Muscarinic acetylcholine receptor signalling in model cells and smooth muscle.

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This Thesis is dedicated to my late grandfather, Doug.

Abstract

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Muscarinic acetylcholine receptor signalling in model cells and smooth muscle

In this Thesis, experiments are described in which the signalling of M_2- and M_3-mACh receptors as homogeneous receptor populations and as a co-expressed population in mammalian cell-lines have been studied. These experiments set out to investigate the hypothesis that M_2-mACh receptors can influence signalling by M_3-mACh receptors via 'cross-talk' at the level of second messenger signalling. This may have particular significance with respect to mACh receptors in smooth muscle. In many smooth muscle tissues mACh receptor subtypes are co-expressed, with the major population often being M_2-mACh receptors and the minor population being M_3-mACh receptors. It is the M_3-mACh receptors which have been implicated in the direct mediation of contraction via activation of phosphoinositide metabolism. Whilst it is known that M_2-mACh receptors signal via inhibition of adenylyl cyclase, it is not known whether M_2-mACh receptors can modulate signalling via M_3-mACh receptors to influence smooth muscle tone. By studying 'cross-talk' of these two receptors in a model cell system, this possibility can be evaluated.

Chinese hamster ovary (CHO) cells expressing M_2-, M_3- or co-expressing M_2- and M_3-mACh receptors were characterised with respect to agonist-stimulation of inhibition of forskolin-stimulated cyclic AMP levels, and agonist-stimulation of inositol 1,4,5-trisphosphate ($InsP_3$; time-course and concentration-dependence). These two responses have been shown to be representative of M_2- and M_3-mACh receptor-mediated responses, respectively. Whilst inhibition of cAMP production in CHO-SLM2 cells was pertussis toxin-sensitive and was blocked by tripitramine (30 nM), neither stimulation of cAMP, nor $InsP_3$ production in CHO-M3 cells was sensitive to either pretreatment.

In CHO-M_2/M_3 cells, the second phase of agonist-stimulated $InsP_3$ production was enhanced above that seen in CHO-M3 cells. This represented a 'cross-talk' event between M_2- and M_3-mACh receptors, as it was sentitive to both pertussis toxin and tripitramine at a M_2-mACh receptor-selective concentration. This enhancement may be due to $\beta\gamma$-subunits of M_2-mACh receptor-coupled G_i G-proteins, increasing the activation of PLC stimulated by M_3-mACh receptor activation. Alternatively, it may be due to M_2-mACh receptors increasing Ca^{2+} influx across the plasma membrane via activation of non-selective cation channels.

Muscarinic acetylcholine receptor-activation of contraction was studied in guinea-pig isolated uterus preparations. It has been previously reported that this tissue lacks M_3-mACh receptor expression, and therefore it was thought that this tissue may offer an insight into possible mechanisms of activation of contraction by M_2-mACh receptors. An atypical antagonist affinity profile was however recorded in this tissue, using the most M_2/M_3-mACh receptor-selective antagonists currently available. This affinity profile was not altered by selective alkylation/protection procedures, ruling out the synergistic activation of M_2- and M_3-mACh receptors. However, competition radioligand binding studies in uterus membranes implicated the presence of a homogeneous population of M_2-mACh receptors. Therefore, contraction is mediated by an atypical mACh receptor-subtype, or is mediated via M_2-mACh receptors coupling via phosphoinositide hydrolysis to cause contraction directly.

These studies indicate that cross-talk can occur between the signalling pathways of M_2- and M_3-mACh receptors in model cells. Further investigations to establish the mechanism of this cross-talk should include studies of signalling in a smooth muscle tissue which co-expresses these mACh receptors, to establish whether similar cross-talk occurs in a more physiological context.

Abbreviations

ARF	ADP ribosylation factor
CHO cells	Chinese Hamster ovary cells
CMP-PA	CMP-phosphatidic acid
cAMP	cyclic AMP
4-DAMP	4-diphenyl-acetoxy-N-methylpiperidine methiodide
DAG	diacylglycerol
ERK	extracellular signal regulated kinase
IP_3	inositol 1,4,5 trisphosphate
MAPK	mitogen activated protein kinase
mACh receptors	muscarinic acetylcholine receptors
p-F-HHSiD	para- fluorohexahydrasiladiphenidol
PTx	pertussis toxin
PBZ	phenoxybenzamine
PC-PLC	phosphatidylcholine-preferring PLC
PIP_2	phosphatidylinositol 4,5-bisphosphate
PI	phosphoinositides
PLA_2	phospholipases A_2
PLC	phospholipases C
PLD	phospholipases D
PKC	protein kinase C

Contents

Chapter 1 - Introduction

In this Thesis, the signalling of mACh receptors in a model cell system and smooth muscle has been investigated. In this Chapter the aspects of the field of mACh receptor pharmacology which led to the conception of this study and to the design of the subsequent experiments are introduced. The putative role of the M_2-mACh receptor in smooth muscle contraction is an area of heated debate (which is discussed in Section 1.3). Whilst being widely co-expressed with M_3-mACh receptors in many smooth muscle tissues of most mammalian species, M_2-mACh receptors have only been shown to have a direct role in contraction under specific experimental conditions and only in certain tissues. This Thesis sets out to ascertain whether there is an underlying modulatory role in the regulation of smooth muscle tone for M_2-mACh receptors, which involves cross-talk between the signalling pathways of M_2- and M_3-mACh receptors, and not solely between M_2-mACh and β_2-adrenoceptors, as has been previously suggested. An essentially reductionist approach has been used and much of what is discussed in this Thesis arises from studies in a model cell-line expressing M_2- and M_3-mACh receptors. This is in order that the signalling can be studied in detail in a simple, but mammalian, cell context, to evaluate the validity of this hypothesis, before studies in smooth muscle cells themselves can be designed. The Introduction to this work begins with a summary of the current and historical classification of mACh receptors, and continues with a description of the signalling pathways currently ascribed to each subtype. The relevance of this study of mACh receptor-signalling to smooth muscle pharmacology, physiology and pathology is then discussed, concluding with an overview of current work in the area of G_q/G_i-receptor-linked cross-talk.

1.1 Classification of Muscarinic Acetylcholine receptors

1.1.1 mACh receptor subtypes

Muscarinic acetylcholine receptors belong to a large superfamily of G-protein-coupled receptors, which are characterised by seven putative hydrophobic transmembrane helical domains joined by alternating intracellular and extracellular loops, an extracellular amino-terminal and a cytoplasmic carboxyterminal (Wess, 1993). The evidence that there was more than one mACh receptor subtype came in 1980 (Hammer *et al.*, 1980), long after the discovery of muscarine and atropine as naturally occurring compounds selective for muscarinic over nicotinic acetylcholine receptors, and it was only in the late 1980s that molecular biology and pharmacology were used definitively to demonstrate the existence of multiple subtypes of mACh receptor. By cloning techniques, five mACh receptor subtypes (m1-m5) have been characterised (Bonner *et al.*, 1987; Bonner *et al.*, 1988; Kubo *et al.*, 1986; Kubo *et al.*, 1986; Peralta *et al.*, 1987; Peralta *et al.*, 1987). Also, M_1-M_4 mACh receptors have been pharmacologically characterised, and three of these (M_1-M_3) in human tissues (Birdsall *et al.*, 1989). Generally, the cloned receptor subtypes m1-m4 correlate with pharmacologically defined M_1-M_4 mACh receptors, although some discrepancies exist. The five mammalian mACh receptors cloned display a large degree of sequence homology, sharing about 145 invariant amino acids. All have a large intracellular 3rd cytoplasmic loop, which has little sequence identity between subtypes, and is thought to be involved in G-protein binding (Wess, 1993). See Figure 1.1A for primary amino acid structures of M_2- and M_3-mACh receptors, including areas of homology. The 3rd intracellular loops are omitted for clarity as there is almost no homology in this region.

Due to the lack of receptor subtype-specific ligands, the pharmacological classification of mACh receptors relies on the use of receptor selectivity profiles i.e. rank orders of affinities, using as extensive a range of ligands as is possible. Measurements of antagonist equilibrium constants (by radioligand binding analysis) or apparent dissociation constants (by Schild

Figure 1.1A – Amino acid sequence of m3 and m2 mACh receptors

m2- and m3-mACh receptor sequences are deduced from the cloned nucleotide sequence of the porcine myocardial mACh receptor (Peralta *et al.*, 1987) and the rat M3 mACh recptor (Bonner, 1989) respectively. Numbers at front and end of sequences indicate amino acid positions. Transmembrane domains (I-VII) are indicated by underline. Homology is indicated in red. The total lengths of the m2 and m3 amino tails are 21 and 66 amino acids, and the third intracellular loop 182 and 240 amino acids, respectively.

1 (NH_2)MTT ... LGGHTIWQVVFIAFLTGFFLALVTIIGNILVIVAFKVNKQLKTVNNYFLFSLAC
1 (NH_2)MNN ... TSPYKTFQVVFIVLAVAGSLSLVTIIGNILVMVSILVNRHLQTVNNYFLFSLAC

114 ADLIIGVISMNLFTTYIIMNRWALGNLACDLWLSIDYVASNASVMNLLVISFDRYFSITRPLYYR
68 ADLIIGVFSMNLYTLYTVIGYTPLGPVVCDLWLALDYVVSNASVMNLLIISFDRYFCVTKPLTYP

180 AKRTTKRAGVMIIGLAWVISFVLWAPAILFWQYFVGKRTVPPGECFIQFLSEPTITFGTAIAAF
134 VKRTTKMAGMMIIAAAWVLSFILLWAPAILFWQFIVGVRTVQDGQCYIQFFSNAAVTFGTAIAAF

242 YMPVTIMTILYWRIYKETEKRTK...SLIKEKKAAQTLSAILLAFILTWTPYNIMVLVNTFCDSC
199 YLPVIIMTVLYWHISRASKSRIK... PPSNQLLVTRTILAILLAFIITWAPYNVMVLINTPCAPC

519 IPKTYWNLGYWLCYINSTVNPVCYALCNKTFRTTFKTLLLCQCDKRKRRKQQYQQRQSVIF
417 IPNTVWTIGYWLCYINSTINPACYALCNATFKKTFKHLLMCHYKNIGATR(COOH) 466

581 HKRVPEQAL(COOH) 589

analysis) are routinely used to distinguish subtypes of mACh receptor, with the caveat that some mACh receptor agonists (including carbachol) also stimulate nicotinic ACh receptors. Agonists are not generally useful for this purpose, as they do not differ in terms of affinity by more than one order of magnitude between subtypes. It should be noted that if large numbers of antagonists that do not discriminate well between receptor subtypes are used to determine whether the receptor subtypes in two tissues are different for example, correlations may be good regardless of whether the receptors are in fact distinct from each other. Therefore, it is antagonist affinities that do not fit this line of correlation that should be noted. In the very first proposal of heterogeneity, the observation that the affinity for pirenzepine at different sites did not fit on the regression line comparing cerebral cortex affinity with apparent affinity at receptors mediating contraction of guinea pig ileum resulted in the definition of M_1- and M_2-mACh receptor subtypes (Hammer *et al.*, 1980). However, due to the fact that many tissues co-express more than one mACh receptor subtype, and the lack of subtype selective ligands, atypical affinity profiles may not necessarily indicate a distinct subtype and care should be taken in the interpretation of such data. The expression of cDNA for each cloned receptor subtype in mammalian cells has enabled the study of ligand affinities at pure and homogeneous populations of receptors. Reassuringly, these affinity estimates in cloned cell-lines agree in most cases with functional and radioligand data at endogenously expressed receptors (see Table 1.1).

There are certain trends in mACh receptor ligand affinity profiles which can be generally anticipated, in terms of each receptor subtype (see Caulfield, 1993). M_1-mACh receptors are characterised by a high affinity for pirenzepine and 4-DAMP (4-diphenyl-acetoxy-N-methylpiperidine methiodide), intermediate affinity of *p*-F-HHSiD (*para*-fluoro-hexahydrosiladiphenidol) and low affinity for methoctramine, tripitramine and himbacine. M_2-mACh receptors exhibit high affinity for methoctramine, tripitramine and himbacine, but low affinity for pirenzipine, *p*-F-HHSiD, and 4-DAMP. M_3-mACh receptors are characterised by high affinity for 4-DAMP and *p*-F-HHSiD, but low for pirenzipine, methoctramine, tripitramine and himbacine. Zamifenacin and darifenacin are selective for

M_3-mACh receptors over other receptor subtypes, but also show some selectivity for M_3-mACh receptors (as they are currently defined) expressed in gut, over other those expressed in other tissues. M_4-mACh receptors are more difficult to define. Pirenzepine has a relatively high affinty for M_4-mACh receptors, and whilst most compounds show a similar affinity for M_2- as M_4-mACh receptors, tripitramine can discriminate to some degree, with its affinity for M_4-mACh receptors being approximately one magnitude lower than that for M_2-mACh receptors. MTx-3, a toxin derived from the green mamba (*Dendroaspis augusticeps)* venom has been reported to be selective for M_4-mACh receptors (Jolkkonen *et al.*, 1994), however functional data are limited for this compound (Olianas *et al.*, 1996), partly due to the paucity of robust and accepted bioassays exhibiting M_4-mACh receptor pharmacology, apart from recombinant systems. The m5 mACh receptor gene product has yet to be assigned a functional correlate as no ligands bind preferentially to this receptor, and it has been defined purely on structural and cloning data.

The use of subtype-selective antibodies may be useful when employed alongside classical pharmacology to further characterise mACh receptor subtype expression (Levey *et al.*, 1991; Wall *et al.*, 1991; Yasuda *et al.*, 1993). Another method of characterisation, demonstrated by Waelbroeck and colleagues, utilises the differences in dissociation rates of [^{3}H]-NMS from M_1-M_4 receptors to discriminate between subtypes of mACh receptor (Waelbroeck *et al.*, 1992; Waelbroeck *et al.*, 1990), but it is not clear if or how these rates are affected by coupling of the receptors to specific G-proteins. It is also known that some antagonists may act allosterically with another site on mACh receptors (e.g. gallamine and methoctramine at M_2-mACh receptors) which may act to decrease the dissociation rate for competing radioligands, and hence interfere with results gained in this way (Caulfield, 1993).

It has been shown that mACh receptors can be subject to post-translational modification e.g. phosphorylation and glycosylation, and that different cells have different complements of effector moieties, and therefore differences may occur in receptors and their signalling between those expressed in native cells and cloned cell lines (Caulfield, 1993). So, although

the use of cell lines expressing a homogeneous population of cloned receptors is a useful pharmacological tool, care should be taken when comparing the receptor-G-protein coupling for example, in cloned cell-lines to that in native cells.

Table 1.1 - Operational characterisation of mACh receptors using selected antagonists
The values are apparent affinites defined functionally and are expressed as pK_B. Values in parentheses are K_Ds determined by radioligand binding studies at cloned human receptors in cell-lines. Data from Caulfield, (1993); Eglen & Watson, (1996). n.d. = not determined

Receptor gene:	m1	m2	m3	m4	m5
Receptor subtype:	M_1	M_2	M_3	M_4	
Pirenzepine	8.3 (8.2)	6.8 (6.7)	6.9 (6.9)	7.7 (7.4)	(7.1)
Methoctramine	6.5 (7.3)	7.9 (7.9)	6.0 (6.7)	7.6 (7.5)	(7.2)
4-DAMP	8.6 (9.2)	7.8 (8.4)	9.1 (9.3)	n.d. (8.9)	(9.0)
Himbacine	7.2 (7.0)	8.5 (8.0)	7.6 (7.0)	8.8 (8.0)	(6.3)
p-F-HHSiD	7.2 (7.7)	6.0 (6.9)	7.9 (7.8)	n.d. (7.5)	(7.0)
Tripitramine	n.d. (8.8)	9.7 (9.6)	6.5 (7.4)	n.d. (7.9)	n.d.

1.1.2 G-protein coupling

G-protein coupled receptors are a large superfamily of proteins that allow transduction of a signal across the membrane. The receptors bind a ligand at the external surface of the cell and activate a G-protein which then alters the ability of an effector within the cell to initiate a signal cascade, or directly changes the gating of an ionic current. On agonist binding, the receptor catalyses exchange of GDP for GTP on the G-protein α subunit, which activates the heterotrimer and causes its dissociation both from the receptor, and into G_α and $\beta\gamma$ subunits. Both G_α-GTP and $\beta\gamma$ can activate or inhibit effectors such as adenylyl cyclases, phospholipases and ion channels, either independently or synergistically. The activity of G-

proteins is brought to an end by the GTPase activity of the G_α subunit itself, a process facilitated by some effectors (Berstein *et al.*, 1992) and a growing family of RGS (regulators of G-protein signalling) proteins (Berman & Gilman, 1998), returning the G_α subunit to its inactive form, and causing reassociation with $\beta\gamma$ subunits to be facilitated.

Given that there are hundreds of different receptors linked to G-proteins, over 1000 possible combinations of G-protein $\alpha\beta\gamma$-heterotrimers known thus far, and an unknown number of effectors, it has not yet been established how specific these interactions must be to ensure correct transmission of a signal through a receptor to result in a particular physiological effect (Birnbaumer, 1992; Rahmatullah *et al.*, 1995). It is possible by intranuclear injection of antisense to particular β and γ subunits to inhibit synthesis of specific subunits, and it has been shown in this way that the M_2-mACh receptor can signal through $\alpha_{o1}\beta_3\gamma_4$ and not other $\alpha_{o1}\beta_2$- or $\alpha_{o1}\beta_3$-containing forms. Since a receptor -G-protein interaction may be specific to one of many possible trimeric combinations and therefore the $\beta\gamma$ subunits generated upon receptor activation may be a small subset of the total and possibly may even be unique, this could have profound influence on the specificity of the signalling of a receptor. Although, for instance, it may be possible that different $\beta\gamma$ subunits are capable of activating effectors to a different extent, $\beta\gamma$ subunits have been referred to in general in this Thesis, without reference to specific G-protein combinations. The discussions later in this Thesis therefore should be considered with reference to the possible heterogeneity of $\beta\gamma$ responses.

Using cloned receptors stably expressed in cell-lines and the use of pertussis toxin to determine G-protein selectivity, it has been agreed that m2-and m4-mACh receptors preferentially couple to G_i G-proteins in a pertussis toxin-sensitive manner to cause inhibition of adenylyl cyclase (Buckley, 1990; Burford *et al.*, 1995; Dell'Acqua *et al.*, 1993) and that m1, m3 and m5-mACh receptors couple preferentially to pertussis toxin-insensitive G_q G-proteins to activate phosphoinositide hydrolysis (Caulfield, 1993; Lambert *et al.*, 1992). Studies using chimaeric receptors demonstrated that interchanging the 3rd intracellular loops of m2 and m3 mACh receptors allowed coupling of the m3 mACh

receptors to inhibition of adenylyl cyclase and coupling of m2 mACh receptors to PLC activity (Wess *et al.*, 1989), implicating this region as a major determinant of receptor-G-protein coupling.

However, the specificity of mACh receptor functional coupling is dependent both on the expression levels of the receptors and the cell-type in which they are expressed (Ashkenazi *et al.*, 1987; Burford *et al.*, 1995; Milligan, 1993; Richards, 1991). It has been shown that cloned m2- and m4-mACh receptors may also cause stimulation of the phosphoinositide cycle via a pertussis toxin-sensitive G-protein, although with lower efficiency than the m1-, m3- and m5-mACh receptor subtypes (Ashkenazi *et al.*, 1987). This may be by stimulation of PLC-β2 or β3 by βγ subunits derived from pertussis toxin-sensitive G-proteins (Carozzi *et al.*, 1993; Katz *et al.*, 1992). Signals transduced via the less abundant $G_{q/11}$ family of G-proteins have been shown to be able to activate all isoforms of PLC-β, but preferentially activate PLC-β1 (Smrcka *et al.*, 1991). Due to the low potency of the m2-mACh receptor-mediated response, it is possible that these PLC enzymes may only be activated by locally high concentrations of βγ subunits (Berstein *et al.*, 1992). Also, m3-mACh receptors have been shown to stimulate adenylyl cyclase at high agonist concentrations in a clonal cell-line (Burford *et al.*, 1995). Taken together, this evidence suggests that mACh receptor-G-protein interactions may not be exclusive, and each receptor may interact with more that one G-protein in a given environment, or that each receptor subtype could be further subdivided according to G-protein binding. The idea that each receptor may bind to more than one G-protein depending on a given stimulus or G-protein complement seems the most likely explanation.

Additionally, mACh receptors can also activate phospholipase A_2, phospholipase D, tyrosine kinase activity, Ca^{2+} influx, activation of K^+-channels and non-selective cation channels and other transduction pathways (Felder, 1995), presumably, but not necessarily in all cases, via G-proteins, and this is dependent not only on the receptor-G-protein coupling but also the effector complement of the cell in which the receptors are expressed.

1.2 mACh receptor signalling pathways

Although M_2-mACh receptor activation may modulate M_3-mACh receptor-mediated smooth muscle contraction by sensitising the contractile machinery downstream of any signalling events, e.g. by increasing myosin light chain kinase activity, other modulation could occur at the level of second messenger signalling of the two receptor subtypes (i.e. cross-talk of M_2-mACh receptor signalling with that of M_3-mACh receptors may occur). This Thesis concentrates on the interactions between the signalling pathways of M_2- and M_3-mACh receptor subtypes when co-expressed in a model cell system, and therefore the signalling pathways usually ascribed to these receptors are described below.

1.2.1 Receptor-mediated phosphoinositide hydrolysis

Introduction

Historically, the first experiments observing agonist stimulation of inositol phospholipid hydrolysis were performed by Hokin & Hokin, (1953) who showed that acetylcholine (ACh) stimulated the turnover of phosphoinositides in pancreas. Experiments using rabbit iris, showed that ACh could increase the breakdown of [^{32}P]-labelled phosphatidylinositol 4,5-bisphosphate (PIP_2) in smooth muscle tissue (Abdel-Latif *et al.*, 1977). In 1983, Berridge and colleagues reported that inositol 1,4,5-trisphosphate ($InsP_3$) had a second messenger function (Berridge, 1983; Streb *et al.*, 1983) and later hypothesised that $InsP_3$ was the 'missing link' between plasma membrane receptors and the mobilisation of Ca^{2+} from intracellular stores (Berridge & Irvine, 1984; Berridge & Irvine, 1989). In response to membrane stimuli (neurotransmitters, hormones and growth factors) both $InsP_3$ and diacylglycerol (DAG) are formed by the hydrolysis of PIP_2 by the activation of phosphoinositide-specific phospholipases C (PLC). DAG also performs signalling functions (Nishizuka, 1988).

