC₅₀ values of 20.8 and 23.8 μM were obtained for methacholine in filtration- and immunoprecipitation-based [³⁵S]-GTPγS binding assays respectively. Therefore for human receptors expressed in CHO cells the M₁ muscarinic receptor subtype displays a more potent activation of G_{q11}α than the M₃ receptor subtype, when compared directly in two independent studies. The potencies of either carbachol or methacholine in activating different receptor subtypes have not been systematically compared in many studies. However, one of the earliest comparisons of the muscarinic receptor subtypes, comparing them at the level of PI hydrolysis, revealed a 4-16 fold lower EC₅₀ value for carbachol acting at the M₁ subtype when compared directly to the M₃ subtype (Peralta et al., 1988). For Rat receptors expressed in CHO-cells, the order of potency with respect to PI turnover was M₁ > M₃ (Gurwitz et al., 1994). Again the order of potency with respect to PI turnover was found to be M₁ > M₃ in A9L cells expressing human muscarinic receptors (Richards and van Giersbergen, 1995). Even at the level of L-type calcium channel inhibition the M₁ receptor subtype has been evaluated as a more potent mediator than the M₃ receptor subtype (Pemberton and Jones, 1997). However, other studies have reported no differences between human M₁ and M₃ receptor subtypes (Sandmann et al., 1991; Offermanns et al., 1994; Burford et al., 1995a). Alternatively carbachol has been reported to be 10 times more potent when activating M₃ receptors compared to M₁, with respect to IP release (Bujo et al., 1988) and Ca²⁺ elevation (Neher et al., 1988) in cells expressing the porcine receptor subtypes. So although there is a large supporting body of evidence at the second messenger level for the agonist potency order M₁ > M₃, there appears to be an overall lack of consensus. It must be borne in mind in this discussion of the potency of methacholine or carbachol, that the activation of PI hydrolysis or the mobilisation of Ca²⁺ are not
solely indicators of $G_{q11}\alpha$ activation and other pathways may be involved. Therefore, results taken at the second messenger level may not be rigid indicators of single G protein coupling events. Variations in the potency of carbachol at muscarinic receptor subtypes have been shown to be a function of receptor expression levels (Kukkonen et al., 1996). Also the stoichiometry of coupling to G proteins is expected to affect the sensitivity of receptor-mediated responses to the concentration of ligand (Kenakin, 1997). We have addressed both of these points in our study as the levels of expression in the CHO-M$_1$ and CHO-M$_3$ cells are equal, with both cell types showing equivalent levels of G proteins. Therefore the differences observed, using two separate assays, are likely to reflect true coupling differences between the M$_1$ and M$_3$ receptor subtypes.

Atropine blockade of agonist-stimulated G$\alpha$ [$^{35}$S]-GTP$\gamma$S binding was obtained for all muscarinic acetylcholine receptor subtypes when atropine was included at 10 $\mu$M. However when atropine was incubated alone it did not mediate a reduction in the basal level of [$^{35}$S]-GTP$\gamma$S binding to either $G_{ii3}\alpha$ or $G_{q11}\alpha$. This is in contrast to the results outlined in Chapter Four where it was observed, by a reduction in basal [$^{35}$S]-GTP$\gamma$S binding, that atropine acts as an inverse agonist at the M$_2$ and M$_4$ receptor subtypes. One explanation may be that the experimental conditions utilised unintentionally biased the assay towards the evaluation of agonist behaviour. This assay system may also be insensitive to the small reductions in basal [$^{35}$S]-GTP$\gamma$S binding mediated by atropine due to the levels of GDP and Na$^+$ present. The inhibition of [$^{35}$S]-GTP$\gamma$S binding by atropine in cardiac membranes was found to be modulated by both NaCl and GDP (Hilf and Jakobs, 1992). The inhibition mediated by atropine was maximal in the absence of NaCl and with the inclusion of 0.3 $\mu$M GDP. The inhibition of basal [$^{35}$S]-GTP$\gamma$S binding was reduced in a concentration-dependent manner by increasing the Na$^+$ and GDP concentrations. A reduction in agonist-independent activity mediated by an inverse agonist involves preferential binding to the inactive receptor species (R) altering the position of the equilibrium between inactive (R) and active (R*) forms, and in turn this reduces the level of R*. Na$^+$ and GDP both influence the equilibrium between R and R* by increasing the level of R, so they would be expected to minimise the level of constitutive activity which can be
observed. Thus, Na\(^+\) and GDP make it harder to observe inverse agonist activity so in
this assay system, with the inclusion of 100 mM Na\(^+\) and either 1 or 10 \(\mu\)M GDP, we
are unable to measure the inverse properties of atropine. An alternative explanation is
that the inverse properties of atropine may be mediated by other G proteins not tested
for e.g. G\(_{q/11}\). Yet this is unlikely as atropine was reported to act as an inverse agonist
at all the PLC-coupled muscarinic receptor subtypes in studies using a G\(_{q/11}\) G
protein overexpression system by Burstein et al. (1995, 1997). Also Jakubik et al.
(1995, 1996) reported atropine-mediated increases in cAMP in both M\(_2\) and M\(_4\)
expressing CHO-cells, and M\(_2\) expressing rat cardiomyocytes, and atropine-inhibition
of basal inositol phosphate production in M\(_1\) and M\(_3\) expressing CHO-cells. This
evidence would suggest that atropine mediates its inverse agonist activity by
uncoupling the expected classes of G proteins from the respective muscarinic receptor
subtypes.

Most of the current studies were performed with a single concentration of agonist,
GDP and a single concentration of antiserum. Although this approach has been
traditionally accepted as valid, subtle differences in coupling may have been missed
by this approach. Another potential problem with our approach is that some degree of
cross-reactivity among the antisera may be possible. Again as stated before it has to
be borne in mind that GDP may affect the activation state of specific G\(\alpha\) subunits
differently, so the concentration of GDP has to be stated and evaluated for each G\(\alpha\)
subunit. Varying the GDP concentrations may have revealed activation of different
G\(\alpha\) subunits. This approach does allow comparisons of the abilities of different
receptors to activate various G\(\alpha\) subunits, however this methodology does not allow
comparisons between different G\(\alpha\) subunits due to the potential differences in antisera
affinity.

In conclusion this study shows that immunoprecipitation of \(^{35}\)S-GTP\(\gamma\)S-bound G\(\alpha\)-
subunits can be used as both a qualitative and quantitative measure of agonist activity
at muscarinic acetylcholine receptors. Also human muscarinic acetylcholine receptor
subtypes expressed in Chinese hamster ovary cells display differing G protein
activation profiles, and that activation of distinct G protein subtypes by different
muscarinic acetylcholine receptors occurred with different speeds, magnitudes and efficacies. From the results obtained we can show that marked differences exist between the human muscarinic acetylcholine receptor subtypes in their abilities to activate Gα proteins.
Chapter Seven.