Phosphoinositide synthesis and inositol (poly)phosphate metabolism

Phosphoinositides are minor components of plasma membranes of eukaryotic cells and constitute less than 3-5% of the total phospholipids. PtdIns is synthesised in the endoplasmic reticulum or plasma membrane by the recycling of CMP-PA and *myo*-inositol by PtdIns synthase. PtdIns can then be transported from its site of synthesis to the plasma membrane where it may be phosphorylated by various kinases into its derivatives PtdIns(4)P and PtdIns(4,5)P_2 (Figure 1.1).The phosphorylated forms may also be dephosphorylated by phosphomonoesterases so that the levels of phosphoinositides are maintained by specific kinases and phosphatases (Figure 1.1; Berridge & Irvine, 1984). Inositol may be obtained via (i) synthesis *de novo* from glucose 6-phosphate, (ii) by facilitated diffusion across the membrane, or (iii) by sequential dephosphorylation of inositol phosphates (Figure 1.1). Therefore, inositol phosphates formed by hydrolysis of phosphoinositides are recycled into phosphoinositide 'stores' in the membrane. $InsP_3$ may also be phosphorylated by $InsP_3$ 3-kinase in the presence of Mg^{2+} and ATP to Ins(1,3,4,5)P_4, another putative second messenger. This molecule can also be sequentially dephosphorylated, and hence recycled in the same way.

Phospholipase C isoforms and their regulation

Phosphoinositide-specific phospholipase C (PLC) enzymes are phosphodiesterases which hydrolyse the glycerophosphate bond of membrane phospholipids generating DAG and the inositol phosphate headgroup. There are three distinct isoforms of PLC (β,γ,δ) which are all single proteins, are discrete gene products and have been described based on their sequence homology, size and immunological cross-reactivity (Rhee & Choi, 1992). There are two regions of 40-60% homology (designated X and Y), which are assumed to be the catalytic regions of the proteins, whilst there is little homology in the remaining portions of the polypeptides between isoforms. All hydrolyse PIP_2 and all exhibit some degree of Ca^{2+}

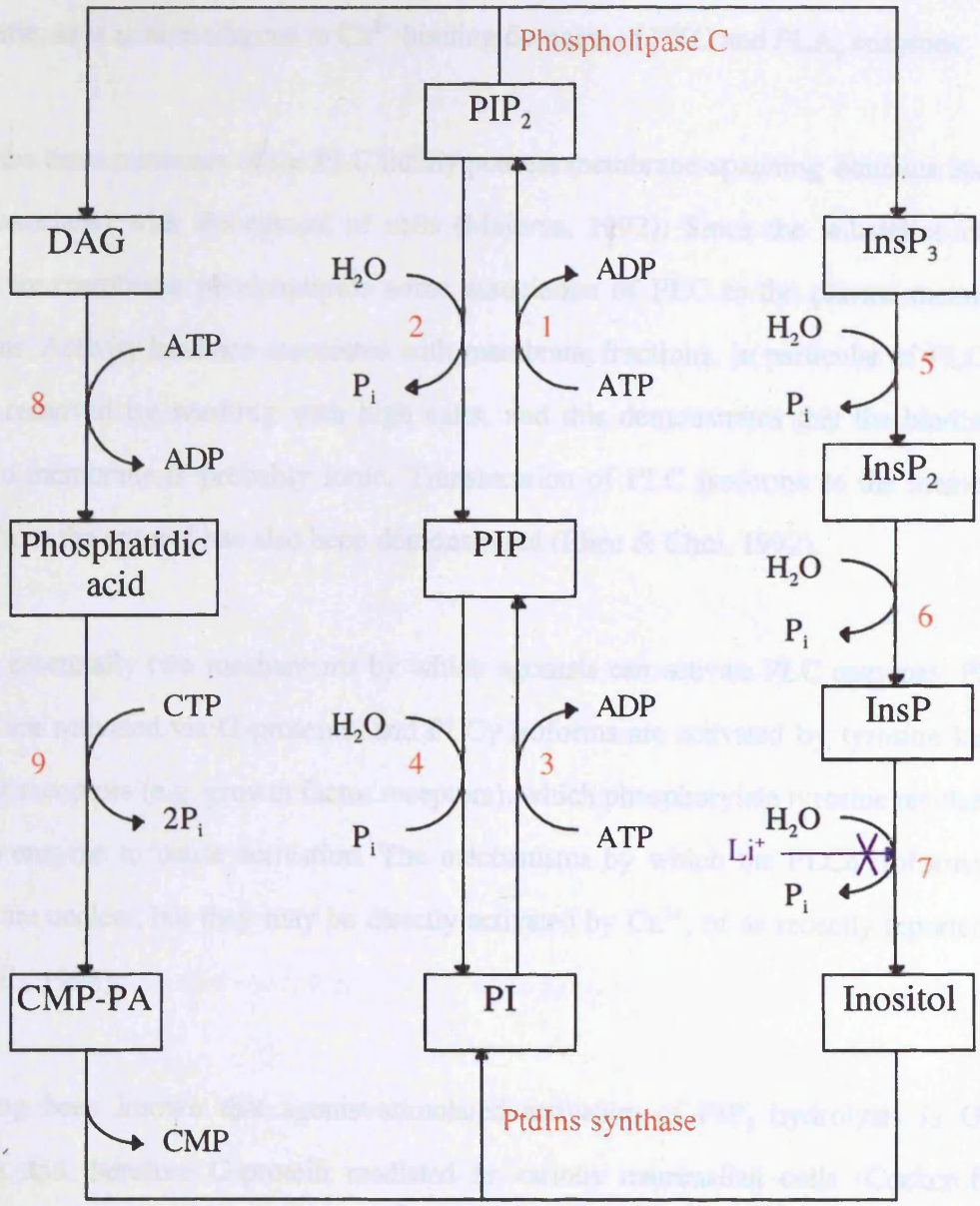


Figure 1.1 - Recycling of inositol and CMP-PA into phosphoinositides from Ins(1,4,5)P_3 and DAG production via PLC activation

KEY - 1- PtdIns(4)P 5-kinase; 2- PtdIns(4,5)P_2 phosphomonoesterase; 3- PtdIns 4-kinase; 4- PtdIns(4)P phosphomonoesterase; 5- InsP_3 5-phosphatase; 6- InsP_2 1-phosphatase; 7- InsP monophosphatase; 8- DAG kinase; 9- CTP-phosphatidate cytidyl transferase.

Li^+ blocks inositol monophosphatase activity

sensitivity. The carboxy-terminal end of the Y region is thought to be a possible Ca^{2+}-binding site, as it is homologous to Ca^{2+}-binding domains of PKC and PLA_2 enzymes.

None of the three members of the PLC family possess membrane-spanning domains and are mostly associated with the cytosol of cells (Majerus, 1992). Since the substrates of the enzymes are membrane phospholipids some association of PLC to the plasma membrane must occur. Activity has been associated with membrane fractions, in particular of PLC-β1, which is removed by washing with high salts, and this demonstrates that the binding of enzyme to membrane is probably ionic. Translocation of PLC isoforms to the membrane fraction from the cytosol has also been demonstrated (Rhee & Choi, 1992).

There are essentially two mechanisms by which agonists can activate PLC enzymes. PLCβ isoforms are activated via G-proteins, and PLCγ isoforms are activated by tyrosine kinase activity of receptors (e.g. growth factor receptors), which phosphorylate tyrosine residues of the PLCγ enzyme to cause activation. The mechanisms by which the PLCδ isoforms are activated are unclear, but they may be directly activated by Ca^{2+}, or as recently reported G_h (Park *et al.*, 1998).

It has long been known that agonist-stimulated activation of PIP_2 hydrolysis is GTP-dependent and therefore G-protein mediated in various mammalian cells (Cockcroft & Gomperts, 1985) and numerous studies have demonstrated that GTP, its non-hydrolysable derivative GTPγS and aluminium fluoride (AlF_4^-) stimulate PI turnover in permeabilised cells, crude membrane preparations or partially purified enzyme preparations (Rhee & Choi, 1992). PLCβ1, PLCβ3 and to a lesser extent PLCβ2 are activated by $G_q\alpha$ (Rhee & Choi, 1992), but also agonist-mediated PLC stimulation may be pertussis toxin-sensitive, and it has been shown that PLCβ isoforms, particularly PLCβ2 and β3 can also be activated by βγ subunits, derived from pertussis toxin-sensitive G-proteins (Camps *et al.*, 1992; Katz *et al.*, 1992). It is possible that PLCγ isoforms may be activated downstream of activation of PLCβ isoforms. Agonist stimulation of PLCβ leads to increases in $[Ca^{2+}]_i$ via activation of IP_3

receptors, and this may activate Ca^{2+}-sensitive tyrosine kinases which can phosphorylate and stimulate PLCγ.

PLC activity is dependent on cytosolic Ca^{2+} concentrations. Ca^{2+} influx, initiated by Ca^{2+} ionophores or K^+, can initiate or enhance phosphoinositide hydrolysis. In some cells the plateau phase of $Ins(1,4,5)P_3$ generation has been shown to be entirely dependent on Ca^{2+} influx across the plasma membrane, whilst the peak phase is also regulated by $[Ca^{2+}]_i$. Also, stimulation of PLC by $G_q\alpha$ has been studied in the presence of varying concentrations of Ca^{2+}. It was found that not only was the maximal activity of PLC increased, $G_q\alpha$ increased the apparent affinity of the enzyme for Ca^{2+} (Rhee & Choi, 1992).

So, it has been shown that activation of PLC is complex; not only may there be activation of a variety of effector isoforms by agonist stimulation of a single receptor subtype, but also each enzyme isoform may be activated by various concurrent intracellular stimuli, such as $G_q\alpha$, βγ subunits and Ca^{2+} for PLCβ isoforms.

Inositol 1,4,5-trisphosphate, Ca^{2+} mobilisation and contraction

$InsP_3$ receptors and ryanodine receptors are intracellular Ca^{2+} channels responsible for mobilising stored calcium ions. They share considerable structural homology, but $InsP_3$ receptors are activated by $InsP_3$ binding whilst the activity of ryanodine receptors is independent of $InsP_3$ and dependent on Ca^{2+} concentration, hence being responsible for 'Ca^{2+}-induced Ca^{2+} release'. The majority of $InsP_3$ receptors are found in the membrane of the endoplasmic reticulum of cells, which are believed to act as intracellular Ca^{2+} stores. There are at least three subtypes of $InsP_3$ receptor, from three distinct genes, and splice variants of the gene products also leads to further heterogeneity (Berridge, 1993; Mikoshiba, 1993). The use of cloned $InsP_3$ receptors has shown that these proteins may be considered as $InsP_3$-gated Ca^{2+} channels, even though the primary sequence of the receptor has no sequence homology with any of the Ca^{2+} channels found in the plasma membrane

(Mikoshiba, 1993). The receptor is likely to have six transmembrane domains and is expressed at the membrane as a tetramer, with each subunit being able to bind one molecule of $InsP_3$. There are phosphorylation sites on the receptor for cyclic AMP-dependent protein kinase (PKA), protein kinase C (PKC) and calmodulin-dependent protein kinase II, and ATP binding sites which can act to modulate receptor function. This modulation and the affinity for $InsP_3$ may vary between $InsP_3$ receptor subtypes. Therefore, the presence and relative distribution of different subtypes and splice variants of the $InsP_3$ receptor may determine, at least in part, the unique Ca^{2+} mobilisation profiles of different cells in response to agonist-induced activation of phosphoinositide hydrolysis.

Generally, the resting levels of intracellular cytoplasmic [Ca^{2+}] are maintained at concentrations of ~50 - 200 nM by transporter proteins within the plasma membrane (i.e. Ca^{2+}/Mg^{2+} ATPases and Na^{+}/Ca^{2+} antiporters) and the membranes of intracellular stores (i.e. sarcoplasmic / endoplasmic reticulum Ca^{2+} ATPases (SERCA pumps)) (Berridge, 1993). These transporters thereby maintain a steep Ca^{2+} concentration gradient between the cytoplasm of the cell and the extracellular space (extracellular free [Ca^{2+}] is ~1 mM) and intracellular stores. This physiological concentration gradient ensures that although the pore formed by the tetramer is relatively non-selective for cations, whether monovalent or divalent, the majority of the current produced when the channel is in an open configuration is due to Ca^{2+} flux across the membrane. $InsP_3$ receptors and ryanodine receptors may be co-localised on endoplasmic reticulum. Alternatively, $InsP_3$- and ryanodine-sensitive Ca^{2+} stores may be separate, but interact with each other (Berridge, 1993). This may be determined by the cell-type in which $InsP_3$- and ryanodine receptors are expressed.

Ca^{2+} contained within the stores is released into the cytoplasm of the cell when $InsP_3$ binds to its receptor. However there is some debate as to whether this binding is co-operative and requires 4 molecules of $InsP_3$ to be bound (Meyer *et al.*, 1990), whether there is an absence of co-operativity of binding, with one molecule of $InsP_3$ being sufficient to open the channel (Finch *et al.*, 1991), or whether Ca^{2+} is released quantally in response to the binding of each

$InsP_3$ molecule to the tetramer, resulting in four conductance states of the channel (Ehrlich & Watras, 1988). In non-excitable cells, the transient increase in $InsP_3$ correlates with a rapid release of Ca^{2+} from intracellular stores, and this is generally accompanied by a second phase of increase in $[Ca^{2+}]_i$ involving Ca^{2+} entry across the plasma membrane via voltage-dependent and-independent mechanisms. Receptor-induced Ca^{2+} release has been shown to be quantal in nature, with increasing concentrations of $InsP_3$ causing incremental increases in Ca^{2+} release from intracellular stores, suggesting that Ca^{2+} stores are extensively compartmentalised (Muallem *et al.*, 1989). Meyer & Stryer, (1990) reported that after addition of $InsP_3$, a rapid efflux of Ca^{2+} from internal stores was followed by a slower release, which could not be explained by changes in the pump rate of the Ca^{2+}-ATPase. They also reported that the slower phase was responsive to further additions of $InsP_3$. They therefore proposed that there were multiple Ca^{2+} stores which could be activated in an all-or-none fashion with each store being unloaded over a narrow concentration range of $InsP_3$. It has also been shown in rat hepatocytes that liberation of additional $InsP_3$ at the top of a $[Ca^{2+}]_i$ spike, delivered by photolysis of caged $InsP_3$, failed to increase $[Ca^{2+}]_i$ further, although photolysis of caged $InsP_3$ in an unstimulated cell caused an increase in $[Ca^{2+}]_i$ which was graded according to the amount of $InsP_3$ released (Chiavaroli *et al.*, 1994). Although the presence of discrete Ca^{2+} stores is an attractive interpretation of the available results, and seems possible, if not likely, given the heterogeneity of $InsP_3$ receptors, it should be noted that experiments such as these require permeabilisation of the cells, which may disrupt the intracellular Ca^{2+} pools themselves.

As has been previously mentioned, agonist-induced increases in Ca^{2+} concentrations within the cell are also due, in part, to Ca^{2+} influx across the plasma membrane. This may be due to changes in activation of voltage-sensitive Ca^{2+} channels or via receptor-operated channels, or may be due to capacitative entry induced by depletion of intracellular stores (Putney, 1993; Putney & Bird, 1993). The channels involved in capacitative entry have been designated Ca^{2+}-release-activated Ca^{2+} or CRAC channels, generating the current I_{CRAC}. It has been suggested that there may be one or several diffusible messengers which indicate depletion of

stores, and calcium influx factor, small GTPases and inositol 1,3,4,5-tetrakisphosphate have been implicated in this role. Also, it has been suggested that the cytoskeleton of the cell may transmit this information to the plasma membrane directly (Putney, 1993; Putney & Bird, 1993). There is also some evidence using antibodies against the $InsP_3$ receptor that $InsP_3$ binding proteins exist in the plasma membrane, which could also be implicated in Ca^{2+} entry (Khan *et al.*, 1992).

There is much evidence showing that $InsP_3$-induced Ca^{2+} release is responsible for agonist induction of contraction of smooth muscle (reviewed in Bárány & Bárány, 1996). For example, $InsP_3$ causes Ca^{2+} release and development of contraction in permeabilised rabbit pulmonary arterial smooth muscle in a dose-dependent manner (Somlyo *et al.*, 1985). There is reasonable agreement in the biochemical kinetics involved in $InsP_3$-induced Ca^{2+} release and initiation of contraction in smooth muscle (Somlyo *et al.*, 1992).

Diacylglycerol and protein kinase C

Another possible second messenger involved in maintenance of contraction is diacylglycerol, also produced by phosphoinositide hydrolysis. This messenger may also be produced at least in part from other sources such as PLD and phosphatidylcholine-specific PLC activation. DAG activates PKC to cause phosphorylation of other cellular proteins i.e. activates a protein kinase cascade. Agonist-stimulated increases in DAG have been correlated with increased translocation of PKC to the plasma membrane (see Nahorski *et al.*, 1994), which is on a time-scale consistent with $InsP_3$ production and Ca^{2+} mobilisation in smooth muscle and also consistent with its activation by DAG (Langlands & Diamond, 1994; Langlands & Diamond, 1992). Phorbol esters, which directly activate PKC can cause slow contraction of smooth muscle (Chatterjee & Tejada, 1986), supporting the idea that PKC is involved in maintenance of contraction and not its rapid initiation. PKC may also be involved in negative-feedback mechanisms on PLC-activated phosphoinositide hydrolysis (Huwiler *et al.*, 1997; Watanabe *et al.*, 1996), and so may have an additional role in cross-

talk between receptor-activated transduction pathways, as well as in the maintenance of contraction in smooth muscle.

1.2.2 Receptor-mediated inhibition of adenylyl cyclase activity

Introduction

The most extensively studied of the G-protein linked receptor-mediated signalling cascades is that involving adenylyl cyclase. Not only has the signalling pathway involving activation of cAMP-dependent protein kinase been shown to control glycogenolysis, lipolysis and other synthetic cellular pathways, but the importance of cAMP in the control of tone in smooth muscle is certain. The G-proteins G_s and G_i bimodally modulate the activity of adenylyl cyclases, but different isoforms of the enzyme may also be differentially regulated e.g. by Ca^{2+}. It has been suggested that β-adrenoceptors and M_2-mACh receptors physiologically antagonise each other via their opposing actions on adenylyl cyclase, to alter smooth muscle tone. This is the main argument for the role of M_2-mACh receptors in smooth muscle contraction, and therefore adenylyl cyclase and mACh signalling are discussed in further detail below.

Adenylyl cyclase isoforms and their regulation

In 1989, adenylyl cyclase was sequenced from bovine brain (Kuprinsky *et al.*, 1989). It was described as being 120 kDa in size, 1134 amino acids in length and consisting of 12 transmembrane domains, split into two regions by a large cytoplasmic loop. Adenylyl cyclase has homology with ion channels and some transporter proteins, but most notably considerable homology of the large carboxyl terminal and central cytoplasmic loops with those of guanylyl cyclase. It was therefore assumed that the catalytic sites occur in these regions, and suggested that $G_s\alpha$ and forskolin may regulate interactions between these two loops, thus regulating the catalytic activity of the enzyme, by binding to other areas of the molecule to cause a conformational change in the case of $G_s\alpha$, and possibly by changing the membrane fluidity around the enzyme in the case of forskolin. Since 1989, eight other mammalian forms of adenylyl cyclase have been cloned, all of which conform to the basic

structure but which have different regulatory properties. All are activated by $G_s\alpha$ and forskolin, which stimulates the effector independently of $G_s\alpha$ activation. Types I, III and VIII are also activated by Ca^{2+}/calmodulin and therefore by increases in $[Ca^{2+}]_i$, whilst types V and VI are inhibited by increased $[Ca^{2+}]_i$. There is differential regulation of isoforms by $\beta\gamma$ subunits. Type I is directly inhibited by $\beta\gamma$ subunits, whilst types II and IV are stimulated by $\beta\gamma$ subunits when concurrently stimulated by $G_s\alpha$. $\beta\gamma$ subunits have no effect on types III, V and VI. PKC is also involved in the regulation of Types II and VII, since phorbol esters can activate these isoforms, in some cases more effectively than $G_s\alpha$. Types VII, VIII and IX have been cloned only partially, but it is thought that type VII is regulated in a similar manner to types II and IV, type VIII is regulated by Ca^{2+}/calmodulin, whilst the regulation of type IX is different from the other isoforms (for reviews see Cooper *et al.*, 1995; Iyengar, 1993). Some isoforms appear to have a highly localised (often neuronal) expression e.g. types I, III, VIII and IX, whereas others appear to be widespread. However, the reported distribution of adenylyl cyclases has in most cases been based on mRNA levels for each cloned isoform and should therefore be interpreted accordingly. There is little information as yet available regarding the expression of isoforms in smooth muscle tissues, however it has been reported that those expressed probably include Types II and IV (Yoshimura & Cooper, 1993) and types V and VI (Murthy & Makhlouf, 1997).

Receptor-mediated inhibition of adenylyl cyclase activity

Although much work has been carried out on the stimulation of adenylyl cyclase and subsequent relaxation of smooth muscle, little work has been done to demonstrate receptor-mediated inhibition of adenylyl cyclase and its consequences in smooth muscle. This is despite the knowledge, for example, that M_2-mACh receptors profoundly inhibit forskolin-stimulated adenylyl cyclase activity in recombinant systems via G_i G-protein activation, and that M_2-mACh receptors form the major component of the mACh receptor population in smooth muscle tissues (Caulfield, 1993).