Summary and Concluding Discussion.
The aims of this study were to compare and contrast the agonist-mediated G protein responses elicited by individual muscarinic receptor subtypes, expressed as homogeneous populations in CHO-cells. In this system, other than the receptor subtypes, all other signalling molecules were endogenous to the CHO-cells including the heterotrimeric G proteins and effector molecules, such as adenylyl cyclase and phosphoinositidase C.

The major advantage of using recombinant receptors expressed in a single cell type is the ability to examine a single receptor subtype in isolation. Generally in tissues a mixed population of acetylcholine muscarinic receptors are expressed and this complicates the interpretation of agonist-stimulated G protein activation as the muscarinic receptor agonists used are relatively non-selective. Also, comparisons of responses between single populations of muscarinic receptor subtypes expressed in two different tissues may be complicated by differences in the levels of receptor expression, the subtypes of G proteins present and their relative abundance, and the level and the subtypes of effector molecules expressed. Therefore, by expressing recombinant muscarinic receptor subtypes in CHO cells the G protein and effector complement should, in theory, be equivalent. Another advantage of this system is that one can equalise the receptor expression levels and by expressing similar densities of muscarinic receptor subtypes in different CHO cell clones, agonist-mediated responses can be directly compared between receptor subtypes.

However, there are disadvantages attached to such a system. CHO cells do not normally express muscarinic receptor subtypes and therefore it could be argued that the responses elicited by muscarinic receptor stimulation in CHO cells may not be physiologically relevant. Also, the expression levels in the recombinant cell system can be high in comparison to expression levels of muscarinic acetylcholine receptors in certain tissues. The variety of expression levels of various muscarinic receptor subtypes in tissues which also possess heterogeneous pools of G proteins and effector molecules, suggests that muscarinic receptor agonist-mediated responses may vary between tissues with no single response defining the activity of a particular muscarinic receptor subtype. Therefore it can be argued that, although not ideal,
recombinant muscarinic receptors expressed in CHO cells offer several advantages over tissues for comparing agonist-mediated responses between the different muscarinic receptor subtypes.

Many of the techniques for G protein analysis discussed in this study and others, utilise cell free systems, relying on the production of cell plasma membranes. The high concentrations of G proteins at the plasma membrane allows the measurement of receptor-stimulated activation. However, with the use of membranes comes a number of problems and criticisms, the key criticism being the loss of microdomains in which the concentrations of receptors, G proteins and other signalling components are potentially constant. There is increasing evidence that targeting and compartmentation of signalling molecules may play a role in the specificity and fidelity of transmembrane signalling observed in intact systems. Information is emerging that caveolin and PDZ domain-containing proteins both play a role in cellular organisation. Many G protein signalling related molecules reside within plasma membrane caveolae including Go proteins (Chang et al., 1994; Li et al., 1995; Song et al., 1996; Nomura et al., 1997), agonist-activated G protein-coupled receptors (Raposo et al., 1987; 1989; Raposo and Benedetti, 1994) and down stream effectors such as Ins(1,4,5)P3 receptors and the plasma membrane Ca2+-ATPase (Schnitzer et al., 1995; Fujimoto et al., 1995). The precise role for the caveolae in the regulation of G protein signalling remains to be elucidated but with the concentration of signalling components and the fact that the interaction of Go with caveolin appears to be a regulated process suggests that caveolin may be an important mediator of cellular compartmentation (Li et al., 1995). The PDZ domain, a membrane targeting system that is important in the central nervous system, has been shown to play an important role in GPCR targeting (Brakeman et al., 1997). This evidence together with the incidence of occurrence of the PDZ binding motif in GPCRs (Neubig, 1998), RGS proteins (De Vries and Farquhar, 1999) and effector molecules such as potassium channels (Kim et al., 1995) suggest a role for PDZ proteins in assembling a signalling complex. Compartmentation of signalling machinery, by caveolin, PDZ proteins or other novel mechanisms could potentially provide localised concentrations of signalling molecules which may lead to enhanced specificity (Kleuss et al., 1993;
Neubig, 1998). Therefore, the loss of microdomains may expose receptors to levels of specific G proteins that they would not encounter in their normal physiological environment, leading to promiscuous coupling events and/or altering agonist efficacy (Jakubik et al., 1998).

Other problems with plasma membranes may occur at the preparation stage. During preparation, membranes must be washed thoroughly to ensure that any remaining serum is removed, as any remaining serum may act as a G protein stimulator and interfere with the final results. After preparation guanine nucleotide concentrations in the micromolar range can exist in washed membranes (Neubig and Szamraj, 1986). These levels could potentially influence the activity state of the G protein complement and/or the specific activity of the radioligand and in turn any final experimental results. However, agonist binding studies performed in this study, and discussed later, indicate that the guanine nucleotide levels present were unlikely to effect the results significantly. Proteolysis of signal transduction components can often be a problem and in all of the cell preparations utilised in this study protease inhibitors were not used. At the outset though it was determined that the inclusion of protease inhibitors in the reaction media had no significant effect on the results obtained. Also the effect of proteases was minimised by carrying out membrane-handling work at 0-4°C and completing the homogenisation stage, in plasma membrane preparation, rapidly. Any variations in batches of membranes were not assessed and perhaps an assessment should be made for purity and activity using a plasma membrane marker such as the ouabain-sensitive \((\text{Na}^+, \text{K}^+)\) ATPase (Cotman et al., 1971).

Alternatively, to overcome the problems associated with using membranes and to maintain a more intact cellular system permeabilised cells were utilised. With the permeabilised cells we decided to utilise a buffer which reflected the intracellular environment, but this differs from the buffer used in the other experimental systems. When using a buffer of a particular composition the concentrations of \(\text{Na}^+\) and \(\text{K}^+\) have to be borne in mind when interpreting experiments in relation to physiological conditions. Methacholine-stimulation of the \(M_1\) and \(M_3\) muscarinic receptors, in \(\beta\)-escin permeabilised cells, resulted in the activation of PLC indicated by \(^{45}\text{Ca}^{2+}\) release
from intracellular calcium stores. Ins(1,4,5)P₃ liberated after receptor-stimulation was capable of eliciting the release of ⁴⁰Ca²⁺ therefore, permeabilisation of the cell suspensions did not prevent receptor-G protein coupling. Although permeabilised cells allowed receptor activation to be measured, maybe the loss of intracellular components should have been assessed further, by perhaps investigating the pore size formed using an indicator such as the efflux of lactate dehydrogenase. The stoichiometry of the signal transduction components may be different in membrane and permeabilised cell preparations, and as it has been shown recently that the EC₅₀ value of carbachol increases with an increasing R/G₆ ratio (Jakubik et al., 1998), comparisons of data from different cellular preparations, where the receptor-G protein interactions are potentially altered, may be invalid.