Theoretically, receptors coupling to G_i G-proteins might inhibit adenylyl cyclase via three different mechanisms. Firstly, $G_i\alpha$ may inhibit the enzyme directly, or, secondly, this may be the function of $\beta\gamma$ subunits. Alternatively, $\beta\gamma$ subunits may interact with $G_s\alpha$ to cause reformation of the inactive heteromer, and hence prevent activation of adenylyl cyclase. This mechanism would only be possible if the expression of G_i was greater than that for G_s, otherwise the eqilibrium would be such that reformation of the heteromer would actually increase the stimulation of the G-protein by increasing the probability that $G_s\alpha$ could be activated by the receptor molecule. This is in fact true for smooth muscle, with G_i generally being expressed at ten-times the concentration of G_s G-proteins. However, in a cyc^- S49 lymphoma cell line, which does not express G_s G-proteins, an agonist may still inhibit forskolin-stimulated adenylyl cyclase (Birnbaumer, 1992), suggesting that this interaction of $\beta\gamma$ subunits from G_i subunits with $G_s\alpha$ is not the main mechanism of agonist-induced inhibition of cyclic AMP. It is perhaps most likely that all three mechanisms operate under different physiological conditions, with the direct effect of $G_i\alpha$ being most important under conditions of stimulation of adenylyl cyclase by G_s.

The first evidence that mACh receptor agonists could inhibit β-adrenoceptor-induced or forskolin-induced stimulation of adenylyl cyclase activity in smooth muscle (Jones *et al.*, 1987) in a pertussis toxin-sensitive manner (Sankary *et al.*, 1989) was subsequently repeated in other smooth muscles (Candell *et al.*, 1990; Griffin & Ehlert, 1992; Reddy *et al.*, 1995). However, despite the pharmacological evidence that mACh receptor-induced inhibition occurs, little is known about how this affects smooth muscle tone. At the moment, results from functional studies of adenylyl cyclase have been demonstrated to be highly tissue-specific. The importance of M_2-mACh receptor-induced inhibition of adenylyl cyclase is as yet unknown. It has been shown, in certain smooth muscles, that increases in tone induced by mACh receptor agonists are relatively resistant to relaxation by β-adrenergic agonists compared to increased tone induced by other spasmogens (Torphy, 1984; Van Amsterdam *et al.*, 1989), and that the relaxant potency of β-adrenergic agonists are increased by concurrent incubation with M_2-mACh selective antagonists, such as methoctramine

(Watson & Eglen, 1994; Watson *et al.*, 1995), or by pertussis toxin pretreatment (Mitchell *et al.*, 1993; Thomas & Ehlert, 1996) in some tissues precontracted with muscarinic agonists. However, it is unknown if adenylyl cyclase inhibition by M_2-mACh receptors has an important role in maintenance of tone in a physiological situation, or if other putative modulatory effects on, for example, the phosphoinositide pathway caused by this inhibition of cAMP production are more important.

1.2.3 Receptor-mediated modulation of ion channels

Acetylcholine is known to depolarise smooth muscle cells with an increase in membrane conductance (Bolton, 1972), and this depolarisation has been shown to result from mACh receptor-activated cation channel activation (Benham *et al.*, 1985). This channel is non-selective between cations, but the current measured is predominantly due to Na^+ ions moving through the channel (Inoue *et al.*, 1987). This would in theory, be sufficient to cause contraction of smooth muscle, since the opening of cation channels, although not in themselves greatly permeable to Ca^{2+} ions, would cause depolarisation of the cells and hence opening of voltage-operated Ca^{2+} channels. The resultant Ca^{2+} influx could be adequate to cause contraction of the smooth muscle. mACh receptors appear to be linked to the cationic channel via a pertussis toxin-sensitive G-protein (Inoue & Isenberg, 1990; Komori *et al.*, 1992), and increases in $[Ca^{2+}]_i$ have been shown to facilitate non-selective cation inward currents, but are unable to activate these channels in the absence of ACh (Inoue & Isenberg, 1990; Inoue & Isenberg, 1990; Pacaud & Bolton, 1991; Zholos & Bolton, 1995). Most recently, evidence has shown that the receptor subtype mediating this response in guinea-pig ileum is the M_2-mACh receptor subtype, but that blockade of the co-expressed M_3-mACh receptor reduces the effects of M_2-mACh receptor activation on the inward current (Zholos & Bolton, 1997). Perhaps, this modulation by M_3-mACh receptors is via the ability to induce increases in $[Ca^{2+}]_i$ via phosphoinositide hydrolysis and $InsP_3$ release, and hence increase the sensitivity of the channels to activation by M_2-mACh receptors, presumably via G_i G-proteins. Alternatively, interactions of the two receptor subtypes at another level may account for this enhancement. In any case, this mechanism has been postulated to account for a direct role of M_2-mACh receptors in smooth muscle contraction, at least in some smooth muscle cell-types.

The ability of mACh receptors to cause Ca^{2+} influx in CHO cells has previously been studied (Singer-Lahat *et al.*, 1996). It was shown that m3-, but not m2-mACh receptors could cause Ca^{2+} influx when expressed in this cell-line, although both types could cause Ca^{2+}-release

from intracellular stores. From this data it was suggested that mACh receptors were coupled more strongly to PLC activation than to the Ca^{2+} influx pathway. By using chimaeric m2/m3 receptors it was shown that the 3rd intracellular loop played a pivotal role in m3-mACh receptor coupling to Ca^{2+} influx, which was in contrast to previous results by the same group in which the 3rd intracellular loop was shown not to be involved in m3-mACh receptor activation of Ca^{2+} influx when expressed in A9 cells. The inability of m2-mACh receptors to activate Ca^{2+} influx in this case may indicate that CHO cells do not express the non-specific cation channels and/or the voltage-sensitive Ca^{2+} channels necessary for the m2-mACh-mediated increases in $[Ca^{2+}]_i$ which are seen in smooth muscle preparations.

In addition, mACh receptors have been shown to inhibit large conductance Ca^{2+}-dependent K^+-channels (BK_{Ca}) in smooth muscle, and this is, at least partially, mediated via a direct pertussis toxin-sensitive mechanism (Cole & Sanders, 1989; Kume & Kotlikoff, 1991). This has been proposed to be by M_2-mACh receptor activation (Kume *et al.*, 1995), in direct opposition to activation of BK_{Ca} by β-adrenergic agonists, which has been proposed to account, in part, for β-adrenergic-induced relaxation (Jones *et al.*, 1990). Studies showing that, in passively sensitised airway smooth muscle, β-adrenergic-induced relaxation is attenuated by activation of M_2-mACh receptors, G_i G-protein expression and coupling (Hakonarson *et al.*, 1995) were subsequently followed by data suggesting the involvement of the BK_{Ca} channel in this interaction (Hakonarson *et al.*, 1996). Thus, the inhibition of BK_{ca} channels offers another explanation as to how M_2-mACh receptor activation may affect smooth muscle tone.

1.2.4 Other signalling pathways regulated by mACh receptors

It is unknown whether M_2-mACh and M_3-mACh receptors interact to regulate other phospholipases, such as phospholipases D or A_2, phosphatidylcholine-preferring-phospholipases C, or the cascades involved in proliferation, such as the ERK pathway, although there is evidence that these activities exist in smooth muscle and may be subject to either direct or indirect regulation by mACh receptors.

Phospholipases D

Phospholipase D catalyses the hydrolysis of phospholipids, to generate phosphatidic acid and the polar head group e.g. choline. The preferred substrate of membrane-associated PLD is phosphatidylcholine, but this enzyme may also hydrolyse phosphatidylethanolamine and phosphoinositides. Whether differences in substrate preference indicate the existence of different isoforms of the enzyme is unclear (Challiss & Blank, (1997), and references therein). PLD activity can be stimulated by a diverse range of molecules, such as growth factors, cytokines, cell adhesion molecules and a variety of transmitters and hormones acting via G-protein-linked receptors, and there is evidence that PLD may be activated indirectly via PKC, as well as directly by G-protein activation. Although heterotrimeric G-proteins may be involved in some cell types, most evidence points to activation of PLD by small monomeric G-proteins such as ARF (ADP ribosylation factor) and Rho (Bowman *et al.*, 1993; Brown *et al.*, 1993), although this only seems to be true for a subset of phospholipases D, and the mechanism of this activation is not fully understood. It has been shown that mACh receptors can activate PLD directly via pertussis toxin-insensitive G-proteins in human embryonic kidney (HEK) cells (Rümenapp *et al.*, 1995), and that M_3-mACh receptors can activate PLD via PLC activation of a PKC/Ca^{2+}-dependent pathway (Schmidt *et al.*, 1994). Most importantly, the majority of studies looking at the activation of PLD have used transfected cell lines, and it is by no means certain that mACh receptors can activate PLD directly in smooth muscle, though activation of PLD by PKC has been demonstrated in intestinal

smooth muscle (Murthy & Makhlouf, 1995), or that PLD is expressed in all smooth muscles, although it seems probable given that PLD expression has also been demonstrated in vascular smooth muscle (Freeman *et al.*, 1995; Plevin *et al.*, 1992). Further studies need to be carried out before the involvement of PLD in mACh receptor-induced activation of smooth muscle contraction can be truly evaluated.

Phospholipases A_2

Phospholipase A_2 catalyses the hydrolysis of membrane phospholipids to the lysophospholipid and a *sn*-2 position fatty acid (often arachidonic acid). There appear to be different requirements for different isoforms of PLA_2 enzymes, whether for a specific substrate, e.g. phosphatidylcholine, or regulation by Ca^{2+}, and this family of phospholipases is growing steadily (Dennis, 1997). It has been shown that G-protein-linked receptors, including M_2-mACh receptors may activate PLA_2, in a pertussis toxin-sensitive (Conklin *et al.*, 1988; Dickerson & Weiss, 1995; Felder *et al.*, 1991; Piomelli *et al.*, 1991; Winitz *et al.*, 1994) or -insensitive (Axelrod, 1990; Dickenson & Hill, 1997) manner. However, studies of receptor activation of PLA_2 in smooth muscle are few. In cat oesophageal circular smooth muscle mACh receptor activation stimulates cytosolic arachidonic acid release, which may synergise with DAG to contribute to the sustained phase of contraction in this tissue (Sohn *et al.*, 1994). Also reported, agonist-stimulated PLA_2 activity in intestinal longitudinal, but not circular, smooth muscle leads to arachidonic acid release and hence to Ca^{2+} influx and mobilisation, via a pertussis toxin-sensitive mechanism (Murthy *et al.*, 1995). In many studies, receptor activation of PLA_2 has been shown to be pertussis toxin-sensitive, and $G\alpha_{i2}$ has been suggested as the G-protein component of the receptor-effector pathway, but direct activation of PLA_2 by $G\alpha_i$ has yet to be demonstrated. It would not be surprising, however, if PLA_2 did have a part to play in modulation of smooth muscle tone, since arachidonic acid, lysophospholipids and the resultant lysophosphatidic acid (produced by PLD hydrolysis of lysophospholipids) have all been implicated as second messengers which can modulate $[Ca^{2+}]_i$ (see Challiss & Blank, 1997). It is also possible that activation of

cytoplasmic PLA_2 may be via tyrosine or serine-threonine kinase activities given the presence of appropriate phosphorylation sites, which may be deduced from its primary sequence (Kramer, 1994), and its phosphorylation and activation by ERK (Lin *et al.*, 1993).

Phosphatidylcholine-preferring Phospholipase C

In some smooth muscles, a rapid accumulation of phosphocholine and phosphatidylcholine-derived DAG can be measured in response to growth factors, and to stimulation of G-protein-coupled receptors, including mACh receptors, and this may involve activation of PC-PLC by mitogenic signalling and PKCζ, or another mechanism (Challiss & Blank, (1997) and references therein). Very little is known about whether direct signalling via G-proteins is involved, although in some cases the response has been shown to be pertussis toxin-sensitive. The only attractive incentive to suggest that PC-PLC may be involved in smooth muscle contraction is that the response appears to be relatively resistant to desensitisation, allowing maintenance of increased DAG production for many minutes, thereby suggesting a mechanism for the sustained phase of contraction. However, in light of the paucity of information about the activation, or consequences of activation of PC-PLC in smooth muscle, it must be considered as only a speculative mechanism for control of smooth muscle tone by M_2-mACh receptors, despite the possibility of direct activation.

1.3 mACh receptors in smooth muscle

1.3.1 mACh receptor expression in smooth muscle

Early contractile studies in smooth muscle carried out to distinguish novel mACh receptor subtypes were hampered by the lack of subtype selectivity of the ligands used, and inevitably this led to various smooth muscle mACh receptor populations being described as homogeneous. With the advent of the use of more selective mACh receptor ligands in radioligand binding studies to assess membrane expression of receptors, and, more recently, the discovery of molecular biological techniques, such as Northern blotting and RT-PCR, to assess mRNA present in cells, it was discovered that, actually, most smooth muscles express more than one form of mACh receptor (see reviews Eglen *et al.*, 1997; Eglen *et al.*, 1994).

This issue was first considered in guinea-pig ileal preparations, since this was a tissue used as a 'standard' in many smooth muscle studies. It had been illustrated that contraction was mediated by M_3-mACh receptors, using measurements of antagonist affinities taken by standard pharmacological methods to distinguish between subtypes, by many different groups. It was therefore extremely surprising for researchers to discover that in radioligand binding studies only a homogeneous M_2-mACh receptor population (as defined at that time) could be demonstrated (Michel and Whiting 1987; Roffel *et al.*, 1988). This was due not only to the poor selectivity of the competing antagonists available e.g. gallamine, but also because M_2-mACh receptors outnumber the M_3-muscarinic receptors by up to 80% to 20% in this tissue, making two site analysis difficult, even with relatively selective ligands (Michel and Whiting 1988, 1990; Giraldo *et al.*, 1987; Entzeroth and Mayer, 1991). Further analysis using a multidisciplinary approach confirmed the co-expression of M_2- and M_3-mACh receptors in guinea-pig ileum, at a ratio of 70% M_2-mACh to 30% M_3-mACh receptors (Ford *et al.*, 1991). Using a variety of methods including radioligand binding,

Table 1.2 - Examples of coexpression of mACh receptors in smooth muscle tissues as demonstrated by competition radioligand binding studies (adapted from Table 8; Eglen *et al.*, 1997)

Tissue	*Species*	*M_2:M_3*	*Antagonists used*	*Reference*
Ileum	Guinea pig	65%:35%	AF-DX 116	(Michel & Whiting, 1987)
		82%:18%	AF-DX 116	(Giraldo *et al.*, 1988)
		77%:23% ; 80%:20%	AF-DX 116; methoctramine	(Michel & Whiting, 1990)
		70%:30%	Methoctramine	(Ford *et al.*, 1991)
Stomach	Human	79%:21%	AF-DX 116	(Bellido *et al.*, 1995)
Colon	Dog	82%:18%	Pirenzepine	(Zhang & Buxton, 1991)
	Human	76%:24%	AF-DX 116	(Gomez *et al.*, 1992)
	Rat	49%:51%	4-DAMP	(Zhang, 1996)
Trachea	Cow	74%:26%; 83%:17%	AF-DX 116; methoctramine	(Roffel *et al.*, 1988)
	Dog	72%:28%	Methoctramine; 4-DAMP	(Yang *et al.*, 1991)
	Guinea-pig	52%:48%; 64%:36%	AF-DX 116; methoctramine	(Haddad *et al.*, 1991)
Bladder	Rat	87%:13%	AF-DX 116	(Monferini *et al.*, 1988)

Northern blot analysis, and antibodies specific to receptor subtypes, it has been demonstrated that most smooth muscles co-express mACh receptors, generally M_2-mACh receptors being the major population and M_3-mACh receptors forming the minor population. Some examples are given in Table 1.2. Exceptions include the following: apparent M_1-, M_2-, M_3- and M_4-mACh co-expression has been detected in rat gastrointestinal smooth muscle, using subtype-specific antibodies (Wall *et al.*, 1991), although M_2-mACh receptors were still shown to be the dominant subtype; M_1- M_2- and M_3-mACh receptor subtype expression has been determined in rat lung using radioligand binding (Fryer & El-Fakahany, 1990); M_2- and M_4- coexpression has been described in guinea-pig uterus using Northern blot analysis (Eglen *et al.*, 1992)(see also Chapter 6 this Thesis); mACh receptors which could not be characterized as either M_1-, M_2-, M_3- or M_4-mACh receptors have been described in rabbit iris sphincter (Bognar *et al.*, 1992); debate continues as to the nature of the mACh receptor population in gall bladder, whether M_3-mACh or also M_4-mACh receptors (Eltze *et al.*, 1997); and rabbit anococcygeus muscle has been described as co-expressing M_2- and M_4-mACh receptors (Gross *et al.*, 1997). It is not known why such a universal co-expression of mACh receptors occurs in smooth muscles, especially since, in most cases, contraction is mediated by M_3-mACh receptors, or, importantly, what is the elusive function of M_2-mACh receptors, given the dominant expression of this subtype in smooth muscle tissues.

Much debate and investigation has occurred to determine whether M_2-mACh receptors have a role to play in mACh receptor-induced smooth muscle tone. It has been shown repeatedly that M_2-mACh receptors couple to the inhibition of adenylyl cyclase via pertussis toxin-sensitive G_i G-proteins (Buckley, 1990; Burford *et al.*, 1995; Dell'Acqua *et al.*, 1993). This would appear to be in opposition to β-adrenoceptor-induced increases in cAMP production and hence relaxation in smooth muscle tissues. A number of groups have therefore studied the ability of muscarinic agonists to antagonise β-adrenoceptor-induced decreases in smooth muscle tone. It was noted that, in guinea-pig isolated trachea, tissues contracted using mACh receptor agonists were more resistant to relaxation by isoprenaline than those contracted using other spasmogens, such as histamine or leukotriene D_4, even when the developed

isometric tension produced by each agonist was closely matched before relaxation was induced with isoprenaline (Torphy, 1984). It has been shown in the same tissue that isoprenaline-induced relaxation is augmented by the use of SDZ ENS 163 (thiopilocarpine) as the spasmogen, which is a partial M_3-mACh receptor agonist, but also displays antagonism at M_2-mACh receptors, compared with the non-selective mACh receptor agonist (+)-*cis*-dioxolane, and is also augmented by the addition of methoctramine, an M_2-selective antagonist (Watson & Eglen, 1994). It has also been proposed that M_2-mACh receptors are responsible for mACh-induced inhibition of isoproterenol-induced relaxation in canine airway smooth muscle (Fernandes *et al.*, 1992; Torphy *et al.*, 1983), which is further supported by the evidence that pertussis toxin pre-treatment increases the potency of isoprenaline to cause relaxation of mACh receptor-induced contraction, but not KCl-induced contraction in canine trachealis (Mitchell *et al.*, 1993). However, no such augmentation of isoprenaline-induced relaxation of mACh receptor-induced tone using M_2-mACh receptor selective antagonists was observed in bovine tracheal smooth muscle (Roffel *et al.*, 1995). In studies using gallamine as the 'M_2-selective' agent in guinea pig tracheal smooth muscle, no involvement of M_2-mACh receptors could be observed in the antagonism of isoprenaline-induced responses (Roffel *et al.*, 1993), however the use of this compound as an M_2-selective antagonist is controversial as its selectivity is poor, and was actually responsible for some of the earlier erroneous claims that mACh receptor populations in smooth muscle tissues are homogeneous. In the same study showing that pertussis toxin pre-treatment augments β-adrenoceptor-induced relaxation in canine isolated trachea, pirenzepine, at a concentration that occupies mostly M_3-mACh receptors and not M_2-mACh receptors, also augmented isoprenaline-induced relaxation of mACh receptor-induced tone (Mitchell *et al.*, 1993), possibly implicating M_3-mACh receptors as well as M_2-mACh receptors in functional antagonism of β-adrenoceptor-induced relaxation.

There is much heated debate which surrounds these issues (e.g. Eglen *et al.*, 1994; Roffel *et al.*, 1994), so attempts have been made to clarify the role of M_2-mACh receptors in smooth muscle using approaches which involve selective removal of one subtype of mACh receptor.

Firstly Thomas *et al.* (1993) demonstrated 're-contraction', thus guinea-pig ileum was contracted using histamine, and then elevation of cAMP levels was induced in the tissue using isoprenaline or forskolin, causing relaxation of the tissue. Following this demonstration of increased cAMP production, mACh receptor agonists were used to 're-contract' the tissue. The resultant concentration-response curve was characterised using the M_2-mACh selective antagonist AF-DX 116 and it was discovered that this analysis of the 're-contraction' was inconsistent with its mediation via a single receptor site. Thomas *et al.* (1993) selectively inactivated M_3-mACh receptors by incubating ileum with 4-DAMP mustard (a receptor-alkylating analogue of 4-DAMP) and AF-DX 116 (1μM) concurrently. This concentration of AF-DX is such that occupation of M_2-mACh receptors is maximised and the occupation of M_3-mACh receptors is minimised, so that M_2-mACh receptors are protected from alkylation whilst M_3-mACh receptors are not. Both agents were then washed from the tissues, to theoretically leave an intact and homogeneous population of M_2-mACh receptors. Under these conditions, AF-DX 116 antagonism of a 're-contraction' response, as before, was now consistent with mediation by a homogeneous population of M_2-mACh receptors, and the Hill slope of the 're-contraction' was increased to nearer unity. This result was taken to indicate that M_2-mACh receptors could be responsible for direct contraction of this tissue under certain specific conditions.

Later experiments in guinea-pig ileum by Thomas & Ehlert (1994) showed that the re-contraction response following selective alkylation was blocked by pre-incubation of the tissue with pertussis toxin, and that the inhibition of adenylyl cyclase was also prevented in this case. They therefore concluded that 're-contraction' was mediated by M_2-mACh receptors acting via pertussis toxin-sensitive G-proteins and that the mechanism of this response involves inhibition of adenylyl cyclase. However, when no prevailing smooth muscle relaxant tone was present, there was no evidence for M_2-mACh receptor involvement in mACh receptor-induced contraction (Eglen & Harris, 1993; Reddy *et al.*, 1995; Thomas *et al.*, 1993). Using the technique of selective M_3-mACh receptor inactivation, it was not possible to demonstrate M_2-mACh receptor-mediated re-contraction of guinea-pig

oesophageal muscularis mucosae, nor was it possible to demonstrate that M_2-mACh receptors contributed to the relative resistance of oxotremorine-M-mediated (non-selective mACh receptor agonist) contraction to isoprenaline-mediated relaxation in this tissue (Watson *et al.*, 1995). The authors suggested that in this tissue, M_3-mACh receptors and not M_2-mACh receptors interacted with β-adrenoceptors. The use of selective inactivation procedures has been inconclusive in guinea-pig tracheal preparations. In one study, although there was no evidence of M_2-mACh receptor-mediated recontraction, after selective alkylation of M_3-mACh receptors, M_2-mACh receptors did have a small inhibitory effect on isoprenaline-mediated relaxations (Watson *et al.*, 1995). In another study carried out by Thomas *et al.*, guinea-pig tracheal re-contractions were shown to be mediated by M_2-mACh receptors, whilst in guinea-pig oesophagus and rat fundus the authors suggested a joint role for M_2- and M_3-mACh receptors in the re-contraction response, despite the inactivation of the major proportion of the M_3-mACh receptors by selective inactivation (Thomas & Ehlert, 1996).

Although the evidence suggests that M_3-mACh receptors have a direct role in contraction of smooth muscle tissue, an influence of M_2-mACh receptor activation on the control of smooth muscle tone cannot be excluded. The mechanism of muscarinic control of tone may be different between tissues, and it has been suggested that M_2-mACh receptors may have a more important part to play in control of tone in situations of high sympathetic activity or where M_3-mACh receptors are dysfunctional. It is clear that control of smooth muscle tone is complex with respect to mACh receptor function, and is likely to differ between different tissues, and different experimental conditions. It is therefore important to ascertain whether, in the absence of clear direct participation of M_2-mACh receptors in smooth muscle contraction, they may have a modulatory function on contraction produced by M_3-mACh activation.

1.3.2 Innervation of smooth muscle

Neural control of smooth muscle initiates contraction, or enhances or depresses the force or rate of spontaneous, myogenic rhythmic contractions (Bolton, 1979). Acetylcholine is released from autonomic nerve terminals onto smooth muscle cells. The precursor of acetylcholine, choline, is synthesised in neuronal cell bodies and transported to the axon terminals where it is acetylated and stored as vesicles, until released either phasically or tonically. Acetylcholine may act on mACh receptors on the smooth muscle surface or pre-junctionally on autoreceptors on the nerve (Bolton, 1979). The activation of autoreceptors may not only effect subsequent release of ACh, but also of other neurotransmitters e.g. release of catecholamines from sympathetic neurones (Racké *et al.*, 1992) and ATP from non-adrenergic-non-cholinergic neurones (Burnstock *et al.*, 1970) in co-innervated smooth muscles. The consequence of prejunctional muscarinic receptor activation is not studied in this Thesis. Model cell-lines have been used or in the case of studies in uterine smooth muscle, care has been taken to exclude the possible involvement of neuronal mACh receptors, thereby simplifying the study to post-junctional effects of mACh receptor stimulation at the smooth muscle or a model of that situation.

1.3.3 Muscarinic acetylcholine receptors in smooth muscle pathology

Muscarinic acetylcholine (mACh) receptor dysfunction has been implicated in smooth muscle pathologies of the respiratory tract, such as chronic obstructive pulmonary disease, gastrointestinal disorders, such as irritable bowel syndrome, and urinary tract disorders, such as urge or stress incontinence, to name but a few. Selective blockade of receptors is an essential aim of mACh receptor drug discovery, as many mACh receptor ligands used therapeutically cause side-effects such as blurring of vision, increased heart rate, heat intolerance, decreased secretions, slowed gastric motility, sedation and confusion (Feinberg, 1993). These can be particularly serious in the elderly, where a side-effect as simple as dry mouth can cause mucosal damage and upper tract respiratory infection, due to the loss of the

anti-microbial action of saliva. Also, these side-effects can cause the drug to be less therapeutically advantageous, for example dry mouth encourages increased fluid intake which exacerbates urge incontinence. Other problems of non-selectivity of antagonists observed include a paradoxical bronchoconstriction during the treatment of chronic obstructive pulmonary disease due to concurrent antagonism of pre-junctional mACh autoreceptors, thereby decreasing the effectiveness of post-junctional mACh receptor antagonism. Unfortunately the side-effects of mACh receptor drugs, which lack subtype selectivity, are seen by many patients as worse than the symptoms of the pathologies which are being treated.

Thus far, the search for compounds which are selective for mACh receptor-induced contraction have mostly centred around M_3-mACh receptor selectivity, although other older mACh receptor selective compounds, such as dicyclomine and pinaverium, work via other properties such as Ca^{2+} channel blockade to decrease contractility of smooth muscle (Downie *et al.*, 1977). Compounds such as zamifenacin and darifenacin which are relatively M_3-mACh selective have been shown to be gut-selective in animal models (Quinn *et al.*, 1993; Wallis, 1995), but the reason for this apparent gut selectivity is unclear and these compounds have been shown to have limited therapeutic use in treating gastrointestinal smooth muscle dysfunction (zamifenacin clinical trials discontinued; darifenacin clinical trials as yet unpublished). Urinary bladder selectivity of some mACh receptor ligands has been shown to be due to preferential accumulation in the bladder, and not due to receptor subtype selectivity (Oyasu *et al.*, 1994). If participation of other mACh receptor subtypes in contraction can be shown to be important, then this may explain the limited success of M_3-mACh selective compounds in the treatment of smooth muscle pathologies. Therefore, there are important clinical implications in the detailed study of mACh receptor signalling and the cross-talk which may occur between subtypes.

1.4 Aspects of receptor G_i/G_q-mediated cross-talk

An increasing number of studies reporting synergism of receptors at the level of phosphoinositide or Ca^{2+} signalling are giving credance to the idea of a universal 'cross-talk' mechanism between receptors signalling via G_q and G_i G-proteins (Selbie & Hill, 1998). This cross-talk is characterised by one kind of receptor which couples via pertussis toxin-sensitive G-proteins to the inhibition of adenylyl cyclase, and exhibits a small or undetectable stimulation of phosphoinositide hydrolysis alone, also enhancing phosphoinositide signalling by so-called Ca^{2+}-mobilising receptors, which signal via G_q G-proteins. The pathways by which G_i-coupled receptors cause inhibition of adenylyl cyclase, and G_q-coupled receptors cause increases in phosphoinositide hydrolysis, are well characterised, and the mechanisms of signalling via each pathway, with regards to mACh receptors, have been described above. This augmentation of PLC and Ca^{2+} signalling has been demonstrated in different cell types, including both CHO cells and a smooth muscle-derived cell-line (DDT_1 MF-2), as well as being demonstrated in some smooth muscle tissues at the level of contraction (Selbie & Hill, 1998).

Following observations that $\beta\gamma$ subunits can augment stimulation of some adenylyl cyclase isoforms by $G_s\alpha$ (Tang & Gilman, 1991), many studies have indicated that this modulation of effector stimulation by $\beta\gamma$ subunits may not be exclusive to adenylyl cyclase activation but may also be involved in the augmentation of PLC activation by G_q G-proteins. Gerwins and Fredholm (1992) reported that the simultaneous activation of ATP receptors and adenosine receptors, by ATP and a selective adenosine A1 receptor agonist respectively, caused a synergistic increase in the formation of $InsP_3$ and mobilisation of intracellular Ca^{2+}. This synergy was sensitive to pertussis toxin pretreatment, and was not dependent on cAMP levels, membrane potential or on the activity of PLA_2 or PKC. Adenosine has also been reported to act synergistically with activation of bradykinin receptors (Gerwins and Fredholm, 1992a), cholecystokinin receptors (Dickenson & Hill, 1996), P_{2Y} purinoceptors (Megson *et al.*, 1995), mACh receptors (El-Etr *et al.*, 1989), α_1 adrenoceptors (Haggblad &

Fredholm, 1987), and histamine receptors (Dickenson & Hill, 1994), in a pertussis toxin-sensitive manner in all cases.

Connor & Henderson (1996) showed that M_3-mACh receptor activation of intracellular Ca^{2+} mobilisation could be modulated by co-activation of δ- and μ-opioid receptors, in SH-SY5Y neuroblastoma cells. They showed that although activation of opioid receptors alone could not induce Ca^{2+} mobilisation from intracellular stores, co-activation of opioid receptors and mACh receptors enhanced the increase in $[Ca^{2+}]_i$ seen with application of muscarinic agonists alone. They showed that this enhancement could not be observed if elevations in $[Ca^{2+}]_i$ equivalent to those produced by M_3-mACh receptor activation were induced by a calcium channel activator, i.e. the enhancement by opioid receptors was dependent on mACh receptor activation and was not revealed simply by an elevation in $[Ca^{2+}]_i$, and this augmentation was pertussis toxin-sensitive. Thus, M_3-mACh receptor signalling can also be modulated by receptors activating G_i-Gproteins, possibly via βγ subunits.

Is there any explicit evidence that βγ subunits are involved directly in this enhancement of phosphoinositide and Ca^{2+} signalling? In membrane preparations of turkey erythrocytes, P_{2Y} purinoceptors cause activation of PLC in the presence of guanine nucleotides. This response was enhanced by 50-100% by administration of purified βγ subunits (Boyer *et al.*, 1989), and the fact that the preparations of βγ subunits did not bind [^{35}S]-GTPγS excluded contamination by α-subunits. However, activation of PLC by ALF_4^- addition was not augmented by the addition of βγ subunits. Also, the direct enhancement of PLC activity by G_i G-proteins is inhibited by the addition of the βγ subunit-binding region of β-adrenergic receptor kinase (Koch *et al.*, 1994). Co-expression of so-called 'βγ subunit scavengers' inhibits both adenosine and NPY receptor augmentation of PLC activity stimulated by addition of ATP in CHO cells (Selbie *et al.*, 1997). In COS-7 cells, adenosine A1 receptors can augment phosphoinositide signalling induced by thyrotropin receptors, in a pertussis toxin-sensitive manner. Over-expression of βγ subunits in these cells enhanced stimulation of inositol phosphate production, without having any effect on the adenosine A1-mediated

inhibition of adenylyl cyclase, but could not further increase the enhancement seen with co-stimulation of adenosine receptors (Tomura *et al.*, 1997). Therefore, these experiments suggest that βγ subunits are responsible for this G_i/G_q cross-talk, but an integrated modulation of PLC activity by βγ subunits and G_q G-proteins is required.

Since it is known that M_2-mACh receptors generally signal via M_2-mACh receptors and M_3-mACh receptors signal via activation of phosphoinositide signalling, it is reasonable to assume that these findings may be relevant to experiments studying M_2- and M_3-mACh receptor co-expression, performed in this Thesis.

1.5 Aims

As discussed above, M_2-mACh receptor expression is an almost universal property of smooth muscles, despite M_3-mACh receptor activation accounting for the rapid onset of contraction mediated by increases in cholinergic transmission. Although the subject of many pharmacological studies, the reason for this co-expression and the contribution made to smooth muscle tone, if any, by M_2-mACh receptors, remains a topic of debate. And yet sense seems to dictate that the conservation of M_2-mACh receptor expression, not only in smooth muscles of one species but seemingly of all mammalian species, must indicate an important function.

So far, work has centred on studies of smooth muscle contraction using various methods of manipulating the mACh receptor population, or looking at the interaction of the whole mACh receptor population with other receptors which affect tone e.g. β-adrenergic receptors, in isolated tissues. This approach has led to some important conclusions, but in general has thrown up many more questions than those which were answered. Extensively, experiments have been performed to look at mACh receptor signalling in smooth muscle or cell-lines. The latter involves the stable transfection of each cloned receptor gene into cell-lines to produce cells, for example CHO-M3 cells, in which the signalling of a particular receptor subtype can be studied. Signalling studies in smooth muscle cells are complex, due to the many 'unknown' factors, such as which other receptors are expressed at the cell membrane, which effectors, G-proteins or signalling pathways are present, and/or utilised, and by how much the mACh receptor population varies between animals. The work presented here attempts to investigate the possible function(s) of M_2-mACh receptors in a novel manner. The main hypothesis of the first part of this Thesis is that M_2-mACh receptors may in some way act to influence the signalling pathways of M_3-mACh receptors when expressed in the same cell. Studies of signalling in cell-lines stably expressing M_2- and M_3-mACh receptors may shed light on 'cross-talk' between the signalling pathways and may suggest how M_2-mACh receptors could influence contraction, illicited by a stimulus at mACh receptors, in

smooth muscle. This may be by synergism, or complete alteration of the usual signalling of the mACh receptor populations.

Since the laboratory was already using Chinese hamster ovary (CHO) cells which stably expressed M_2- or M_3-mACh receptors, these became the starting point for this research. Firstly, the signalling properties of the receptor subtypes, when expressed in this cell type as homogeneous populations, were to be confirmed. The main idea was to then transfect m3 mACh receptor cDNA into CHO cells which already expressed M_2-mACh receptors at the cell surface, at a level not dissimilar to that seen physiologically in smooth muscle tissues. It was hoped that this transfection process would produce cells expressing a heterogeneous population of mACh receptors, with M_3-mACh receptors being expressed at different densities in different clones. It was understood that the population densities must be quantitified so that it could be ascertained that the expression levels were stable, and so that the effect of different receptor densities on signalling could perhaps be investigated. More fundamentally than that, it was by no means certain that introducing cDNA for the m3 mACh receptor into the M_2-mACh receptor-expressing cell-line would result in mACh receptor subtype co-expression. The cDNA could be incorporated, but not translated to a cell membrane protein, or the translation of this receptor gene could prevent the concurrent expression of M_2-mACh receptors. Therefore, initial studies to investigate whether any antagonists available were sufficiently selective to allow quantification of mACh receptor subtypes in a mixed mACh receptor population were designed, using displacement binding studies after physical mixing of plasma membranes prepared from cells expressing known homogeneous mACh receptor populations. These investigations and preliminary signalling studies are described in Chapter 3.

The next stage of the research was to be the transfection of m3-mACh receptor cDNA into CHO-SLM2 cells, and the subsequent characterisation of the cell-lines produced. This characterisation was to be carried out using methodology developed by membrane mixing experiments, and also by other methods, such as antibody detection by Western blotting

techniques. The signalling, both in terms of inositol phosphates and cyclic AMP was to be studied, and any alterations compared to signalling by homogeneous populations was to be noted. This process is described in Chapter 4.

The possibility that M_2- or M_3-mACh receptor signalling pathways may 'cross-talk' with β-adrenoceptor signalling has been addressed by previous research, but the possibility that M_2-mACh receptors may modulate M_3-mACh receptor signalling directly or indirectly has not been studied. This is in part due to the lack of subtype selective ligands which may be used as tools to distinguish between mACh receptors and their function(s). Using data from the first part of the work (as described in Chapter 3), it could be ascertained whether more recent M_2/M_3- 'selective' antagonists could be used to block one subtype in a mixed M_2/M_3-mACh receptor population. Results from experiments using this method of selective removal of one pathway could then be compared with studies using pertussis toxin to block specific subtype signalling. This should shed more light on any 'cross-talk' occuring, by possibly defining the contributions of each receptor subtype to each signalling pathway. These experiments are described in Chapter 5.

Finally, another approach to unravelling the importance of M_2-mACh receptor activation in smooth muscle contraction was to study more closely guinea-pig uterine smooth muscle tissue which has been reported to express only M_2-mACh receptors, with possibly M_4-mACh receptors at a low density, but expresses no detectable levels of M_3-mACh receptor. Preliminary studies in this laboratory (unpublished) using more selective M_2/M_3-mACh receptors had confirmed that contraction was unlikely to be due to M_3-mACh receptors. It was therefore proposed that a larger study of antagonist affinity estimates than had been performed previously should be carried out using studies of guinea-pig isolated uterine smooth muscle, and that this should be compared to affinity estimates made in displacement binding studies in crude membrane preparations, using the same compounds. Finally, signalling at the level of inositol phosphates and cAMP, both of which have been shown to be involved after mACh receptor activation in this tissue, were investigated. If the M_2-mACh

receptor is truly the only receptor involved in stimulation of contraction by mACh receptor agonists, then the study of this tissue may offer clues as to the function of M_2-mACh receptors in other smooth muscle tissues. These experiments and subsequent findings are presented in Chapter 6.

It was hoped that, by these approaches, further light would be shed on the reason for M_2- and M_3-mACh receptor co-expression in most smooth muscles, and that a contribution would be made to the understanding of mACh receptor signalling as a whole.

CHAPTER 2- General Methods

2.1 Cell culture and transfection procedures

Untransfected Chinese hamster ovary cells (CHO-K1) and those stably transfected with cDNA encoding the human m3 mACh receptor (CHO-M3) or cDNA encoding human m2 mACh receptor (CHO-SLM2) were obtained from Dr. N. Buckley and Dr. S. Lazareno, respectively (National Institute for Medical Research, Mill Hill, London, U.K.). CHO cell clones were grown in αMEM (Minimum Essential Medium) supplemented with 10% (v/v) newborn calf serum, 100 IU ml^{-1} penicillin, 100 μg ml^{-1} streptomycin and 2.5 μg ml^{-1} amphotericin B. Cells were maintained at 37°C in 5% CO_2 / humidified air. Cells were grown to confluence in large tissue culture flasks (175 cm^2), harvested using trypsinisation (or EDTA washing in the case of membrane or cell lysate preparation) and reseeded at approximately 1:10 (v:v) into new flasks or into 24 multi-well plates containing 1ml culture medium per well. Cultures used for analysis of free intracellular Ca^{2+} were seeded onto glass coverslips in small petri dishes (3.5 cm diameter) and maintained as above.

To create a transfected cell line containing both M_2- and M_3-mACh receptor subtypes, human m3 cDNA was transfected into CHO-SLM2 cells using a mammalian expression vector. CHO-SLM2 cells had previously been created by transfecting CHO cells with a vector containing the m2-mACh construct and conferring neomycin resistance, therefore in this transfection a vector which conferred hygromycin resistance as a marker of successful transfection was used. The vector pCEP4 was obtained from Invitrogen (see Figure 2.1A) and the m3-mACh receptor construct inserted by the use of Bam HI and Not I restriction sites and subsequent ligation of the m3-mACh sequence with the linearised plasmid. The calcium phosphate-DNA co-precipitation procedure was used, as described by Sambrook *et al.*, (1989), which increases the uptake of cDNA into cultured cells by presenting the nucleic

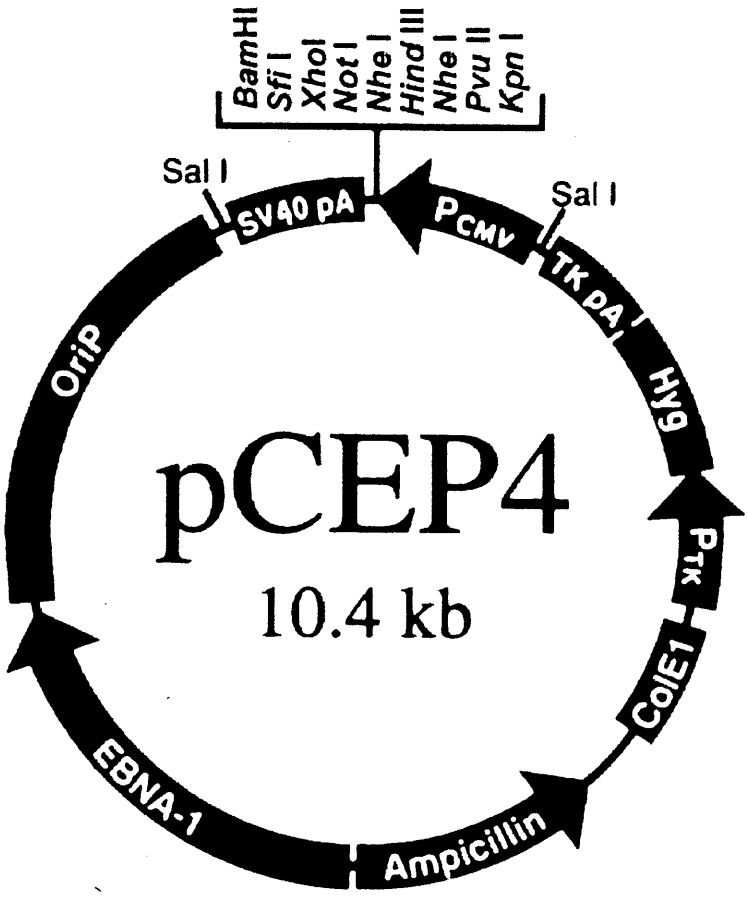


Figure 2.1A- Mammalian expression vector used for transfection of m3-mACh receptor cDNA into SLM2 cells.
m3-mACh receptor cDNA was cloned into the vector using Bam HI and NotI restriction sites. pCEP4 is a human cytomegalovirus immediate-early gene enhancer-promoter. Hygromycin B is an aminoglycosidic antibiotic that inhibits protein synthesis by disrupting translocation and promoting mistranslation at the 70S ribosome. As it has a different mode of action than G418 (neomycin derivative which interferes with function of 80S ribosomes and blocks protein synthesis in eukaryotic cells) it can be used in dual selection experiments. Resistance to hygromycin B is conferred by the *E.coli* hygromycin resistance gene (Hyg).

acid to the cells as a calcium phosphate-DNA precipitate. Twenty-four h before transfection, CHO-SLM2 cells were harvested by trypsinisation and replated onto 5 cm diameter petri dishes at a low cell density (1:20 v:v). At transfection these cells were no more than 50% confluent and were thus still in a stage of exponential growth. 10 μg cDNA was diluted to a final volume of 440 μl in 0.1 M TE (consisting of 1 mM Tris HCl, 0.1 mM EDTA pH 8.0, sterile filtered), and 500 μl of 2 × HBS was added (280 mM NaCl, 10 mM KCl, 1.5 mM Na_2HPO_4, 11 mM glucose, 50 mM HEPES, pH 7.05, sterile filtered). Slowly, 67 μl 2 M $CaCl_2$ was mixed into the solution whilst air was bubbled through the solution using an electric pipetting device to encourage formation of a fine calcium phosphate-DNA precipitate. The mixture was incubated at room temperature for 30 min, during which time a precipitate formed. At the end of the incubation, the precipitate was resuspended and the calcium phosphate-DNA suspension was transferred to the medium above the cell monolayer which was then incubated for 24 h at 37°C in 5% CO_2 / humidified air. The medium was then removed, the cells were washed in sterile buffered saline solution (0.9% NaCl, 10 mM HEPES, pH 7.45) and grown to confluence in complete growth medium. The cells were then seeded at various densities into petri dishes in selection medium (growth medium as before, supplemented with hygromycin B (400 μg ml^{-1} for initial transfection; 800 μg ml^{-1} for second transfection)) to isolate stable transformants. This medium was replaced regularly and dead cell debris removed to allow colonies of antibiotic-resistant cells to grow. When colonies were easily observable above background, individual colonies were isolated and grown separately in selection medium. After 2-4 weeks, clones were screened as described in Chapter 4.