The normal concentration of GTP in a cell is around 0.1 mM, with GDP being present in 10-fold lower concentrations, with Kₘ for GTP of Ga subunits in the range of 0.1-0.5 μM. This means that under normal physiological conditions the G protein could be active at all times. However, it is known that to exchange GDP for GTP the G protein must overcome a highly unstable transition state, with this unfavourable energetic reaction being more commonly initiated by an activated receptor (Iiri et al., 1998). So, in assays designed to quantify G protein activation after receptor-stimulation it is often necessary for GDP to be present such that the receptor can promote the release of this nucleotide and allow G protein activation. Therefore, it is often necessary to perform [³⁵S]-GTPγS binding in the presence of GDP, with the greatest agonist-stimulation being obtained in the presence of millimolar GDP. As well as optimising the agonist-signal GDP has been shown to influence agonist efficacy and potency in a number of systems. Increasing GDP has been shown to magnify efficacy differences between agonists acting at the μ-opioid receptor (Selley et al., 1997). Breivogel et al. (1998) showed that cannabinoid receptor agonist efficacy for stimulating [³⁵S]-GTPγS binding to rat cerebellar membranes correlated with agonist-induced decreases in GDP affinity. Together these studies illustrate that agonist efficacy can be influenced by the activity state of G proteins. The addition of exogenous GDP will affect the activity state of G proteins and therefore all [³⁵S]-GTPγS binding studies are subject to a degree of experimental bias. Therefore,
efficacy differences between the AC-linked and the PLC-linked subtypes are relative to the experimental conditions utilised.

\[^{35}\text{S}\]-GTP\(\gamma\)S binding is extremely useful in allowing the dissection of the initial events in G protein signal transduction pathways. Membrane-filtration based \[^{35}\text{S}\]-GTP\(\gamma\)S binding can be indicative of whether a receptor class functions through the activation of pertussis-toxin sensitive or insensitive G proteins. However, membrane-filtration based \[^{35}\text{S}\]-GTP\(\gamma\)S binding is less useful for G protein identification as \[^{35}\text{S}\]-GTP\(\gamma\)S binding will potentially be dependent on the final concentration of \[^{35}\text{S}\]-GTP\(\gamma\)S utilised and bind to the entire complement of activated G proteins. In this system if one of the muscarinic receptor subtypes activates a G protein that is a minor component of the total number of G proteins present then it becomes extremely difficult to measure the activation of a minor G protein population over the background \[^{35}\text{S}\]-GTP\(\gamma\)S binding. Also, \[^{35}\text{S}\]-GTP\(\gamma\)S binding assays only work when examining G proteins which exchange guanine nucleotides at the same rate as pertussis-toxin sensitive G proteins (\(K_{\text{cat}}\) in the region of \(\sim 4 \, \text{min}^{-1}\)) (Gilman, 1987). Therefore, for G proteins with low intrinsic rates of GTP hydrolysis, such as \(G_z\) or \(G_q/11\alpha\) (Casey et al., 1990; Berstein et al., 1992a; Berstein et al., 1992b), it may not be possible to adequately perform \[^{35}\text{S}\]-GTP\(\gamma\)S binding assays. However, we attempted to address these problems by developing a \[^{35}\text{S}\]-GTP\(\gamma\)S binding-immunoprecipitation-based assay which identifies individual activated \(G\alpha\) proteins. This highly sensitive methodology allows the measurement of activated \(G\alpha\) proteins with lower levels of expression and/or lower intrinsic GTPase activities.

A major criticism of the \[^{35}\text{S}\]-GTP\(\gamma\)S binding-immunoprecipitation-based assay, is related to the ability to define the individual activated \(G\alpha\) proteins. To check that the immunoprecipitates obtained using this methodology contain the expected \(G\alpha\) species SDS-PAGE methodology would be required. However, we have found that the association between the \[^{35}\text{S}\]-GTP\(\gamma\)S moiety and the \(G\alpha\) subunit, under the SDS-PAGE conditions tested, did not allow us to check the composition of the immunoprecipitated material.
Methodological considerations, i.e. the degree of bias introduced when adopting a particular quantitative or qualitative approach, are often underrated in the traditional sciences and these should be borne in mind in all discussions of any overall conclusions (Foucault, 1969).

**Muscarinic acetylcholine receptors expressed in CHO-cells.**

Pharmacological characterisation of muscarinic receptor subtypes expressed in these different CHO-cell clones allowed receptor density and antagonist affinity values to be obtained. The rank order of affinity of a series of antagonists for individual muscarinic receptor subtypes correlated well with those reported by many other groups (Caulfield, 1993). The G protein complement in the individual CHO-cell clones were compared and, as expected, all these cells were shown to express equivalent levels of Go proteins that react with anti- Goα, G11/2α, G6α, Gq11α and Gsα.

In the present study all the muscarinic receptor subtypes displayed guanine nucleotide shifts in the agonist binding studies performed. The PLC-linked muscarinic receptor subtypes showed slightly smaller shifts than the AC-linked receptor subtypes and this pattern has been shown in other studies (Burford et al., 1995b). Not all the agonist binding curves, in the presence of GDP (10 μM) and GTP (70 pM), produced slope factors of unity, indicating that receptors did not form a homogeneous low affinity receptor population under these conditions. A higher concentration of GTP would be required to form homogeneous low affinity receptor populations. These data were not obtained as these experiments were used to compare the affinity and potency of methacholine under the same conditions. The smaller guanine nucleotide shifts observed in the M1 and M3 expressing cells may be due to one or both of two factors. The difference in affinity between the high and low affinity states may be less than the affinity state difference for AC-linked receptors and/or a smaller percentage of M1 and M3 receptors were found to have a high affinity for methacholine under the assay conditions utilised. The reduced level of high affinity state M1 and M3 receptors may be due to the relatively poor efficiency of coupling of PLC-linked muscarinic
receptors to G proteins seen in the $[^{35}S]$-GTPγS binding membrane filtration-based assay system. This suggests the possibility that the coupling of $M_1$ and $M_3$ receptors in membranes may be 'weakened'. The causes of the 'weakened' coupling are not defined, but may relate to PLCβ1 acting as a GTPase activating protein (GAP) on $G_{q/11\alpha}$ (Berstein et al., 1992b) increasing the rate of GTP hydrolysis. It has been suggested that in CHO-cell membrane preparations PLC activity is impaired (Burford et al., 1995b), and that this lack of PLC activity and, hence reduced GTP/GDP exchange may result in minimal high affinity agonist binding. Alternatively there could be significant differences in the levels of different classes of G proteins endogenously expressed in CHO-cells. Finally these differences in guanine nucleotide-modification of agonist-binding may reflect differences in the coupling of the AC-linked and PLC-linked muscarinic receptors with specific G proteins observed in the immunoprecipitation-based $[^{35}S]$-GTPγS binding data.

$M_1$ and $M_3$ muscarinic acetylcholine receptors expressed in CHO-cells.