2.2 Membrane preparation for radioligand binding studies

Transfected cell lines

Cells were harvested using 10 mM HEPES, 0.9% NaCl, 0.02 % EDTA solution (pH 7.4), and the cell suspension homogenised in ice-cold buffer (10 mM HEPES, 10 mM EDTA pH

7.4) using 5 × 4 sec bursts of a Polytron tissue disrupter. The homogenate was centrifuged at 4,000 × *g* for 5 min to remove 'heavy debris' (e.g. complete cells), and then the supernatant was spun at 40,000 × *g* for 20 min at 4°C. The supernatant was then discarded and the pellet resuspended in homogenising buffer. The pellet was re-homogenised using 5 × 4 sec bursts of the Polytron tissue disrupter and the high speed spin step repeated as before. The supernatant was removed and the pellet resuspended in HEPES buffer A (10 mM HEPES, 10 mM $MgCl_2$, 100 mM NaCl, pH 7.45). Protein concentration was determined as described below, and membrane stocks were diluted to approximately 1 mg protein ml^{-1} before being frozen at -80°C for future use. The addition of a variety of protease inhibitors to the buffers used in the preparation and storage of these membranes made no significant difference to the saturation binding isotherms recorded (Results not shown), and therefore it was assumed that no significant receptor degradation occurs in this case. Proteases were therefore omitted from the buffers used during membrane preparations for all binding experiments in this Thesis.

Bovine tracheal smooth muscle

Bovine tracheal smooth muscle (BTSM) membranes were prepared in a similar manner. Bovine tracheal tissue was obtained from young cattle immediately after slaughter at a local abattoir, where the smooth muscle was rapidly removed prior to transport back to the laboratory in ice-cold, pre-oxygenated (95% O_2 / 5% CO_2) KHB buffer (see Appendix 1 for composition). After careful removal of epithelial and connective tissue, the tissue was chopped roughly into small pieces using scissors and homogenised in ice-cold homogenising buffer (as for cell membrane preparation) using 2 × 15 sec bursts of the Polytron tissue disrupter. More homogenising buffer was added and the homogenate was spun at 40,000 × g for 20 min at 4°C. The supernatant was discarded, the pellet resuspended in buffer (as before) and the homogenisation and centrifugation steps repeated as before. The pellet was then resuspended in 10 mM HEPES, 1 mM EDTA (pH 7.4), and the homogenisation and centrifugation steps repeated once more. The membranes were then

filtered through double thickness muslin, resuspended in HEPES buffer A (as for cell membrane preparation), the protein content determined and the membranes diluted to approximately 5 mg protein ml^{-1} before storage at -20°C.

2.3 Radioligand binding studies

Total mACh receptor number was determined by [^{3}H]-NMS saturation binding in intact cells as described by Lambert *et al.*, (1989). Cells were grown to confluence and harvested using 10 mM HEPES, 0.9% NaCl, 0.02 % EDTA solution (pH 7.4). Cells were then pelleted and resuspended in HEPES buffer A (10 mM HEPES, 10 mM $MgCl_2$, 100 mM NaCl, pH 7.45), and protein concentration determined (as below). A range of concentrations of [^{3}H]-N-methylscopolamine (NMS) from 0.03 to 3 nM was incubated with aliquots of the cell suspension (approximately 25 μg protein) in triplicate in the absence and presence of atropine (1 μM; to define non-specific binding) to construct saturation binding curves. Assays were performed at 37°C for 90 min and tubes were gently shaken during this period. Specific binding represented greater than 90% of the total [^{3}H]-NMS binding, and the protein concentrations were such that total [^{3}H]-NMS bound was less than 20 % of total radiolabel added. Values calculated for the B_{max} and K_d of [^{3}H]-NMS in intact cells were not significantly different from those calculated if membrane preparations were used, suggesting that most of the expressed mACh receptors occur at the cell surface in this cell line under these conditions. Saturation binding assays for bovine tracheal smooth muscle were performed in the same way, but BTSM membranes were used in place of cell suspensions. In this case, the non-specific binding also made up <10% of the total [^{3}H]-NMS binding. Antagonist displacement of [^{3}H]-NMS binding was performed in membrane preparations in HEPES buffer A for 90 min at 37°C, using approximately 0.8 nM [^{3}H]-NMS and as wide and detailed a range of antagonist concentrations as possible, in triplicate. For membrane mixing experiments, the membranes were mixed according to receptor number per unit protein to give ratios of 50 : 50 and 70 : 30 M_2- :M_3-mACh receptors.

In all radioligand binding studies, bound and free ligand was separated by rapid vacuum filtration through Whatman GF/B filters followed by 2 × 4 ml washes with ice-cold HEPES buffer A. Filters were removed to scintillation vials containing 5 ml scintillant and radioactivity was quantified > 12 h later by liquid scintillation spectrophotometry.

2.4 Measurements of cyclic AMP mass

Sample generation

Experiments were performed on cells grown to confluence in 24 well multiplates. The growth medium was removed and the cells washed in oxygenated Krebs buffer (KHB) (see Appendix 1) and allowed to acclimatise in 1 ml KHB for 10 min at 37°C before commencing experiments. The experiments were performed in KHB buffer at 37°C in a shaking water bath, with the final experimental volume being 500 µl. The cells were incubated with or without MCh (various concentrations) for 15 min, before being challenged with forskolin (10 µM) for 5 min. An experiment was carried out to determine the order in which forskolin and the mACh receptor agonist should be added to the cells. Cells were incubated as above, with forskolin incubation first or with both agents being added simultaneously (Figure 2.1). There was no significant difference in the levels of cAMP mass measured in each case. In some experiments, pertussis toxin (100 ng ml^{-1} in growth medium) was added for 24 h prior to the experiment. Any antagonists used were incubated 30 min prior to MCh administration. Phosphodiesterase inhibitors were not used as they did not significantly increase the amount of cAMP measured in these experiments. Reactions were terminated by removal of the buffer and the addition of 250 µl of trichloroacetic acid (TCA; 0.5 M). The plates were then left on ice for 15 min, before the solution was removed from the wells and transferred to polycarbonate tubes. 3 ml of water-saturated diethylether was mixed thoroughly with each sample, which were then left to stand for 5 min before the upper phase was removed and discarded. This was repeated 3 times for each sample. Any remaining diethylether was left to evaporate from the samples, and then an aliquot (150 µl) was taken from each sample and neutralised with 50 µl of 60 mM $NaHCO_3$ to pH 7.4. The samples were stored at 4°C for up to one week before cAMP determination. The cell debris left in the plates was analysed for protein content as described below.

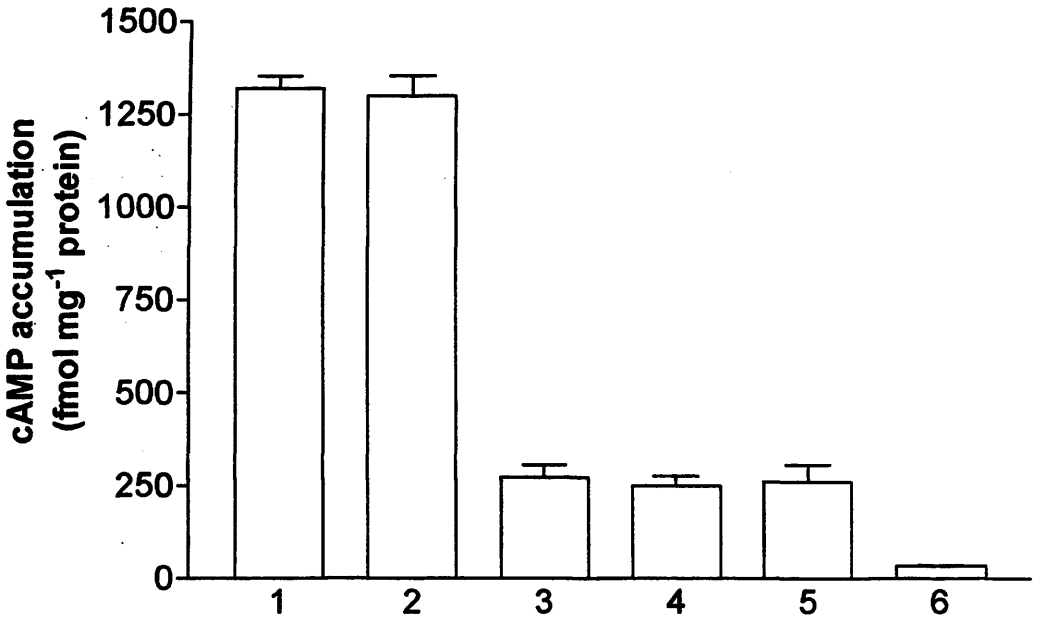


Figure 2.1 - Forskolin stimulation and methacholine-induced inhibition of forskolin-stimulated cAMP accumulation in CHO-SLM2 cells : Effects of different sequences of additions and incubation times

Data are from a single experiment performed in triplicate. Forskolin and MCh were incubated as detailed below in KHB at 37°C in a shaking water bath. Incubations were ended by the removal of buffer and addition of 0.5 M TCA. cAMP was extracted and assayed as described in Methods.

1 = Forskolin (10 μM) 5 min
2 = Forskolin (10 μM) 20 min
3 = MCh (1mM) 15 min followed by forskolin (10 μM) 5 min (MCh total inc. time = 20 min)
4 = Forskolin (10 μM) 5 min followed by MCh (1mM) 15 min (Forskolin total inc. time = 20 min)
5 = Forskolin (10 μM) and MCh (1mM) added simultaneously and incubated 15 min
6 = No addition- stopped after 20 min

Cyclic AMP determination

Cyclic AMP mass was quantified using a radioreceptor assay essentially as described by Brown *et al.*, (1971). Cyclic AMP levels were assessed through competitive binding to a sample of prepared binding protein (a crude preparation of the regulatory subunit of cAMP-dependent protein kinase isolated from bovine adrenal cortex, as described below) with a known concentration of [^{3}H]-cAMP. 50 µl of sample or a known concentration of cAMP standard (2.5 - 200 nM, made up in assay buffer 50 mM Tris-HCl and 4 mM EDTA, pH7.5; NSB = 250 pmol cAMP / 50 µl)) was added to microfuge tubes containing 100 µl of [^{3}H]-cAMP (approximately 4 nM; specific activity 28.9 Ci mmol^{-1}). Binding protein preparation (150 µl; approximately 1mg ml^{-1} in assay buffer) was then added to the tubes to begin the reaction, tubes were capped, vortexed thoroughly and incubated for >90 min at 4°C. Equilibrium was reached by 90 min at 4°C, but longer incubation times (e.g. overnight) were without detriment. Separation of bound and free [^{3}H]-cAMP was achieved by the addition of 250 µl of an ice-cold suspension of activated charcoal (0.25 g / 50ml) in bovine serum albumin solution (0.1 g / 50ml in assay buffer), followed by vigorous mixing. The samples were then left on ice for 7 min prior to centrifugation at 14,000 × *g* for 4 min. Radioactivity in the bound fraction was then determined by counting of 0.4 ml of the supernatant by liquid scintillation spectrophotometry.

Preparation of cyclic AMP binding protein

The cAMP binding protein was partially purified from a bovine adrenal gland preparation according to the method of (Brown *et al.*, 1971). Bovine adrenal glands, obtained from a local abattoir, were cleaned of fat and connective tissue and the medulla of each removed while on ice. The cortex was de-capsulated and finely chopped into 2 volumes of ice cold buffer (50 mM Tris-HCl, 4 mM EDTA, pH 7.5). The tissue was homogenised, filtered through two layers of muslin and centrifuged at 15,000 × *g* for 15 min at 4°C. The supernatant was collected after any contaminating fat had been removed, and was

centrifuged again for a further 15 min. The final supernatant was dispensed into samples and stored at -20°C. New batches were diluted to match previous batches of binding protein.

2.5 Measurements of CMP-phosphatidic acid accumulation

CMP-phosphatidic acid (CMP-PA) accumulation was assayed according to the method described by Kennedy *et al.*, (1990). Cells were grown to confluence in 24 well multiplates, the growth medium removed, the cells washed in KHB (see Appendix 1) and incubated in KHB solution containing 0.3 μCi ml^{-1} [^{3}H]-cytidine at 37°C for 60 min in a shaking water bath. The buffer was then removed, the cells washed with unlabelled buffer and further incubated at 37°C in fresh KHB or KHB containing LiCl (10 mM) for 15 min. The cells were challenged with MCh (in a total volume of 500 μl), and the reaction stopped by the addition of ice cold TCA (500 μl; 1 M). After standing for 15 min on ice, the solution was removed from the wells and discarded, and the wells were washed with 5% TCA containing 1 mM EDTA. CMP-PA was extracted from the wells by the addition of acidified methanol/chloroform (0.94 ml; 2:1 (v/v) containing 0.1 M HCl), scraping the cell debris carefully from each well. The aqueous and organic phases were resolved by addition of 0.31 ml chloroform and 0.56 ml of 0.1 M HCl to each sample. The samples were left to stand for 10 min before centrifugation at 3500 × *g* for 5 min. The aqueous phase was discarded and an aliquot (350 μl) of the organic phase was transferred to a scintillation vial and allowed to evaporate to dryness overnight at room temperature. Incorporation of [^{3}H]-cytidine into CMP-PA was then determined by liquid scintillation counting. Under these conditions at least 95 % of the [^{3}H]-cytidine labelled lipids recovered are in the form of CMP-PA (Godfrey, 1989).

2.6 Measurements of total inositol phosphate accumulation

Labelling and sample preparation

Cells were grown to confluence in 24 well multiplates, and labelled with 1 μCi ml^{-1} [^{3}H]-*myo*-inositol for 48 h prior to the start of the experiments. Cells were washed with oxygenated KHB to remove tissue culture medium, and incubated in KHB ± Li^+ (10 mM) for 15 min in a shaking water bath at 37°C. Lithium has been shown to uncompetitively inhibit inositol monophosphatase activity (Nahorski *et al.*, 1991), thus preventing dephosphorylation of inositol monophosphates and allowing accumulation of inositol phosphates ($InsP_x$) to be assessed as an index of PLC activation (Berridge *et al.*, 1982). Reactions were initiated by the addition of agonist or KHB to give a total volume of 500 μl. Antagonists were incubated for 30 min prior to stimulation. The experiment was terminated with the removal of buffer and the addition of ice-cold TCA (500 μl; 0.5 M). After 15 min on ice, the samples were removed to polypropylene tubes and extracted using 3 washes with 3 ml diethylether, as for cAMP sample preparation. Samples were neutralised with 60 mM $NaHCO_3$ and stored at 4°C for up to 1 week prior to [^{3}H]-$InsP_x$ separations.

[^{3}H]-$InsP_x$ separations

Separation was achieved using a method based on that described by Challiss *et al.*, (1992). Samples were washed over a 0.5 ml bed-volume Dowex AG1-X8 anion exchange resin (Cl^- form; 50-100 mesh) in a glass column with 20 ml water. [^{3}H]-glycerophosphoinositol was removed by washing with 10 ml of 25 mM NH_4COOH (ammonium formate). The bound [^{3}H]-$InsP_x$ fraction was eluted with 10 ml of 1 M HCl, of which 2 ml were taken for scintillation counting. Regeneration of the Dowex resin was achieved by further addition of 10 ml of HCl (1 M) followed by repeated washing with water.

2.7 Measurements of $Ins(1,4,5)P_3$ mass

Sample preparation

Intact cells plated into 24 well multiplates were washed with KHB and allowed to stabilise over 15 min in KHB at 37°C in a shaking water bath. Antagonists were incubated for 30 min prior to challenge by addition of MCh at specific concentrations for defined time periods. If a concentration - peak response curve was being constructed, MCh (at each concentration) was incubated for 10 s before termination of the reaction. Total experimental volume was 500 µl in KHB. Reactions were terminated by addition of an equal volume of TCA (0.5 ml; 1 M). Samples were extracted using diethylether extraction as described previously for cAMP sample preparation, neutralised to pH 7.8 and stored in the same way.

$InsP_3$ mass assay

$Ins(1,4,5)P_3$ mass assays were performed as described by Challiss *et al.* (1988). 30µl aliquots of samples or known concentrations of $InsP_3$ (1.2 - 1200 nM; diluted in neutralised diethylether-extracted TCA) were added to 30 µl assay buffer (100 mM Tris-HCl, 4 mM EDTA, pH 8.0) and 30 µl buffer containing 5,000-10,000 d.p.m. $[^3H]$-$Ins(1,4,5)P_3$. Finally, 30 µl of $InsP_3$ binding protein (15 - 18 mg ml^{-1}; bovine adrenal preparation as below) was added, and the tubes were vortexed thoroughly before incubating on ice for 45 min. Non-specific binding was defined by an excess of $InsP_3$ (10 µM). Separation of bound and free $[^3H]$-$InsP_3$ was achieved by vacuum filtration through Whatman GF/B filters and washing with 2 × 4 ml ice-cold wash buffer consisting of 25 mM Tris-HCl, 1 mM EDTA, 5 mM $NaHCO_3$, pH 8.0. Filters were removed to scintillation vials containing 5 ml scintillant and radioactivity was quantified > 12 h later by liquid scintillation spectrophotometry.

Preparation of Ins(1,4,5)P_3 binding protein

Fresh bovine adrenal glands were obtained from a local abattoir and were cleaned, de-medullated and de-capsulated on ice. 60-80 g of cortex was homogenised in 8 volumes of ice-cold homogenisation buffer (20 mM $NaHCO_3$, 1 mM dithiothreitol, pH 8.0) and centrifuged for 15 min at 5,000 × *g* at 4°C. The supernatant was removed and retained, and the pellet resuspended and homogenised again in 4 volumes of homogenisation buffer prior to centrifugation as above. The supernatant fractions from both spins were pooled and centrifuged at 38,000 × *g* at 4°C for 20 min. The pellet from this final spin was washed twice and resuspended in homogenisation buffer at a concentration of 15-18 mg ml^{-1}. Binding protein was stored in aliquots at -20°C until use.

2.8 SDS-PAGE, Western blotting and immunodetection techniques

Simple cell lysate preparation

One 175 cm^2 flask of cells was harvested using 10 mM HEPES, 0.9% NaCl, 0.02 % EDTA solution (pH 7.4), centrifuged at 4,000 × *g* and the supernatant discarded. On ice, the pellet was resuspended in 5 ml of lysis buffer (20 mM Tris-HCl, 1 mM dithiothreitol, 5 mM EGTA, 2 mM EDTA, 0.5 mM phenylmethylsulphonyl fluoride (PMSF), 1 μg ml^{-1} aprotinin, 10 μM benzamidine-HCl, 5 μM iodoacetamide) and homogenised with 10 passes with a glass Teflon homogeniser. Protein concentration was determined and samples diluted accordingly. 15 μl sample with 15 μl sample buffer (see Appendix 2) was placed in a water bath at ~70°C for 5 min to denature the proteins.

Tris-glycine SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Westen blotting

Samples were resolved by SDS-PAGE analysis using 10% (w/v) polyacrylamide slab gels (see Appendix 2). Denatured samples and markers were loaded onto the gel and run at 200 V

for approximately 45 min. For running buffer see Appendix 2. The molecular weight markers consisted of the following:

MWt (KDa)	Marker
106	Phosphorylase B
80	Bovine serum albumin
49.5	Ovalbumin
32.5	Carbonic anhydrase
27.5	Soybean trypsin inhibitor
18.5	Lysozyme

After gel electrophoresis, proteins were transferred electrophoretically to nitrocellulose membrane by 0.65 mA per cm^2 for about 2 h using a transfer buffer containing 0.037% SDS as described in Appendix 2. Nitrocellulose buffers were blocked with Tris-buffered saline (TBS; 20 mM Tris-base, 500 mM NaCl, pH 7.5) supplemented with 0.05% (v:v) Tween-20 (polyoxyethylene-sorbitan monolaurate) and 5% (w:v) dried milk powder for 24 hr at 4°C. The blocking buffer was replaced and then the human m3-antibody was added at 1 : 1000 (v:v) dilution and incubated for 90 min at room temperature with shaking. The nitrocellulose was then vigorously washed using TBS for 1 × 5 min and 3 × 20 min at room temperature. The wash period can be harsh as the antibody has high affinity for the receptor, therefore only non-specific background binding will be removed even by such vigorous wash steps. Horseradish peroxidase-anti-rabbit IgG conjugate was then added at a 1 : 1000 dilution and incubated at room temperature for 30 - 45 min in the presence of the blocking buffer. Membranes were washed as before but with gentler agitation and then chemiluminescence was detected using ECL Western blotting detection reagents and exposure for varying times to photographic paper.

2.9 Protein concentration determination

Protein concentration was estimated using the method of Lowry *et al.*, (1951). Cell debris was digested from multiwell plates by >2 h incubation with 0.5 ml 0.1 M NaOH solution and the resulting solution was then diluted with 0.5 ml water and vortexed thoroughly. Cell solutions were also added to 0.5 ml 0.1 M NaCl and then made up to a total volume of 1 ml with water. Further dilutions were made if necessary. Lowry C reagent (1 ml; 2 % Na_2CO_3, 0.4 % NaOH, 1 % $CuSO_4$ and 2 % sodium potassium tartrate) was then added to 200 µl of this sample solution, vortexed and left to stand at room temperature for 10 min. Folin/Ciocalteau reagent (100 µl of 1 : 3 dilution) was then added, the samples vortexed and left for another 20 min at room temperature. The samples were diluted with 1 ml water and the absorbance at 750 nm was measured using a spectrophotometer. Protein concentration was calculated by the use of a calibration curve constructed from bovine serum albumin standards, prepared in the same way as the samples, for each assay.