Both the $M_1$ and $M_3$ receptor subtypes displayed the ability to couple with both pertussis toxin-sensitive and -insensitive G proteins, as observed in the membrane filtration-based and immunoprecipitation-based $[^{35}S]$-GTPγS binding data. Results obtained from the CHO-$M_1$ and -$M_3$ mediated Ins(1,4,5)P$_3$ generation in β-escin permeabilised cells after pertussis toxin pre-treatment potentially suggested that the functional significance of such a coupling event may be in the stimulation of PLCβ by $\beta\gamma$ subunits derived from $G_i$-like G proteins. However, the functional significance of the coupling of $M_1$ and $M_3$ muscarinic receptors to pertussis toxin-sensitive G proteins should be established by performing functional assays in the presence of either G protein antiserum or following introduction of antisense oligonucleotides to selectively inactivate members of the $G_i$-like family.

Throughout this study it was shown that the $M_1$ and $M_3$ receptor subtypes display different rates and magnitudes of $[^{35}S]$-GTPγS binding, reflecting differences at the level of G protein activation. Membrane filtration-based $[^{35}S]$-GTPγS binding in the presence of pertussis toxin appeared to indicate that the $M_1$ receptor interacts with
pertussis toxin-insensitive G proteins more rapidly then the M3 receptor subtype. This finding was strengthened using specific \[^{35}\text{S}]\text{-GTPyS}\) binding to G\(_{q/11}\alpha\). Here, the G\(_{q/11}\alpha\)-[^{35}\text{S}]\text{-GTPyS}\) binding mediated by the M1 receptor subtype also occurred more rapidly when compared directly to M3 receptor-mediated G\(_{q/11}\alpha\)-[^{35}\text{S}]\text{-GTPyS}\) binding. \[^{35}\text{S}]\text{-GTPyS}\) binding membrane filtration-based assays in the absence of pertussis toxin indicated that the M1 receptor subtype activates G\(_r\)-like G proteins faster than the M3 receptor subtype, to validate this point time-course evaluation using G\(_{i/3}\alpha\) antisera would be required. If we had these data to hand it would be possible to postulate that the M1 receptor subtype is more efficient in catalysing the GDP/GTP exchange reaction on all G\(\alpha\) proteins and not just G\(_{q/11}\alpha\).

Also in this study it was observed that the activation of G\(_{q/11}\alpha\) through the M1 receptor subtype is greater than that mediated by the M3 receptor subtype. The evidence for this phenomenon comes from both membrane filtration- and immunoprecipitation-based \[^{35}\text{S}]\text{-GTPyS}\) binding and is supported by our findings at the level of Ins(1,4,5)P\(_3\) generation. In all of these experimental results the maximal response mediated by methacholine-stimulation of the M1 receptor subtype was greater then that observed at the M3 receptor subtype.

The M1 subtype produced a significant activation of G\(_{q/11}\alpha\), G\(_{i/3}\alpha\), G\(_{i/3\alpha\,2}\), G\(_{i/3\alpha\,6}\), G\(_q\alpha\) and G\(_s\alpha\) in response to the ‘full’ agonist methacholine, whereas the M3 receptor subtype activates only G\(_{q/11}\alpha\), G\(_{i/3}\alpha\) and G\(_{i/3\alpha\,0}\), to a statistically significant level. The activation of G\(_r\)-like G proteins by the M1 and M3 receptor subtypes can be seen after pertussis toxin pre-treatment in the membrane filtration-based studies and also in the pertussis toxin sensitivity of Ins(1,4,5)P\(_3\) generation, as discussed above. As the receptor expression levels in these two cell lines were closely matched the differential coupling profiles cannot be explained by differences in receptor density. From this study it would appear that the M1 muscarinic receptor subtype can interact with a wider range of G\(\alpha\) proteins than the M3 receptor subtype. To further evaluate the differential coupling of the M1 and M3 receptor subtypes perhaps the GDP concentration should be further reduced in the immunoprecipitation-based assays, although this will become less representative of physiological concentrations.
However the evidence outlined above, from multiple independent assays appears conclusive.

The 'partial' agonist pilocarpine stimulated \(^{35}\text{S}\)-GTP\(\gamma\)S binding to \(G_{q11}\alpha\), \(G_{i3\alpha}\) and \(G_{i3\alpha}\) after activation of the M\(_1\) muscarinic receptor subtype. The M\(_3\) receptor subtype also showed activation of these three sets of G protein \(\alpha\) subunits in response to pilocarpine, but the stimulation of \(G_{q11}\alpha\) by the M\(_3\) receptor subtype was only 23% of that elicited by methacholine and only just attained statistical significance. However, the activation of \(G_{i3\alpha}\) in response to pilocarpine was approximately equivalent to that seen with methacholine for the M\(_3\) receptor subtype, but vastly reduced for the M\(_1\) receptor. These findings reflect marked differences in G protein coupling after pilocarpine-stimulation of the M\(_1\) and M\(_3\) receptor subtypes. Tissue and cell line experiments strongly suggest a lower activity of pilocarpine at M\(_3\) compared to M\(_1\) receptors (Fisher et al., 1983; Brown et al., 1984; Jacobson et al., 1985; McKinney et al., 1985; Fisher and Snider, 1987; Caulfield, 1993; Lazareno et al., 1993a; Wang and El-Fakahany, 1993; Richards and van Giersbergen, 1995). Key studies report pilocarpine as more efficacious in stimulating phosphoinositide hydrolysis linked to the M\(_1\) receptor subtype when compared directly to the M\(_3\) receptor subtype in CHO-cells (Wang and El-Fakahany, 1993; Richards and van Giersbergen, 1995). Both of these studies may reflect the different levels of \(G_{q11}\alpha\) activation after pilocarpine stimulation of the M\(_1\) and M\(_3\) receptor subtypes that we report.

A further difference between the PLC-linked receptor subtypes is that methacholine displays a higher potency for the activation of \(G_{q11}\alpha\) after stimulation of the M\(_1\) muscarinic receptor subtype when compared directly to the M\(_3\) subtype. The EC\(_{50}\) values obtained at the level of \(^{35}\text{S}\)-GTP\(\gamma\)S binding in the presence of pertussis toxin in the filtration-based experiments correlate extremely well with those evaluated by \(^{35}\text{S}\)-GTP\(\gamma\)S-G\(_{q11}\alpha\) binding in the immunoprecipitation-based assays. Therefore for human receptors expressed in CHO-cells at comparable densities the M\(_1\) muscarinic receptor subtype displays a more potent activation of \(G_{q11}\alpha\) than the M\(_3\) receptor subtype, when compared directly in independent studies. Variations in the potency of carbachol at muscarinic receptor subtypes have been shown to be a function of...
receptor expression levels (Kukkonen et al., 1996). Also the stoichiometry of coupling to G proteins is expected to affect the sensitivity of receptor-mediated responses to the concentration of ligand (Kenakin, 1997). We have addressed both of these points in our study as the levels of expression in the CHO-M₁ and CHO-M₃ cells are equal, with both cell types showing equivalent levels of G proteins. Therefore the differences observed, using a range of methodologies, are likely to reflect true coupling differences between the M₁ and M₃ receptor subtypes, which suggest that the intrinsic activity of the M₁ subtype is greater than that of the M₃ muscarinic receptor subtype.

Our current studies illustrate that the M₁ and M₃ muscarinic receptor subtypes can couple to multiple classes of G protein in membranes derived from a single cell type and that significant differences in the G protein coupling exist between these receptor subtypes.

**M₁ and M₃ muscarinic acetylcholine receptors expressed in CHO-cells.**

The M₂ and M₄ receptor subtypes displayed similar time profiles of G protein activation in the time-course data obtained from both the [³⁵S]-GTPγS binding membrane-filtration assays and the immunoprecipitation-based assays. With these two muscarinic receptor subtypes displaying equivalent time-profiles for G₁-like G protein activation, it suggests equivalence of the M₂ and M₄ receptor subtypes at the kinetic level of G protein coupling. Equivalent time profiles for the activation of G proteins after M₂ and M₄ receptor stimulation can be inferred from studies using a luciferase reporter gene under the control of a cAMP-response element (Migeon and Nathanson, 1994; Migeon et al., 1995). To further establish this similarity more studies are required, perhaps using a highly sensitive cAMP inhibition assay. Also it would be interesting to establish the time-course of stimulation for individual G₃α proteins using the immunoprecipitation-based [³⁵S]-GTPγS binding methodology.

The M₂ and M₄ receptor subtypes activated G₁₁-α, G₁₂-α and G₁₃-α in response to methacholine, but showed no statistically significant activation of G₉-α, G₅α or G₄α. As the immunoprecipitation-based [³⁵S]-GTPγS binding assay was standardised to
measure $G_{i2}\alpha$ activation the conditions utilised, especially the inclusion of 10 $\mu$M GDP, may have precluded the measurement of $G_{q11}\alpha$, $G_{o}\alpha$ or $G_{a}\alpha$ activation. As the $M_1$ and $M_3$ receptor subtypes can be seen to activate $G_{q11}\alpha$ in the presence of 1 $\mu$M GDP, perhaps the GDP concentration should be altered to firmly establish that the $M_2$ and $M_4$ receptor subtypes do not activate $G_{q11}\alpha$, $G_{o}\alpha$ or $G_{a}\alpha$ after methacholine-stimulation. As statistically significant levels of agonist-stimulated $[^{35}S]$-GTP$\gamma$S binding to $G_{q11}\alpha$, $G_{o}\alpha$ and $G_{a}\alpha$ has been measured after methacholine-stimulation of the $M_1$ receptor subtype it would suggest that this assay system can be utilised to assess the activation of $G_{i2/2}\alpha$, $G_{i3o}\alpha$, $G_{q11}\alpha$, $G_{o}\alpha$ and $G_{a}\alpha$. However, the low levels of $G_{o}\alpha$ and $G_{a}\alpha$ activity observed may suggest that low levels of $G_{a}\alpha$ and $G_{o}\alpha$ are expressed in CHO-cells or the $G_{o}\alpha$ and $G_{a}\alpha$ antisera may have low affinities for their respective substrates resulting in a reduced immunoprecipitation efficiency which may in turn preclude the measurement of a low level $G_{a}\alpha$ activation.

The activation of $G_{i1}\alpha$, $G_{i2}\alpha$ and $G_{i3}\alpha$ has been reported for agonist-stimulation of the $M_2$ muscarinic receptor subtype (Dell'Acqua et al., 1993; Offermanns et al., 1994a; Migeon et al., 1995), and these studies are in agreement with those reported here. $G_{a}\alpha$ activation by the $M_2$ receptor subtype has been documented in studies by Matesic et al., (1989, 1991) based in heart and cerebellar preparations where the $M_2$ subtype has been shown to mediate interactions with $G_{i1}\alpha$, $G_{i2}\alpha$ and $G_{i3}\alpha$. The lack of detectable $G_{a}\alpha$ activation may reflect the assay conditions utilised as discussed above or agonist-stimulation of the $M_2$ receptor does not result in significant $G_{a}\alpha$ activation as reported here and by Migeon et al. (1995). Studies using recombinant $M_4$ receptors have shown that this receptor subtype preferentially couples to $G_{i2}\alpha$ and $G_{o}\alpha$, with a limited interaction with $G_{i1}\alpha$ and $G_{a}\alpha$ (Migeon and Nathanson, 1994; Migeon et al., 1995). These findings are in agreement with our data for the qualitative coupling of the $M_4$ receptor subtype to $G_{i}$-like G proteins, but again, as for the $M_2$ receptor, no $G_{a}\alpha$ activation was detected in our studies. Again as discussed above this may be due to a low affinity of the antiserum for its substrate resulting in a reduced immunoprecipitation efficiency and/or low levels of $G_{o}\alpha$ expression in CHO-cells, which may in turn preclude the measurement of a low level of $G_{o}\alpha$ activation.
At the level of cAMP accumulation we observed, in conjunction with an inhibitory response, a stimulatory response to M₄ receptor activation at higher methacholine concentrations. A number of studies have shown that the M₄ receptor subtype is capable of stimulating adenylyl cyclase, with this response being dependent upon receptor number and agonist concentration (Jones et al., 1991; Dittman et al., 1994; Migeon and Nathanson, 1994). This process could be mediated by either βγ complexes, increases in intracellular calcium or the activation of G₃α (Sunahara et al., 1996). The activation of G₃α by the M₄ receptor subtype seen in our study approaches statistical significance, yet to fully evaluate this phenomenon further experimentation would be required perhaps using anti-G₃α or anti-Gβγ antibodies to attenuate the rise in intracellular cAMP.

M₂ and M₄ muscarinic receptor stimulation can also mediate phosphoinositide hydrolysis, detected as total inositol phosphate accumulation (Ashkenazi et al., 1987, 1989; Peralta et al., 1988; Caulfield, 1993). However, these findings in conjunction with this study suggest that the activation of PLC is not being mediated by G₄₁α, but the stimulation of PLC by the M₂ and M₄ receptor subtypes is probably being mediated by βγ subunits from Gᵢ-like G proteins (Ashkenazi et al., 1989; Camps et al., 1992; Katz et al., 1992; Lee et al., 1993; Wu et al., 1993), or even activated Gᵢα subunits (Hunt et al., 1994). However, the functional significance of the coupling of M₂ and M₄ muscarinic receptors to PLC should be established by performing functional assays in the presence of Gβγ antisera, as the results linking M₂ and M₄ muscarinic receptors to the activation of PLC were obtained with high receptor expression levels.