2.10 Single cell Ca^{2+} imaging

CHO cells were seeded onto coverslips (22 mm diameter) 16-24 h prior to experiments, in culture dishes (35 × 10 mm) in normal growth medium. On the day of experimentation the coverslips were washed twice with oxygenated KHB buffer (see Appendix 1), and then incubated in the dark at room temperature in KHB supplemented with 2 µM fura-2 acetoxymethyl ester (fura-2 AM) and 1 mg ml^{-1} bovine serum albumin. The coverslips were then washed twice in KHB and incubated for a further 30 min in KHB (22°C), to allow for complete de-esterification of the intracellular dye, before being mounted on the stage of a Nikon Diaphot inverted epifluorescence microscope. KHB (22°C) was continuously perfused over the cells at a perfusion rate of 5 ml min^{-1}. Agonists were applied via the perfusion buffer. After subtraction of background fluorescence, images at wavelengths above 510 nm, after excitation at 340 and 380 nm (40 ms at each wavelength), were collected with an intensified charge-coupled device camera (ICCD camera; Photonic

Science). Experiments were conducted on a Quanticell 700 (Applied Imaging) system (see Figure 2.2).

Ratiometric values were converted to approximate $[Ca^{2+}]_i$ using the equation (Grynkiewicz *et al.*, 1985):

$$[Ca^{2+}]_i = K_d \times \{(R-R_{min})/(R_{max}-R)\} \times \{F_{min}(380\ nm)/F_{max}(380\ nm)\}$$

where K_d is the dissociation constant of Ca^{2+} for fura-2 free acid (135 nM at 22°C), R_{min} and R_{max} are the minimal and maximal fluorescent ratios obtained in a cell-free solution (containing fura-2) in the presence or absence of a saturating concentration of Ca^{2+} (see below), and F_{min} (380nm) and F_{max} (380 nm) are the fluorescent intensities after excitation at 380 nm in the absence and presence of Ca^{2+}, respectively.

For calibration using a cell-free solution, 5 µl drops of fura-2 in 100 mM KCl, 10 mM HEPES, 1 mM $MgCl_2$ and 10 mM EGTA pH 7.2 at 22°C were applied to a cover slip. One drop also contained Ca^{2+} at a concentration which is able to saturate the EGTA in the solution (10 mM). Measurements of fluorescent ratios of these two drops gave R_{max} (+ Ca^{2+}) and R_{min} (-Ca^{2+}).

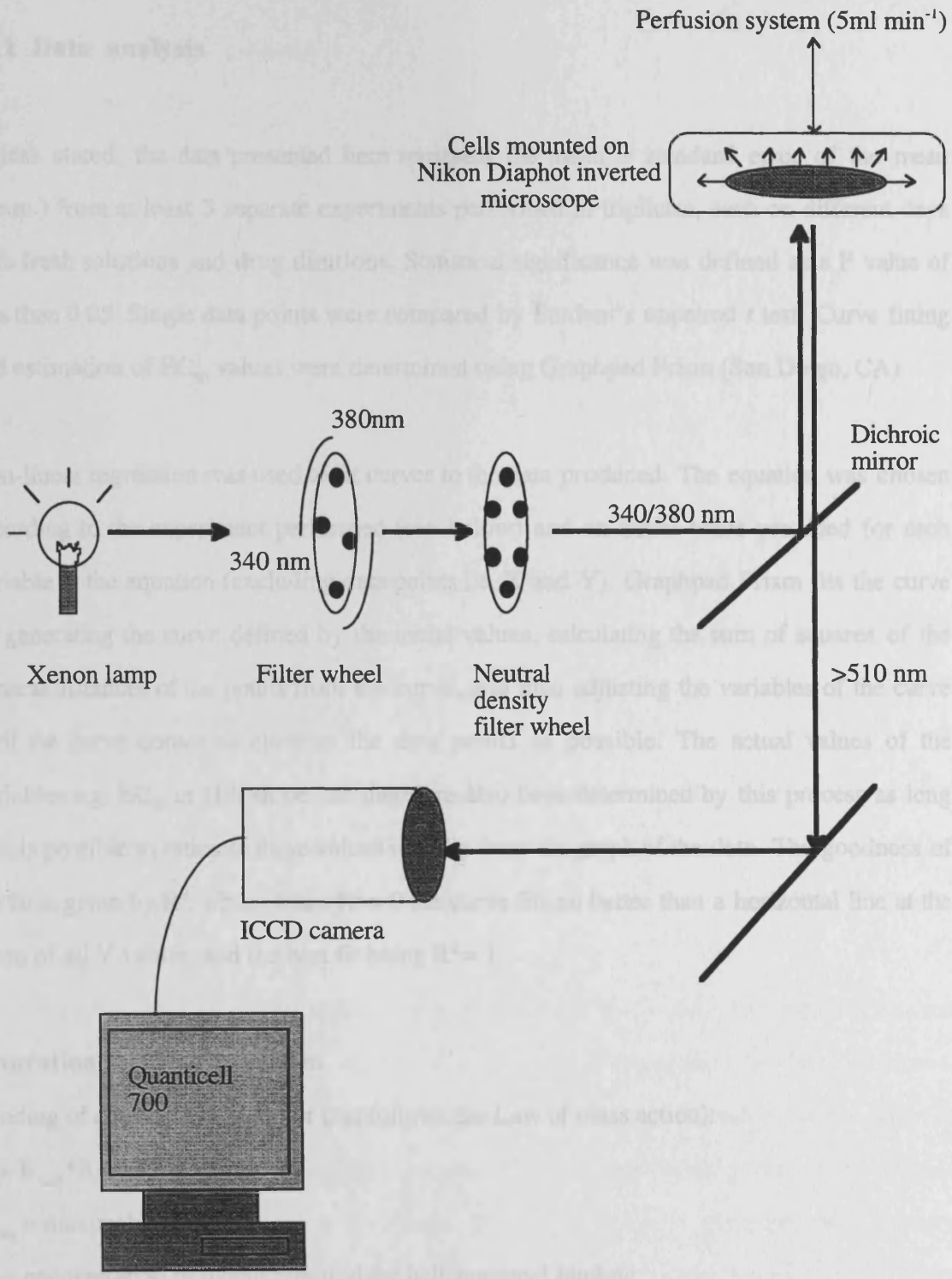


Figure 2.2 - Schematic diagram of single cell Ca^{2+} imaging aparatus

2.11 Data analysis

Unless stated, the data presented here represent the mean ± standard error of the mean (s.e.m.) from at least 3 separate experiments performed in triplicate, each on different days with fresh solutions and drug dilutions. Statistical significance was defined as a P value of less than 0.05. Single data points were compared by Student's unpaired *t* test. Curve fitting and estimation of EC_{50} values were determined using Graphpad Prism (San Diego, CA).

Non-linear regression was used to fit curves to the data produced. The equation was chosen according to the experiment performed (see below) and an initial value provided for each variable in the equation (excluding data points i.e. X and Y). Graphpad Prism fits the curve by generating the curve defined by the initial values, calculating the sum of squares of the vertical distances of the points from the curve, and then adjusting the variables of the curve until the curve comes as close to the data points as possible. The actual values of the variables e.g. EC_{50} or Hill slope can therefore also been determined by this process as long as it is possible to estimate these values initially from the graph of the data. The goodness of the fit is given by R^2, where when $R^2 = 0$ the curve fits no better than a horizontal line at the mean of all Y values, and the best fit being $R^2 = 1$.

Saturation binding isotherm

(binding of a ligand to a receptor that follows the Law of mass action):

$Y = B_{max}*X/K_d+X$

B_{max} = maximal binding

K_d = concentration of ligand required for half-maximal binding

1 site Competition binding

$Y = Bottom + [Top-Bottom]/1+ 10\text{^}(X-logEC_{50})$

X = log concentration of unlabelled ligand

Y = binding

2 site Competition binding

Span = Bottom + Top

Part 1 = Span*Fraction1/1+10^(X-logEC_{50}1)

Part 2 = Span*(1-Fraction1/1+10^(X-logEC_{50}2)

Y = Bottom + Part 1 + Part 2

Fraction 1 + fraction of sites that have affinity 1

X = log concentration of unlabelled

Y = binding

EC_{50}1 and 2 = EC_{50} values for each site

Sigmoidal curves with variable slopes

Y = Bottom + (Top-Bottom)/1+ 10^((logEC_{50}-X)*Hill slope)

X = log concentration

Y = response

When generating a theoretical curve, Graphpad Prism generates the curve which would have been produced had the initial values of the variables selected been true. This can be done from the "New analysis" options from a results sheet or data table.

To compare two fits to decide which is more appropriate for the data, Graphpad Prism can again be used. Goodness of fit is quantified by the sum of the squares as described above, but can only be used to define the best equation fit if the number of variables are the same. In the case, for example of 1 and 2 site competition curves, more often curves with different numbers of variables are being compared. The curve which is generated by the more complicated equation will nearly always come closer to the points because it has more inflection points. The F test assesses whether the decrease in the sum-of-squares is worth the "cost" of the additional variables (degrees of freedom). It does so by calculating a P value which answers the question : If the simpler model is really correct, what is the chance that the data which fits the more complicated model could have been obtained randomly? If the P value is low, then the more complicated equation fits the data significantly better than

the simpler model. The results of the F test are only strictly valid if the simpler equation is a special case of the more complicated model e.g. one and two binding site curves. The F ratio quantifies the relationship between the relative increase in the sum-of-squares and the relative increase in degrees of freedeom such that :

$$F = \frac{(SS1 - SS2)/SS2}{(DF1-DF2)/DF2}$$

If the simple model is correct then the F ratio is near to 1.0. If the ratio is much greater than 1 then either the more complicated model is correct, or the simple model is correct but random scatter led the more complicated equation to be a better fit. The chances of the second option being true are assessed by analysis of variance to produce a P value. If the P value is low then model 2 is significantly better than model 1.

CHAPTER 3 - Quantification of M_2- and M_3-mACh receptor numbers in a mixed receptor-subtype population and determination of second-messenger signalling of homogeneous mACh receptor subtype populations in Chinese hamster ovary cells

3.1 Introduction

The existence of two main classes of acetylcholine receptors, the muscarinic and nicotinic ACh receptors, were first defined by the specific agonism of muscarine and specific antagonism of atropine for muscarinic receptors (for review see Burgen, (1995)). These naturally occurring compounds are highly selective for muscarinic acetylcholine receptors over nicotinic receptors, however the pharmacological classification of the individual subtypes of muscarinic receptors has been more problematic. A major aim of research in this area has been to identify subtype specific ligands for the treatment of Alzheimer's disease, stress incontinence, irritable bowel syndrome and other disorders involving specific mACh receptor subtype activity deficit or augmentation. This has failed thus far, especially regarding the search for agonists that can discriminate between subtypes (Caulfield, 1993). Selectivity is essential for therapeutic use given the incredibly widespread expression of muscarinic ACh receptors throughout the human body and their varying function. Advances have been made in the identification of subtype selective antagonists, although each mACh receptor subtype must still be defined according to an affinity profile of antagonists, usually using a number of key ligands with limited specificity. Therefore, although it is known from cloning work that five mACh receptor subtypes encoded by five distinct genes exist (Bonner *et al.*, 1987; Bonner *et al.*, 1988; Kubo *et al.*, 1986; Kubo *et al.*, 1986; Peralta *et al.*, 1987; Peralta *et al.*, 1987) and that these can be distinguished using antisera (Tobin & Nahorski, 1993; Wall *et al.*, 1991; Yasuda *et al.*, 1993), the operational classification of mACh receptor subtypes is by no means clear cut (Caulfield, 1993).

In general, M_1-, M_3-, and m5-mACh receptors preferentially couple to the $G_{q/11}$ family of pertussis toxin-insensitive G-proteins to augment phosphoinositide hydrolysis via phosphoinositide-specific phospholipase C, and hence mobilisation of intracellular Ca^{2+} via IP_3 production and protein kinase C activation via diacylglycerol liberation. M_2- and M_4-mACh receptors couple via the G_i family of pertussis toxin-sensitive G-proteins to the inhibition of adenylyl cyclase activity (see Introduction Chapter). However, mACh receptors can promiscuously couple to several signalling systems (for review see Fukuda *et al.*, (1989)) and other signalling systems have been identified, including activation of certain membrane ion channels, phospholipase A_2 and D activation and tyrosine kinase activity (Felder, 1995). Therefore classification of subtypes based solely on signal transduction pathway activation is also inadequate.

This difficulty in pharmacological discrimination of mACh receptor subtypes meant that early binding studies in smooth muscle tissues failed to report any heterogeneity of the mACh receptor population. This was due to both poor M_2/M_3-mACh receptor selectivity of the ligands used (e.g. N,N-dimethyl-4-piperidinyl diphenylacetate (4-DAMP) and gallamine), and the fact that M_2- outnumber M_3-mACh receptors by up to 80%: 20% in most smooth muscle tissues making two-site analyses difficult. One of the objectives of this Thesis is to investigate possible roles of M_2-mACh receptor function to help to understand why M_2/M_3 mACh receptor co-expression is a recurring motif of smooth muscle. Transfection of mACh receptor cDNA into cell lines which do not normally express muscarinic receptors allows the creation and study of a known population of mACh receptors. As part of the work described here M_3-mACh message has been introduced into a Chinese Hamster ovary cell line already stably expressing M_2-mACh receptors (CHO-SLM2) in order to create a mixed population of mACh receptors and therefore it is imperative to be able to quantify the number of each receptor subtype expressed in the new cell lines created. Since total mACh receptor numbers are calculated using saturation binding of [^{3}H]-N-methylscopolamine which is not selective between subtypes, an increase in saturation binding does not necessarily indicate the successful introduction of the M_3-mACh

receptor subtype into the cell, but could indicate the sub-cloning of a cell line expressing a higher level of M_2-mACh receptors than the original 'parent' cell line.

This chapter describes the methodology developed to assess accurately the receptor densities of the two mACh receptor subtypes when co-expressed in a cell line derived from a single parent cell. This involves the study of displacement of specific binding of [^{3}H]-N-methylscopolamine by certain M_2/M_3-'selective' mACh receptor antagonists in CHO cell membranes and in smooth muscle preparations. The signalling properties of the two receptor subtypes has also been studied when each receptor subtype is expressed alone in CHO cells. This has provided ways in which characterisation of the mACh receptor populations of co-transfects can be performed on the basis of antagonist affinity and receptor function.

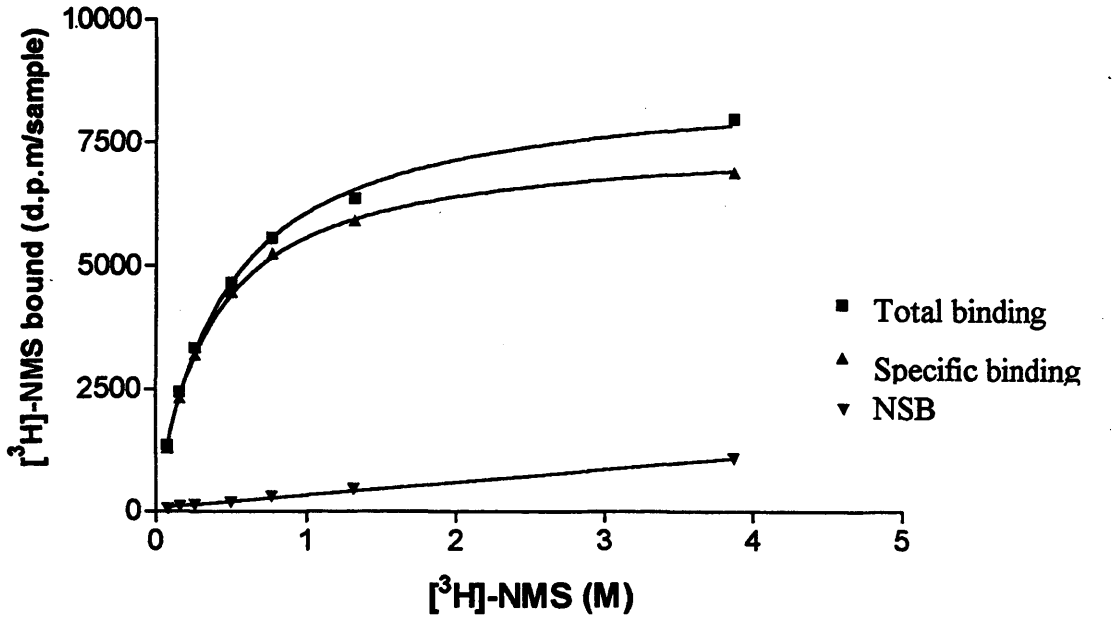
3.2 Results

3.2.1 [^{3}H]-N-methylscopolamine saturation binding and antagonist displacement in CHO-SLM2 and CHO-M3 cell membranes

[^{3}H]-N-methylscopolamine saturation binding data from CHO cell membranes expressing M_2-mACh receptors (CHO-SLM2) and M_3-mACh receptors (CHO-M3) were analysed using an iterative non-linear curve fitting procedure of binding isotherms using Graphpad software (Graphpad Prism, San Diego CA) and according to the method of Scatchard, (1949). There was no statistical difference in estimates of K_D or B_{max} by each method of analysis. Examples of [^{3}H]-NMS saturation binding isotherms are shown in Figure 3.1. B_{max} and K_D values for [^{3}H]-NMS in CHO-SLM2 membranes and CHO-M3 membranes from 5 separate experiments are shown in Table 3.1. The non-specific binding for this ligand is low, allowing specific binding to be easily measured. The incubation time of 90 min at 37°C is sufficient time for equilibrium to be reached for this ligand at these receptors. K_Ds for [^{3}H]-NMS were not significantly different at mACh receptors in CHO-SLM2 compared to CHO-M3 membranes, indicating the lack of specificity for mACh receptor subtypes of [^{3}H]-NMS. The K_D values did however agree with values previously reported for [^{3}H]-NMS at mACh receptors. Binding isotherms for both membranes show clear saturation and therefore the B_{max} can be taken to indicate the density of mACh receptor expressed in each cell type (total number of binding sites for this ligand), assuming that the [^{3}H]-NMS is not being metabolised. The Hill coefficient of approximately 1 cannot conclusively indicate that there is a homogeneous population of binding sites in each cell type given the lack of selectivity of [^{3}H]-NMS.

Antagonist displacement of [^{3}H]-NMS binding in CHO-SLM2 and CHO-M3 cell membranes is shown in Figures 3.2-3.5. A concentration approximately equal to the K_D of [^{3}H]-NMS was displaced from mACh receptors by increasing concentrations of the M_2/M_3-'selective' antagonists methoctramine, tripitramine, zamifenacin and para-fluorohexahydrosiladiphenidol (p-f-HHSiD). IC_{50} values for displacement were adjusted to

a)



b)

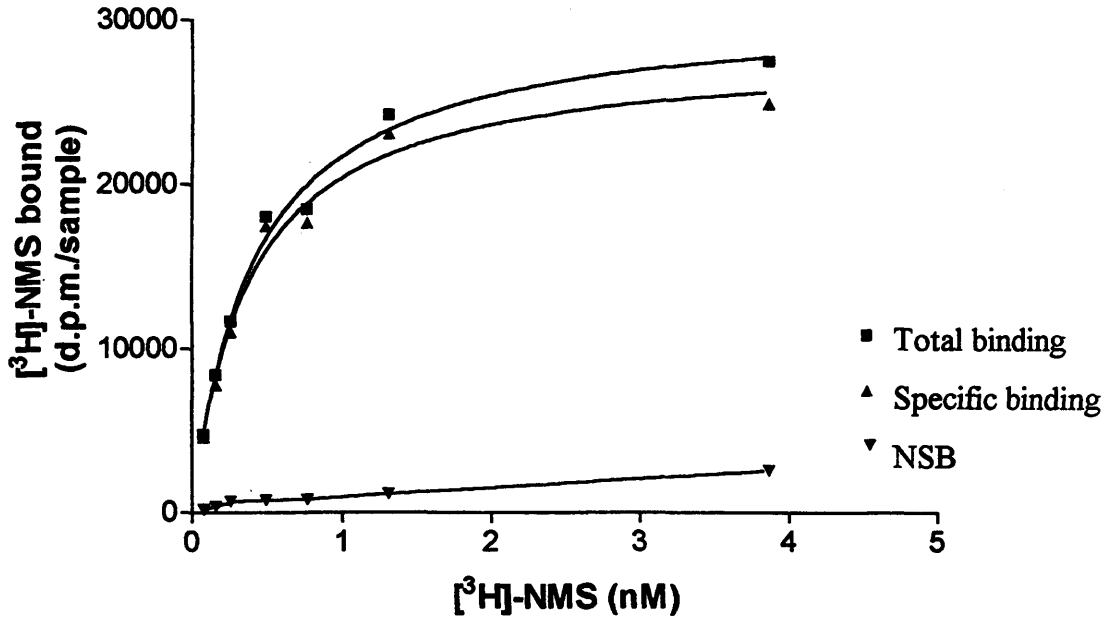


Figure 3.1 - [^{3}H]-N-methylscopolamine saturation binding in a) CHO-SLM2 and b) CHO-M3 cell membrane preparations

Cell membranes were prepared as described in Methods. Approx. 25 μg membanes were incubated in Hepes buffer A (see Methods) with increasing concentrations of [^{3}H]-N-methylscopolamine, in the absence and presence of atropine (1μM; to define NSB) at 37°C for 90 min. Bound and free ligand was separated using rapid vacuum filtration. The data are taken from single typical experiments.