In the membrane filtration-based assays pilocarpine showed reduced levels of [³⁵S]-GTPγS binding when activating the M₄ receptor in comparison to the M₂ receptor subtype. The differential levels of G protein activation after pilocarpine stimulation of the M₂ and M₄ receptor subtypes were also seen in the G₁₃α immunoprecipitation-based assay. This was further verified by a loss of G₁₃α activation after pilocarpine-stimulation of the M₄ receptor subtype, with the signal being transduced solely by the G₁₃α isoform(s). However, the activation of both G₁₃α and G₁₃α remained intact
after M₂ receptor stimulation. These findings suggest that different agonists acting at receptor subtypes may be capable of inducing relatively selective coupling of the occupied receptor to available G proteins. Selective activation of inhibitory G protein α-subunits by partial agonists of the 5-HT₁A receptor have been observed using G protein immunoprecipitation-based studies (Gettys et al., 1994), suggesting that agonist-selective G protein coupling, termed agonist trafficking, may be a more universal G protein-coupled receptor phenomenon (Kenakin, 1995a, 1995b). Agonist trafficking of receptor signals involves receptor promiscuity and separate active receptor states selectively promoting G protein coupling in response to activation by different agonists (Kenakin, 1995b). Alternatively if only one active receptor state exists the differential of coupling M₄ receptor subtype after pilocarpine stimulation suggests that loss of activation of G₁₁ᵣα is due to the reduction in the strength of signal. If a single receptor differentially couples to multiple G proteins, agonists of high efficacy activate multiple G proteins, whereas agonists of low efficacy activate only the most efficiently coupled G protein. If the loss of G₁₁ᵣα stimulation after M₄ activation, upon switching from methacholine to pilocarpine, is interpreted as a change in the strength of signal it leads to a different conclusion based upon the intrinsic activities of the M₂ and M₄ receptor subtypes. As the M₂ receptor subtype activates both G₁₁ᵣα and G₁₃ₒα with either methacholine or pilocarpine the M₂ receptor subtype appears to possess a greater intrinsic activity than the M₄ muscarinic receptor subtype. The findings reported here, for these receptor subtypes, could have substantial physiological significance for receptors that couple to multiple G proteins in a single cell. With the major implication being the potential for selective-G protein activation in the development of therapeutic compounds based upon either agonist-specific activation states or varying intrinsic activities of receptor subtypes.

Methacholine showed very little difference in potency when activating the M₂ and M₄ receptor subtypes in the membrane filtration-based [³⁵S]-GTPγS binding assays. However, when the activation of specific Gα proteins, by the M₂ and M₄ receptor subtypes, was evaluated by constructing concentration-effect curves, the ‘full’ agonist methacholine appeared more potent when activating G₁₃ₒα through the M₂ compared to the M₄ muscarinic receptor. To investigate these differences concentration-response
evaluation using the general $G_{i1,3}\alpha$ antiserum and the more specific $G_{i1/2}\alpha$ would be required. If we had these data it could be possible to postulate that the membrane filtration-based [$^{35}$S]-GTPγS binding results reflect an aggregate potency for methacholine at the $M_2$ and $M_4$ receptors.

Pertussis toxin pre-treatment of CHO-cells, in [$^{35}$S]-GTPγS binding experiments, resulted in a decrease in the basal levels of binding in the CHO-cell clones. In the presence of NAD, pertussis toxin will catalyse the incorporation of an ADP-ribose moiety into $G_i$-like G proteins. This event occurs without affecting the intrinsic functions of the G protein i.e. GTPase activity and subunit dissociation (Haga et al., 1985; Enomoto and Asakawa, 1986, Huff and Neer, 1986; Katada et al., 1986), and acts to impede the interaction between the receptor and the G protein. Therefore, these results suggest that muscarinic receptors can interact and activate G proteins even in the absence of receptor-agonist in CHO-cells. These results are in agreement with the modified ternary complex model of agonist-receptor-G protein interactions. This model suggests that an activated form of the receptor (R*) can exist without agonist occupation, and that this species can still interact with, and activate, G proteins (Lefkowitz et al., 1993; Samama et al., 1993; Milligan et al., 1995; Leff et al., 1997, 1998). This model of receptor behaviour was further supported by evidence for inverse agonist activity in $M_2$ and $M_4$ muscarinic receptor expressing cell lines, where atropine-mediated reductions in basal [$^{35}$S]-GTPγS binding were observed. [$^{35}$S]-GTPγS binding can provide a good indicator of this phenomenon at the level of guanine nucleotide exchange as this event is pivotal in the extended ternary complex model (Milligan et al., 1995). In this study, 10-20 % of control [$^{35}$S]-GTPγS binding was inhibited by a maximal concentration of atropine, and full concentration-effect analyses revealed similar IC$_{50}$ values closely correlated to the reported affinity constant for atropine (Caulfield, 1993). The finding that atropine can act as an inverse agonist at muscarinic receptors is in agreement with previously reported data by Hilf and Jakobs (1992b) where atropine-inhibition of [$^{35}$S]-GTPγS binding in cardiac membranes was observed, with atropine mediating a decrease of around 20 %. Also other groups have reported the inverse agonist activity of atropine (Jakubik et al.
1995, 1996; Burstein et al., 1995, 1997), and this evidence taken together illustrates that atropine can potentially act as an inverse agonist at muscarinic receptors.

From this study it can be seen that human muscarinic acetylcholine receptor subtypes expressed in Chinese hamster ovary cells display differing G protein activation profiles, and that activation of distinct G protein subtypes by different muscarinic acetylcholine receptors can occur at different rates, different magnitudes and at different potencies.

The discussion above, together with all the results of this study and those discussed herein, suggest that stimulation of a particular muscarinic receptor subtype may elicit a variety of cellular responses depending on the environment in which a receptor is expressed, together with the levels and types of cellular signalling molecules present. The receptor density, for example, may influence receptor-G protein coupling events which in turn will affect the potency and maximal responsiveness of the agonist. Also, the presence and expression levels of particular Gα proteins may also influence the pattern of receptor-G protein coupling and hence the final cellular response(s) observed and the magnitude of such a response. In turn, selective activation of G protein α-subunits by agonists may also affect the final effector system activated and the level of the respective cellular response. The present study, therefore, provides some evidence for potentially diverse physiological responses to pharmacological agents acting on muscarinic receptors, and potentially other G-protein coupled receptors, depending on the cells/tissues utilised and the therapeutic agent employed. Hence, in any evaluation of potential therapeutic targets detailed knowledge of the signal transduction machinery is required.
Appendices.
Appendix one.