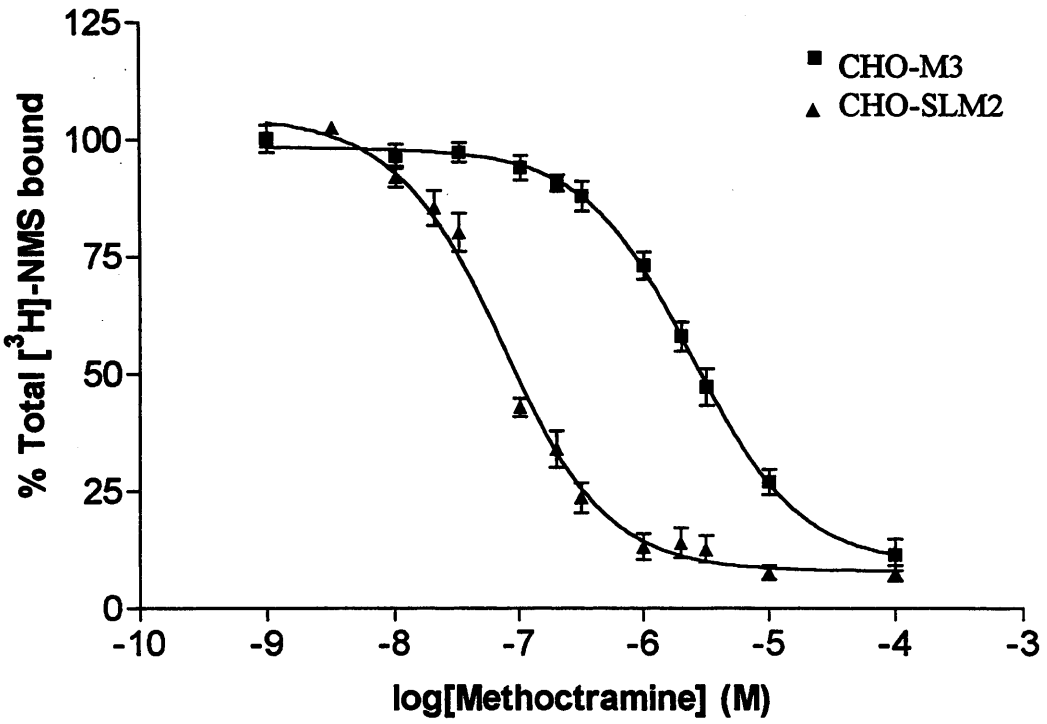


Figure 3.2 - Methoctramine displacement of [^{3}H]-N-methylscopolamine binding in CHO-SLM2 and CHO-M3 cell membranes.

Membranes were prepared as described in Methods. Approx. 25 μg protein was incubated in HEPES buffer A, with 0.8nM [^{3}H]-NMS in the presence or absence of varying concentrations of methoctramine for 90 min at 37°C. The data shown are mean ± s.e.m. of six experiments performed in triplicate.

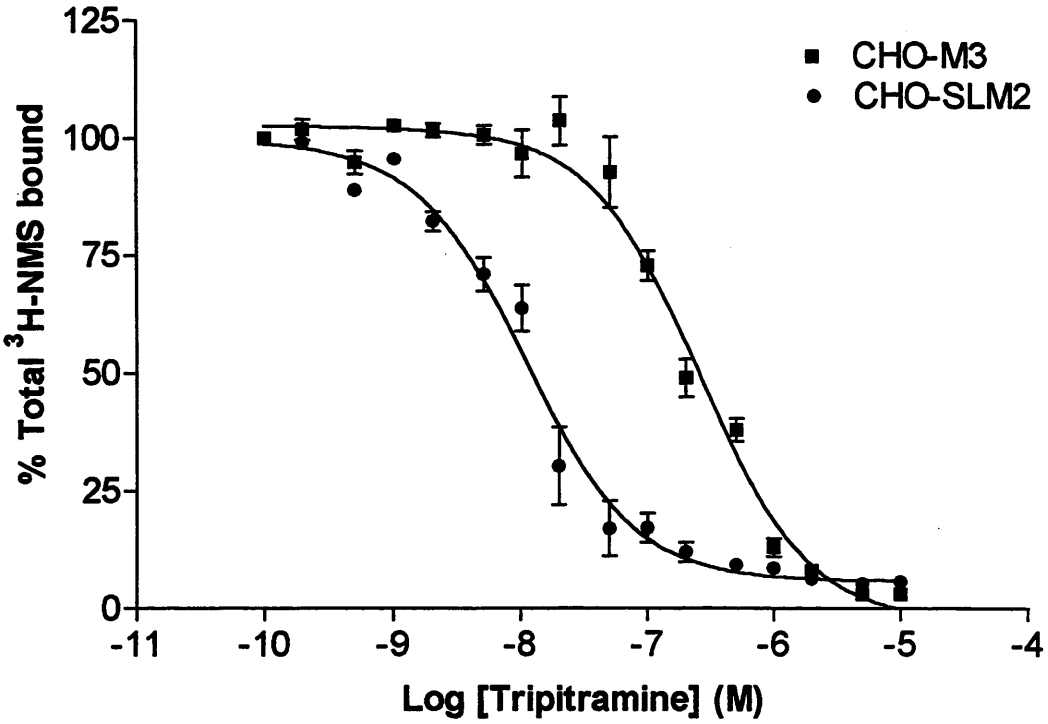


Figure 3.3 - Tripitramine displacement of [^{3}H]-N-methylscopolamine binding in CHO-SLM2 and CHO-M3 cell membranes.

Membranes were prepared as described in Methods. Approx. 25 μg protein was incubated in HEPES buffer A, with 0.8nM [^{3}H]-NMS in the presence or absence of varying concentrations of tripitramine for 90 min at 37°C. The data shown are mean ± s.e.m. of three experiments performed in triplicate.

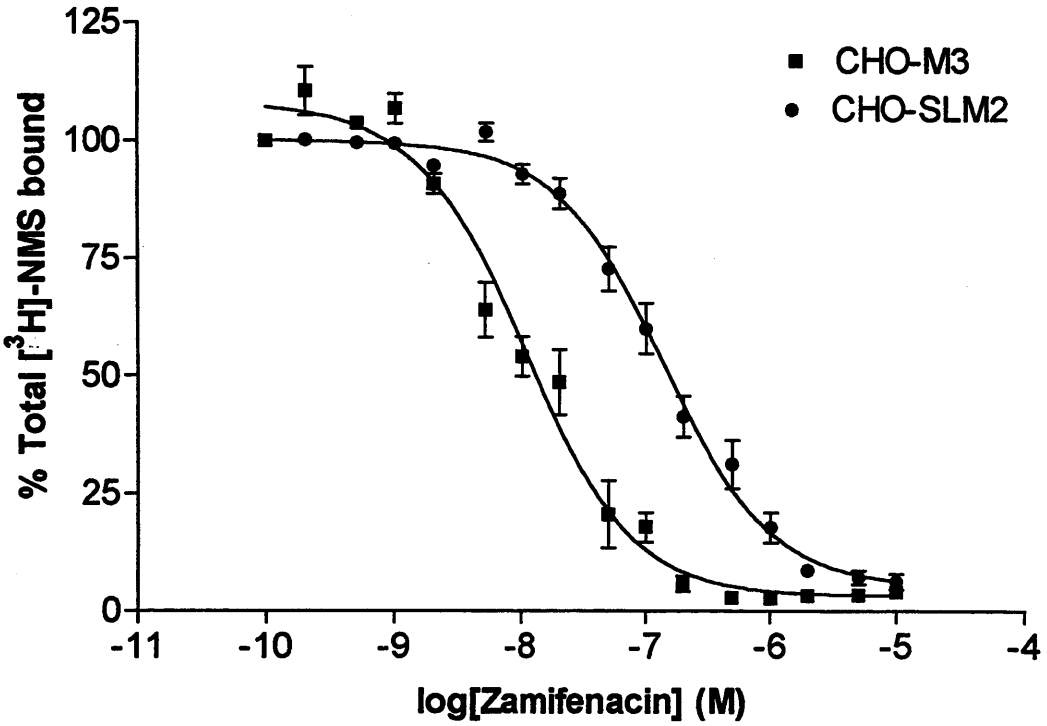


Figure 3.4 - Zamifenacin displacement of [^{3}H]-N-methylscopolamine binding in CHO-SLM2 and CHO-M3 cell membranes.

Membranes were prepared as described in Methods. Approx. 25 μg protein was incubated in HEPES buffer A, with 0.8nM [^{3}H]-NMS in the presence or absence of varying concentrations of zamifenacin for 90 min at 37°C. The data shown are mean ± s.e.m. of three experiments performed in triplicate.

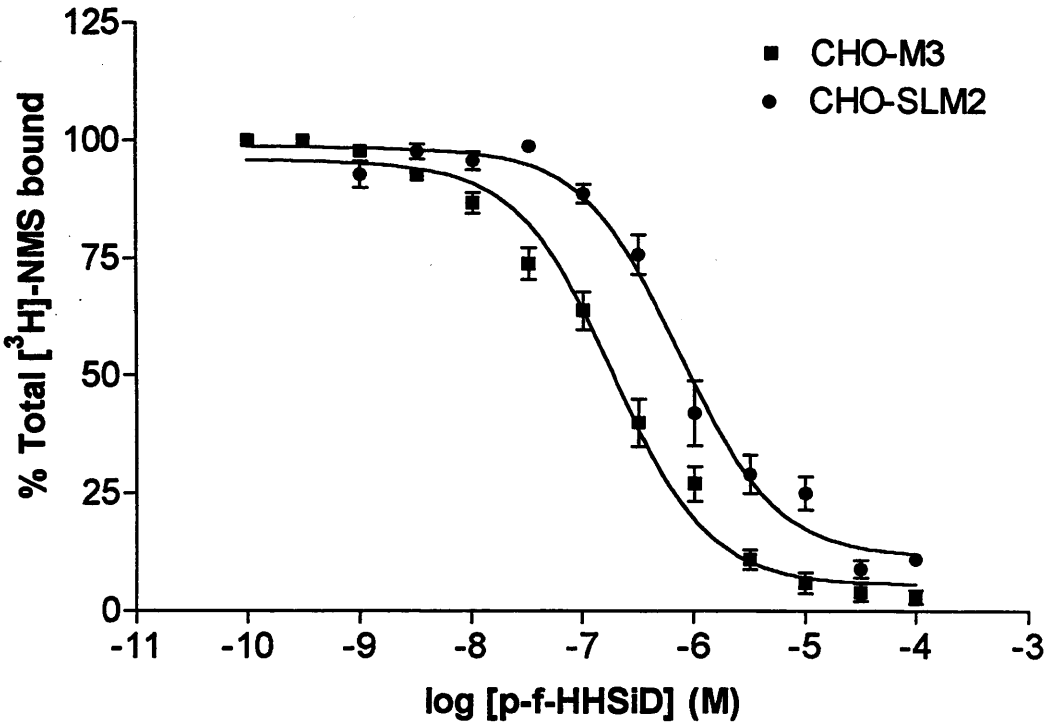


Figure 3.5 - p-f-hexahydrosiladiphenidol (p-f-HHSiD) displacement of [^{3}H]-N-methylscopolamine binding in CHO-SLM2 and CHO-M3 cell membranes.

Membranes were prepared as described in Methods. Approx. 25 μg protein was incubated in HEPES buffer A, with 0.8nM [^{3}H]-NMS in the presence or absence of varying concentrations of p-f-HHSiD for 90 min at 37°C. The data shown are mean ± s.e.m. of three experiments performed in triplicate.

true affinity (K_i) values, taking into account the concentration of [^{3}H]-NMS used and the K_D of [^{3}H]-NMS for the different receptor subtypes, according to the method of Cheng & Prusoff, (1973). Antagonist displacement curves for CHO-SLM2 and CHO-M3 cell membrane preparations were best-fitted by a one-site analysis of the curves, using computer assisted curve fitting (Graphpad Prism, as before), with slopes approximately equal to unity. This is an indication of receptor homogeneity as these antagonists do show some selectivity between M_2- and M_3-mACh receptors. The estimated affinities for the antagonists are given in Table 3.1. Under these experimental conditions, methoctramine has an approximately 30 fold greater affinity for binding sites in CHO-SLM2 than in CHO-M3 cell membrane preparations. This indicates that methoctramine or antagonists with equal or greater selectivity between M_2/M_3 mACh receptors may be a useful tool in the quantification of the densities of M_2- and M_3-mACh receptors when co-expressed in the same cell. The antagonists zamifenacin and p-f-HHSiD under these experimental conditions show less selectivity between M_2- and M_3-mACh receptor subtypes and may therefore be less useful in distinguishing these receptor subtypes in mixed populations. The affinity estimates for these antagonists in CHO-SLM2 and CHO-M3 cell membrane preparations agree with those previously reported at M_2- and M_3-mACh receptors respectively (Caulfield, 1993).

3.2.2 Methoctramine displacement of [^{3}H]-NMS binding in CHO-SLM2 and CHO-M3 membrane mixtures.

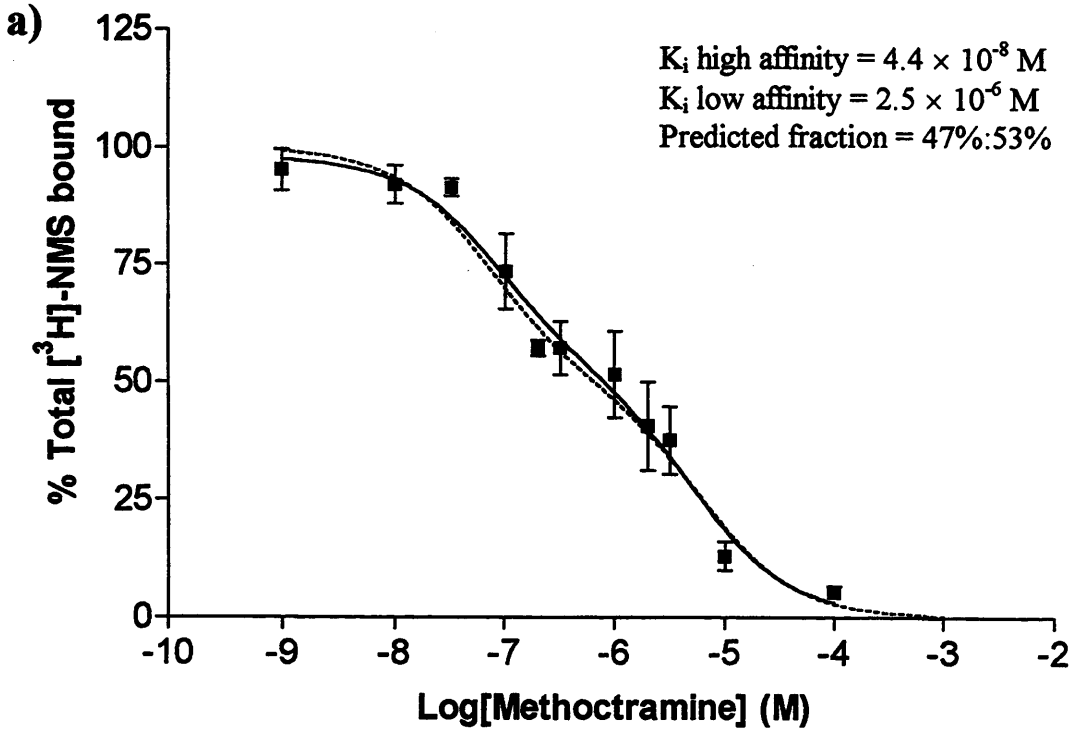
Methoctramine displacement of [^{3}H]-NMS binding to various mixtures of CHO-SLM2 and CHO-M3 membranes was used to assess whether methoctramine would be a useful tool for distinguishing receptor numbers of different mACh receptor subtypes in co-transfected CHO-M_2/M_3 cell lines. Displacement curves were fitted using computer-assisted curve-fitting as before, and the same computer program used to assess whether one-site or two-site modelling best-fitted the experimental data. For these membrane mixing experiments the CHO-SLM2 and CHO-M3 membranes were mixed according to receptor (binding site) number per unit protein to give *receptor* ratios of 50:50 and 70: 30 M_2:M_3 mACh receptors.

Methoctramine displacement of [^{3}H]-NMS in 50/50 (Figure 3.6a) and 70/30 (Figure 3.7a) M_2/M_3 mACh receptor mixes produced curves that were best-fitted using two-site analysis. From this analysis, the K_i for methoctramine for each site and the fractions of each receptor subtype could be estimated. The K_i values for the high affinity site in both membrane mixes were not significantly different to that for M_2-mACh receptors, as described previously in this Chapter using homogeneous populations. Furthermore, the K_i values for low affinity sites were not statistically significantly different to those made for a homogeneous population of M_3-mACh receptors. The fractions of receptors of each subtype of mACh receptors predicted using this analysis of the data agreed with those expected according to the amounts of membranes mixed (Figures 3.6a and 3.7a).

Theoretical curves were modelled, again using Graphpad software (Graphpad Prism, San Diego CA). Affinity estimates for M_2-mACh receptors and M_3-mACh receptors made in homogeneous populations as described previously in this Chapter, and receptor numbers predicted from the amounts of membranes mixed were used to construct theoretical curves for 50:50 (Figure 3.6b) and 70:30 (Figure 3.7b) mixes. These curves accurately model the profile of those fitted to the real data. These data suggest that methoctramine or an antagonist of equal or greater selectivity between M_2- and M_3-mACh receptors can be used to distinguish and quantify M_2- and M_3-mACh receptors in a mixed population of mACh receptors.

3.2.3 Saturation and displacement of [^{3}H]-NMS binding in bovine tracheal smooth muscle membranes

Bovine tracheal smooth muscle (BTSM) membranes were used to test whether receptor heterogeneity could be demonstrated in a smooth muscle tissue using M_2/M_3-mACh receptor selective antagonists, as described for CHO cell membrane mixtures. [^{3}H]-NMS saturation binding data from BTSM membranes was analysed by iterative non-linear curve fitting using Graphpad Prism software and by Scatchard analysis as before, producing a B_{max} of 609 ± 98



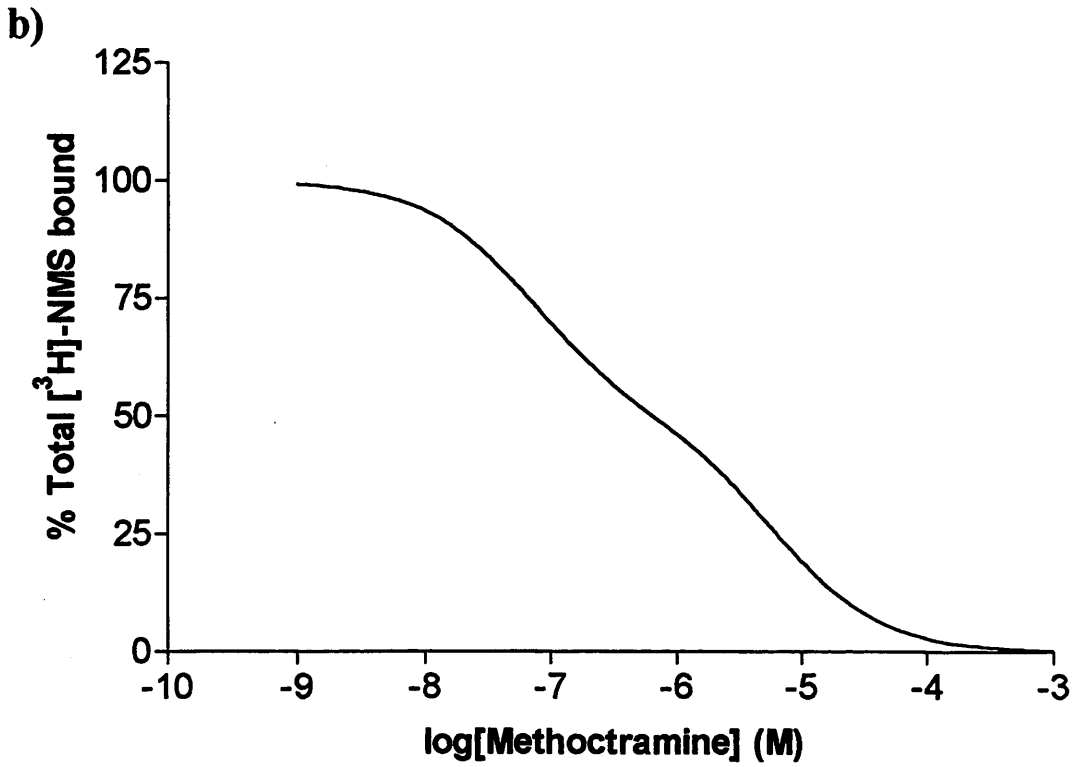


Figure 3.6 - a) Methoctramine displacement of [^{3}H]-N-methylscopolamine in a 50/50 M_2/M_3 mACh receptor mix from CHO-M3 and CHO-SLM2 cell membranes. Data shown represent mean ± s.e.m. from 3 experiments performed in triplicate. Dotted line indicates overlay of theoretical curve as in b)

b) Theoretical curve predicted for 50/50 mix of M_2/M_3 mACh receptors as predicted using known receptor ratio, and affinities of methoctramine for mACh receptors. Curve was modelled using Graphpad Prism.