**ABBREVIATIONS LIST**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
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<tbody>
<tr>
<td>AC</td>
<td>Adenylyl Cyclase</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine 5'-diphosphate</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine 5'-triphosphate</td>
</tr>
<tr>
<td>B_{max}</td>
<td>Maximal specific binding</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>cAMP</td>
<td>Adenosine 3',5'-cyclic monophosphate</td>
</tr>
<tr>
<td>CHO-cells</td>
<td>Chinese hamster ovary cells</td>
</tr>
<tr>
<td>CLB</td>
<td>Cytosol-like buffer</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CPM</td>
<td>counts per minute</td>
</tr>
<tr>
<td>DAG</td>
<td>sn-1,2-diacylglycerol</td>
</tr>
<tr>
<td>DPM</td>
<td>disintegrations per minute</td>
</tr>
<tr>
<td>EC_{50}</td>
<td>The agonist concentration which produces 50% of the maximal response</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetra-acetic acid</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>EGTA</td>
<td>Ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid</td>
</tr>
<tr>
<td>GAP</td>
<td>GTPase-activating protein</td>
</tr>
<tr>
<td>GDP</td>
<td>Guanosine-5'-diphosphate</td>
</tr>
<tr>
<td>G protein</td>
<td>Guanine nucleotide binding protein</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine-5'-triphosphate</td>
</tr>
<tr>
<td>GTP\gamma S</td>
<td>Guanosine-5'-O-(thiotriphosphate)</td>
</tr>
<tr>
<td>IC_{50}</td>
<td>The concentration of a competing ligand which displaces 50% of the specific radiolabel</td>
</tr>
<tr>
<td>Ins(1,4,5)P_3</td>
<td>D-my o-Inositol 1,4,5-trisphosphate</td>
</tr>
<tr>
<td>K_d</td>
<td>Equilibrium dissociation constant</td>
</tr>
<tr>
<td>K_i</td>
<td>Inhibition constant</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>--------------------------------------------</td>
</tr>
<tr>
<td>mAChR</td>
<td>Muscarinic acetylcholine receptor</td>
</tr>
<tr>
<td>NAD⁺</td>
<td>β-nicotinamide-adenine nucleotide</td>
</tr>
<tr>
<td>NGF</td>
<td>Nerve growth factor</td>
</tr>
<tr>
<td>nH</td>
<td>Hill coefficient</td>
</tr>
<tr>
<td>NMS</td>
<td>N-methyl scopolamine</td>
</tr>
<tr>
<td>NSB</td>
<td>Non-specific binding</td>
</tr>
<tr>
<td>PDE</td>
<td>Phosphodiesterase</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet derived growth factor</td>
</tr>
<tr>
<td>PI</td>
<td>Phosphatidylinositol</td>
</tr>
<tr>
<td>PIP</td>
<td>Phosphatidylinositol 4-phosphate</td>
</tr>
<tr>
<td>PIP₂</td>
<td>Phosphatidylinositol 4,5-bisphosphate</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase A</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PLA₂</td>
<td>Phospholipase A₂</td>
</tr>
<tr>
<td>PLC</td>
<td>Phospholipase C</td>
</tr>
<tr>
<td>PLD</td>
<td>Phospholipase D</td>
</tr>
<tr>
<td>PTx</td>
<td>Pertussis Toxin</td>
</tr>
<tr>
<td>TCA</td>
<td>Trichloroacetic acid</td>
</tr>
<tr>
<td>TM</td>
<td>Transmembrane</td>
</tr>
</tbody>
</table>
Appendix two.

MATERIALS

List of reagents and suppliers.

From Sigma Chemical Company Limited, Poole, Dorset, England.

Adenosine 3'-5'-cyclic monophosphate (cAMP)
Adenosine-5'-triphosphate (ATP)
Arecoline hydrobromide (C₈H₁₃NO₂.HBr)
Atropine sulphate
Bordatella pertussis toxin (PTx)
Bovine serum albumin (BSA)
β-Escin
D-myo-inositol 1,4,5-trisphosphate (Ins(1,4,5)P₃)
Dithiothreitol (DTT)
Ethylenediaminetetra-acetic acid (EDTA) ([CH₂N(CH₂.COOH)₂]₂)
Ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA)
Forskolin (FSK)
Guanosine-5'-diphosphate (GDP)
Guanosine-5'-O-(thiotriphosphate) tetralithium salt (GTPyS)
HEPES (C₈H₁₈N₂O₄S)
Methacholine chloride (C₅H₁₈ClNO₂)
Pilocarpine dihydrochloride (C₁₁H₁₄N₂O₂.HCl)
Pirenzepine dihydrochloride (C₁₉H₂₁N₃O₂)
1,1,2-Trichlorotrifluoroethane (Freon)
Tri-n-octylamine
Tropicamide (C₁₇H₂₀N₂O₂)


Cupric sulphate (CuSO₄.5H₂O)
D-Glucose (C₆H₁₂O₆)
Magnesium chloride hexahydrate (MgCl₂.6H₂O)
Hydrochloric acid (HCl)
Potassium chloride (KCl)
Potassium dihydrogen orthophosphate (KH₂PO₄)
Potassium hydroxide (KOH)
Potassium sodium tartrate (KNaC₄H₄O₆.4H₂O)
Sodium chloride (NaCl)
Sodium carbonate (Na₂CO₃)
Sodium dodecyl sulphate C₁₂H₂₅O₄S.Na
Sodium hydrogen carbonate (NaHCO₃)
Sodium hydroxide (NaOH)
Sodium succinate hexahydrate ((NaH₂COONa)₂.6H₂O)
Trichloroacetic acid (TCA) (CCl₃.COOH)
Tris-(hydroxymethyl)-methylamine (NH₂.C(CH₂OH)₃)

From Calbiochem Novchem Ltd, Nottingham, England.

Fura-2
Anti G₁₁₂α (Rabbit Polyclonal Catalogue Number 371723)
Anti G₁₃₀α (Rabbit Polyclonal Catalogue Number 371726)
Anti G₉₁₁α (Rabbit Polyclonal Catalogue Number 371751)

From GIBCO. BRL, Paisley, Scotland.

Alpha MEM medium
New born calf serum
Fungizone
Penicillin
Streptomycin

[^35]S]-Guanosine-5’-O-(3-thiotriphosphate) ([^35]S]-GTP\gamma S)
AS/7 Anti G\textsubscript{i,2}α (Rabbit antisera Catalogue Number NEI-801)
EC/2 Anti G\textsubscript{i,3,0}α (Rabbit antisera Catalogue Number NEI-803)

From Santa Cruz Biotechnology Inc, Santa Cruz, California, USA.

Anti G\textsubscript{i,3}α (Rabbit Polyclonal IgG Catalogue Number sc-262)
Anti G\textsubscript{i,α} (Rabbit Polyclonal IgG Catalogue Number sc-387)
Anti G\textsubscript{q,11}α (Rabbit Polyclonal IgG Catalogue Number sc-392)
Anti G\textsubscript{i,α} (Rabbit Polyclonal IgG Catalogue Number sc-823)

From Amersham International PLC, Aylesbury, Bucks, England.

[^45]CaCl\textsubscript{2}
D-myo-[^3]H inositol-1,4,5-triphosphate ([^3]H]Ins(1,4,5)P\textsubscript{3})

From B.D.H. Limited, Poole, Dorset, England.

Folin-Ciocalteu’s phenol reagent

From Bio-Rad, Laboratories, Hercules, California, U.S.A.

Kaliedoscope Prestained Markers

From Pharmacia Biotech, St Albans, Herts, England.

Protein A sepharose (CL-4B)
From Research Biochemicals International, Poole, Dorset.

Methoctramine tetrahydrochloride (\((\text{C}_{18}\text{H}_{31}\text{N}_2\text{O})_2\cdot 4\text{HCl}\))
Appendix three.

PREPARATION OF BUFFERS

Membrane binding buffer.

The membrane binding buffer was utilised for both \([^{35}S]-GTP\gamma S\) binding and \([^3H]-NMS\) binding in cellular membranes and consisted of the following ingredients:

<table>
<thead>
<tr>
<th>Chemical name</th>
<th>Chemical formula</th>
<th>Concentration (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEPES (free acid)</td>
<td>(C_8H_{18}N_2O_4S)</td>
<td>10</td>
</tr>
<tr>
<td>Magnesium chloride hexahydrate</td>
<td>(MgCl_2)</td>
<td>5</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>(NaCl)</td>
<td>100</td>
</tr>
</tbody>
</table>

When all the salts were dissolved the pH was adjusted to 7.4 at room temperature with 5M NaOH.

Cytosol-like buffer (CLB).

CLB was prepared as a five times stock and stored in plastic (not glass) to avoid contamination by calcium. 10 ml of the 5 x stock was diluted down by the addition of 40 ml of distilled water to give rise to the actual salt concentration. The final calcium concentration in the 1 x stock was buffered using EGTA (10 mM) to a final concentration of 100-200 nM. The process for calcium calibration is as follows. A 2 ml sample of EGTA-buffered 1 x stock was placed in a fluorimeter cuvette with 2 μl of fura-2 free acid (100 μM). The fluorescent intensity of the buffer was determined at
excitation and emission wavelengths of 340 nm and 480 nm respectively. The addition of 2 µl of CaCl₂ solution (1 M) allowed a value for Fₘₐₓ to be obtained and Fₘᵢₙ was defined by the addition of 50 µl of EGTA (200 mM/400 mM KOH). Free [Ca²⁺] in the EGTA-buffered CLB was determined using the equation below, where Kₐ = 139 nM at room temperature and represents the dissociation constant for Ca²⁺ binding to fura-2:

\[
\text{Free [Ca}^{2+}\text{]} \text{nM} = \left( F - F_{\text{min}} / F_{\text{max}} - F \right) \times K_a
\]

Free calcium levels were monitored carefully and always kept within the 100-200 nM range.

The ingredients for CLB are as follows:

<table>
<thead>
<tr>
<th>Chemical name</th>
<th>Chemical formula</th>
<th>Concentration (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEPES (free acid)</td>
<td>C₅H₈N₂O₄S</td>
<td>20</td>
</tr>
<tr>
<td>Magnesium chloride hexahydrate</td>
<td>MgCl₂.6H₂O</td>
<td>2.4</td>
</tr>
<tr>
<td>Potassium chloride</td>
<td>KCl</td>
<td>120</td>
</tr>
<tr>
<td>Potassium dihydrogen orthophosphate</td>
<td>KH₂PO₄</td>
<td>2</td>
</tr>
<tr>
<td>Sodium succinate hexahydrate</td>
<td>(NaH₂COONa)₂.6H₂O₅</td>
<td></td>
</tr>
</tbody>
</table>

ATP was added just prior to use due to the relative instability of ATP under neutral pH conditions. 2 mM ATP (final concentration) was added giving rise to a ATP/Mg²⁺ ratio of 6:5. This ratio helps shift the equilibrium of two forms of ATP (ATP₅⁻ and
ATP$^+$ in the direction of ATP$^2$. This form is the active one in terms of driving the Ca$^{2+}$-ATPase and allowing the uptake of calcium.

The pH of the solution was adjusted to pH 7.2 using KOH (20% w/v).

**Krebs-Henseleit buffer (KHB)**

This buffer has a salt composition as detailed below:

<table>
<thead>
<tr>
<th>Chemical name</th>
<th>Chemical formula</th>
<th>Concentration (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium chloride dihydrate</td>
<td>CaCl$_2$·2H$_2$O</td>
<td>1.3</td>
</tr>
<tr>
<td>HEPES (free acid)</td>
<td>C$<em>8$H$</em>{18}$N$_2$O$_4$S</td>
<td>10</td>
</tr>
<tr>
<td>D-Glucose</td>
<td>C$<em>6$H$</em>{12}$O$_6$</td>
<td>11.7</td>
</tr>
<tr>
<td>Magnesium sulphate hexahydrate</td>
<td>MgSO$_4$·6H$_2$O</td>
<td>1.2</td>
</tr>
<tr>
<td>Potassium chloride</td>
<td>KCl</td>
<td>4.7</td>
</tr>
<tr>
<td>Potassium dihydrogen orthophosphate</td>
<td>KH$_2$PO$_4$</td>
<td>1.2</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>NaCl</td>
<td>118.6</td>
</tr>
<tr>
<td>Sodium hydrogen carbonate</td>
<td>NaHCO$_3$</td>
<td>4.2</td>
</tr>
</tbody>
</table>

The final pH was adjusted to 7.4 using 5M NaOH.
**Immunoprecipitation solubilisation buffer.**

The solubilisation buffer consisted of the following ingredients:

<table>
<thead>
<tr>
<th>Chemical name</th>
<th>Chemical formula</th>
<th>Concentration (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDTA</td>
<td>([\text{CH}_2\text{N(CH}_2\text{COOH)}_2]_2)</td>
<td>1</td>
</tr>
<tr>
<td>Igepal CA 630</td>
<td>Variable</td>
<td>1.25 % (v/v)</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>NaCl</td>
<td>200</td>
</tr>
<tr>
<td>Sodium dodecyl sulphate</td>
<td>(\text{C}<em>{12}\text{H}</em>{25}\text{O}_4\text{S.Na})</td>
<td>0.2 % (w/v)</td>
</tr>
<tr>
<td>Tris</td>
<td>(\text{NH}_2\text{C(CH}_2\text{OH)}_3)</td>
<td>100</td>
</tr>
</tbody>
</table>

This buffer is utilised both with and without SDS. Once all the constituents are dissolved the buffer is stored at 4°C until it is cold, then the pH is adjusted with 1 M HCl to 7.4.
Appendix Four.

Effect of 'snap freezing' on CHO-M₁-M₄ cell membranes.

Effect of freezing CHO-M₁-M₄ cell membranes upon 1 mM methacholine stimulated [³⁵S]-GTPγS binding.

A. CHO-M₁

![Graph A]

B. CHO-M₂

![Graph B]
C. CHO-SLM₂

D. CHO-M₃
Stimulation of $[^{35}S]$-GTPγS binding to fresh and frozen CHO-cell membranes containing Hm1-Hm4 receptors by methacholine. The data are expressed as c.p.m bound for an individual experiment representative of 3 separate experiments. CHO-cell membranes which had been prepared just prior to experimentation were either used fresh or after freezing in liquid nitrogen and thawed for comparison. These were incubated in the presence and absence of 1 mM methacholine for the time indicated at 30°C. The assay buffer also included 10 μM GDP and 70 pM $[^{35}S]$-GTPγS.

These figures show that the 'snap freezing' process, described in the Methods section entitled 'Cell Membrane Preparation' has no effect upon methacholine-stimulated $[^{35}S]$-GTPγS binding.
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