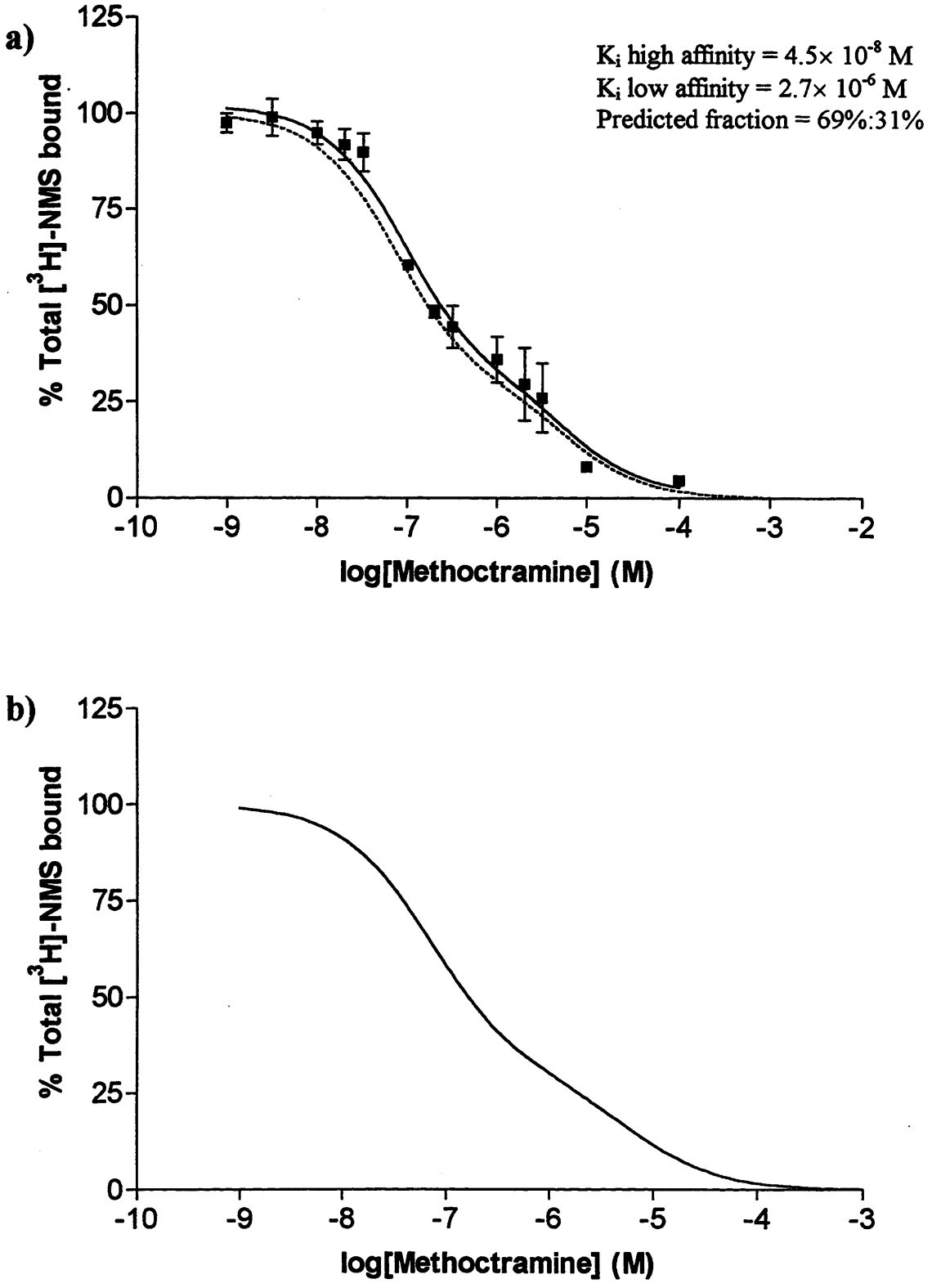


Figure 3.7 - a) Methoctramine displacement of [^{3}H]-N-methylscopolamine in a 70/30 M_2/M_3 mACh receptor mix from CHO-M3 and CHO-SLM2 cell membranes. Data shown represent mean ± s.e.m. from 3 experiments performed in triplicate.
b) Theoretical curve predicted for 70/30 mix of M_2/M_3 mACh receptors as predicted using known receptor ratio, and affinities of methoctramine for mACh receptors. Curve was modelled using Graphpad Prism. (Also shown in a) as dotted line).

fmol/mg protein and a K_D value of 0.61nM ± 0.09 (Figure 3.8). The K_D of [^{3}H]-NMS estimated is not significantly different from that expected for mACh receptors. Methoctramine (Figure 3.9) and para-fluoro-hexahydrosiladiphenidol (p-f-HHSiD) (Figure 3.10) displacement curves were both best-fitted by a two-site analysis of the data. p-f-HHSiD is approximately 10-fold more selective for M_3- over M_2-mACh receptors and was therefore expected to be a less useful tool in this instance. An estimate of K_i for methoctramine and p-f-HHSiD at each affinity site and of the fractions of receptor subtypes could be calculated from the parameters of the best-fit curves. For methoctramine, the high affinity site had a K_i value of 1.4 x 10^{-8}M which is not significantly different for that previously calculated for a homogeneous population of M_2-mACh receptors. The low affinity site had a K_i value of 1.1 x 10^{-6}M which is not statistically significantly different from that at M_3-mACh receptors. As expected for p-f-HHSiD the low affinity site represented the M_2-mACh receptor population whilst the high affinity site represented the M_3-mACh receptor population, but the distinction between affinity sites was more difficult given the lower selectivity of this antagonist. Using both curves, the fractions of receptor subtypes could be estimated as ~80% M_2:20% M_3-mACh receptors. When used in conjunction with saturation data giving total mACh receptor number, actual values for receptor subtype number of approximately 450 fmol mg protein^{-1} M_2-mACh receptors and 150 fmol mg protein^{-1} M_3-mACh receptors could be estimated (Figures 3.8 and 3.9).

3.2.4 Effect of methacholine stimulation on forskolin-stimulated cAMP accumulation in CHO-SLM2 and CHO-M3 cells

In Chinese hamster ovary cells, forskolin caused ~450-fold increase in cAMP accumulation over basal values (Figure 3.11). Basal values of cAMP mass were 3.6 ± 1.5 pmol mg protein^{-1} in CHO cells, and this is stimulated by forskolin to a maximal value of 1619 ± 322 pmol mg protein^{-1}. Forskolin directly stimulates adenylyl cyclase activity via a receptor-independent mechanism. In CHO-M2 cells, methacholine causes a concentration-dependent decrease in forskolin-stimulated cAMP production. Inhibition of up to 95% of the forskolin-

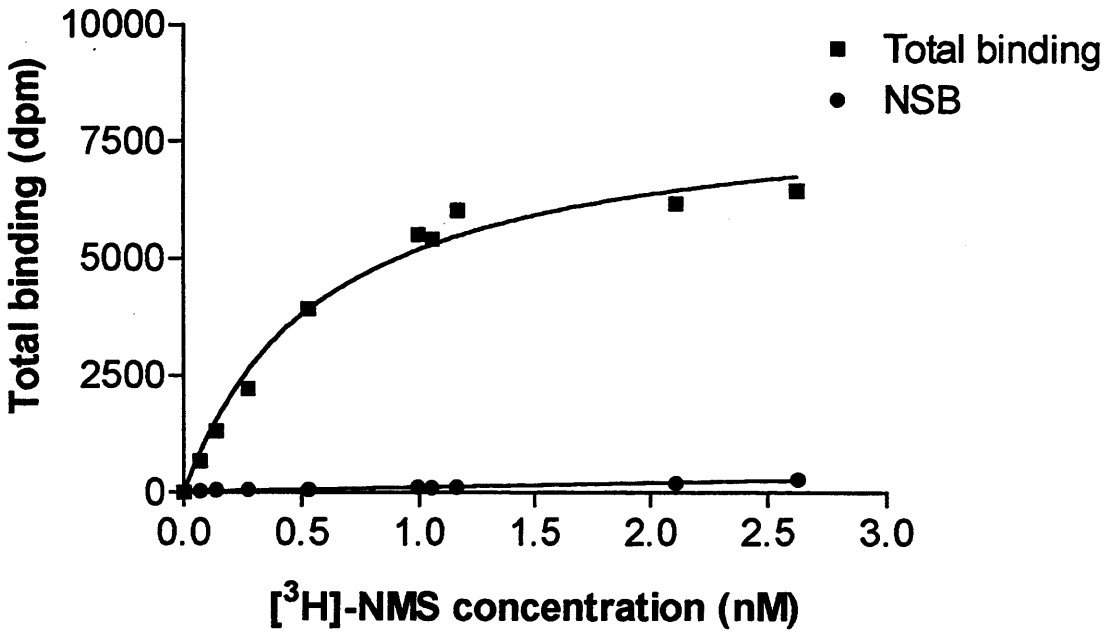


Figure 3.8 - [^{3}H]-N-methylscopolamine saturation binding in bovine tracheal smooth muscle membrane preparation

BTSM membranes were prepared as described in Methods. Approx. 25 μg membanes were incubated in Hepes buffer A (see Methods) with increasing concentrations of [^{3}H]-N-methylscopolamine, in the absence and presence of atropine (1μM; to define NSB) at 37°C for 90 min. Bound and free ligand was separated using rapid vacuum filtration. The data are taken from a single typical experiment.

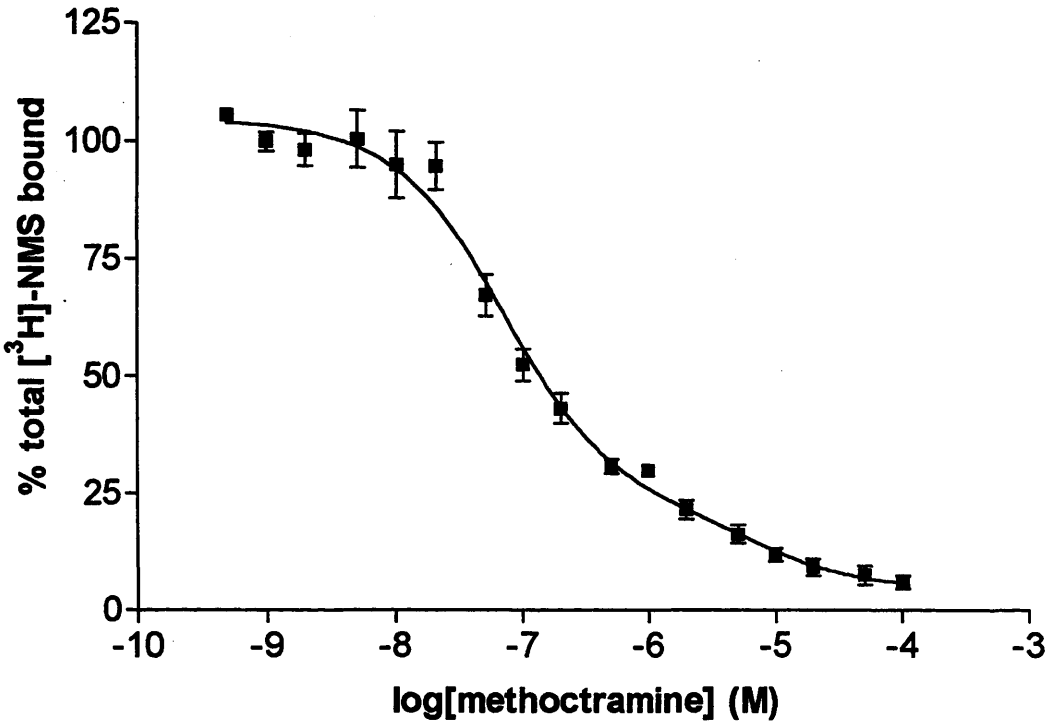


Figure 3.9 - Methoctramine displacement of [^{3}H]-N-methylscopolamine binding in bovine tracheal smooth muscle membrane preparation

BTSM membranes were prepared as described in Methods. Approx. 25 μg membranes were incubated in Hepes buffer A (see Methods) with approx. 0.8nM [^{3}H]-N-methylscopolamine, in the absence and presence of methoctramine at varying concentrations, at 37°C for 90 min. Bound and free ligand was separated using rapid vacuum filtration. The data represent mean ± s.e.m. for 3 separate experiments performed in triplicate.

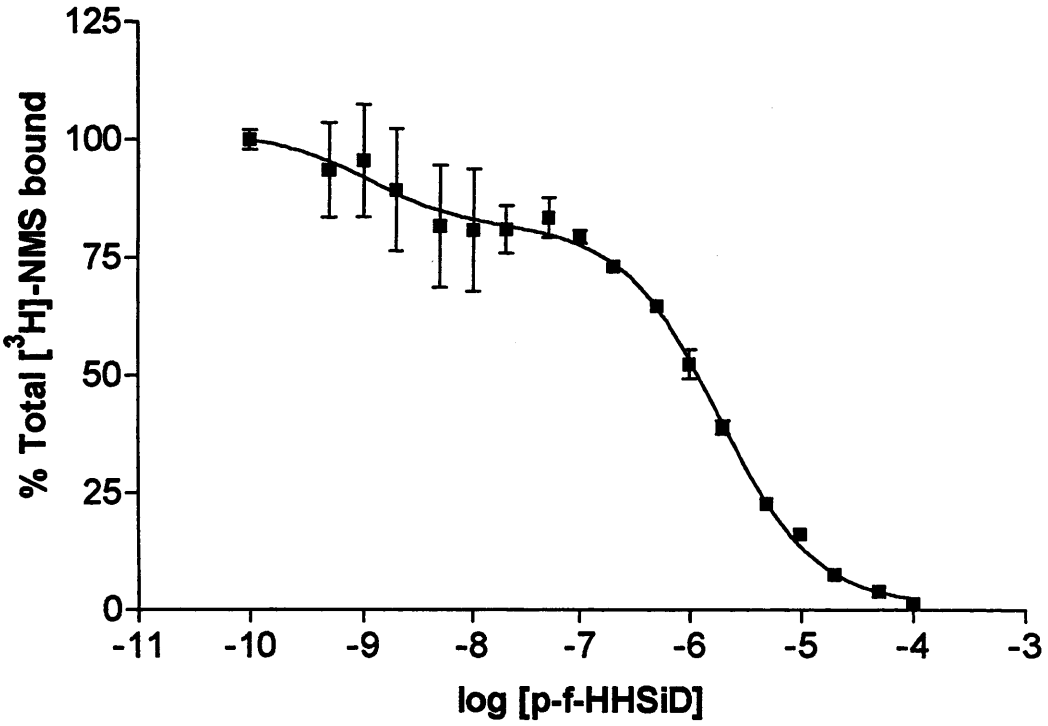


Figure 3.10 - p-f-HHSiD displacement of [^{3}H]-N-methylscopolamine binding in bovine tracheal smooth muscle membrane preparation

BTSM membranes were prepared as described in Methods. Approx. 25 μg membanes were incubated in Hepes buffer A (see Methods) with approx. 0.8nM [^{3}H]-N-methylscopolamine, in the absence and presence of p-f-HHSiD at varying concentrations, at 37°C for 90 min. Bound and free ligand was separated using rapid vacuum filtration. The data represent mean ± s.e.m. for 3 separate experiments performed in triplicate.

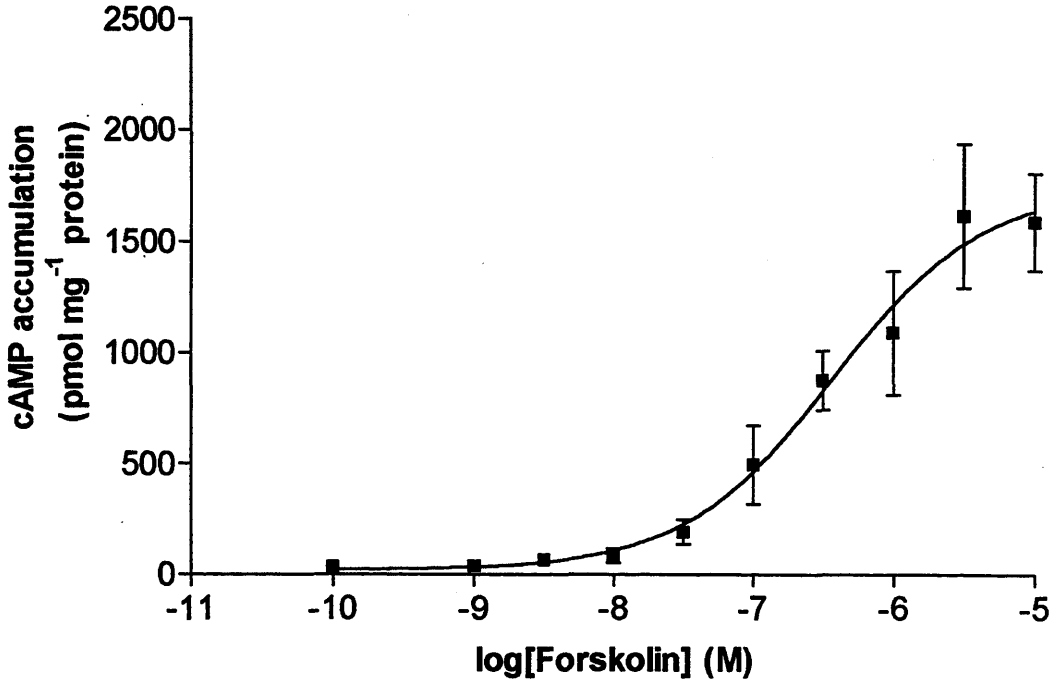


Figure 3.11 - Forskolin stimulation of cAMP accumulation in CHO-SLM2 cells

Cells were washed and incubated with KHB for 10 min, before the addition of forskolin at various concentrations. After 5 min the reaction was stopped by removal of buffer and addition of 0.5 M TCA. Cyclic AMP levels were then analysed as described in Methods. Basal levels of cAMP were 3.6 ± 1.5 pmol mg^{-1} protein. Data represent mean ± s.e.m. of 3 separate experiments performed in duplicate.

stimulated (10μM) adenylyl cyclase response could be achieved by M_2-mACh receptor stimulation in these cells (Figure 3.12). The IC_{50} of this response to MCh was 2.8×10^{-8}M. In CHO-M3 cells, in contrast to CHO-SLM2 cells, there was no such inhibition of response, but rather an additional stimulation of adenylyl cyclase activity at high concentrations of MCh (>1 μM; Figure 3.13). Therefore, these receptor subtypes when stably expressed in CHO cells differ in their coupling to adenylyl cyclase, with the M_2-mACh receptor being inhibitory and the M_3-mACh receptor being stimulatory at high concentrations of agonist.

3.2.5 Effect of methacholine stimulation on total [^{3}H]-inositol phosphate accumulation in CHO-M3 and CHO-SLM2 cells

Experiments in CHO-M3 cells using methacholine to stimulate mACh receptors, indicated that the agonist-induced increase in total [^{3}H]-inositol phosphate accumulation is concentration-dependent (Figure 3.14a) and time-dependent (Figure 3.14b). Methacholine-stimulation was carried out in the presence of 10 mM LiCl and induced a concentration-dependent increase from basal values of 1713 ± 291 d.p.m./well to a maximal value of 15338 ± 3885 d.p.m./well with an EC_{50} value of 2.3 μM. Time-courses showed that an initial rapid rate of total [^{3}H]-inositol phosphate accumulation over the first 10 min was followed by a plateau phase of the response up to 30 min i.e. the mACh receptor agonist-induced accumulation of total [^{3}H]-inositol phosphates was not linear with time. Time-matched controls using LiCl (10 mM), but no agonist-stimulation did not show any increase in [^{3}H]-inositol phosphate production over basal values. In CHO-SLM2 cells under the same experimental conditions increases in total [^{3}H]-inositol phosphate production were less than 2-fold over basal values of 251 ± 29 d.p.m/well. The lower basal value indicates a lower rate of inositol phosphate turnover in these cells than in CHO-M3 cells. No statistically significant increase in total [^{3}H]-inositol phosphate accumulation was observed in these cells after 30 min using a maximally effective concentration of methacholine (1 mM).

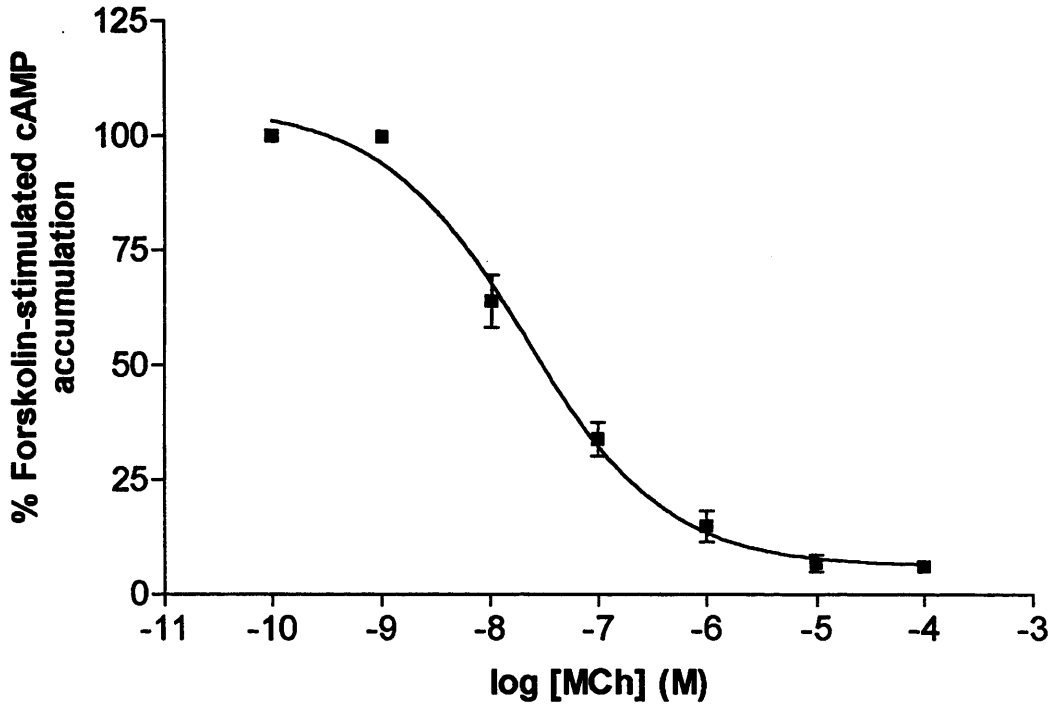


Figure 3.12 - Inhibition of forskolin-stimulated cAMP accumulation in response to methacholine stimulation in CHO-SLM2 cells

Cells were washed and incubated with KHB for 10 min, before the addition of MCh at various concentrations. After 15 min, cells were stimulated with forskolin (10 μM) for 5 min, and then the reaction was stopped by removal of buffer and addition of 0.5 M TCA. Cyclic AMP levels were then analysed as described in methods. Basal levels of forskolin-stimulated cAMP were 1619 ± 322 pmol mg^{-1} protein. Data represent mean ± s.e.m. of three separate experiments performed in duplicate.

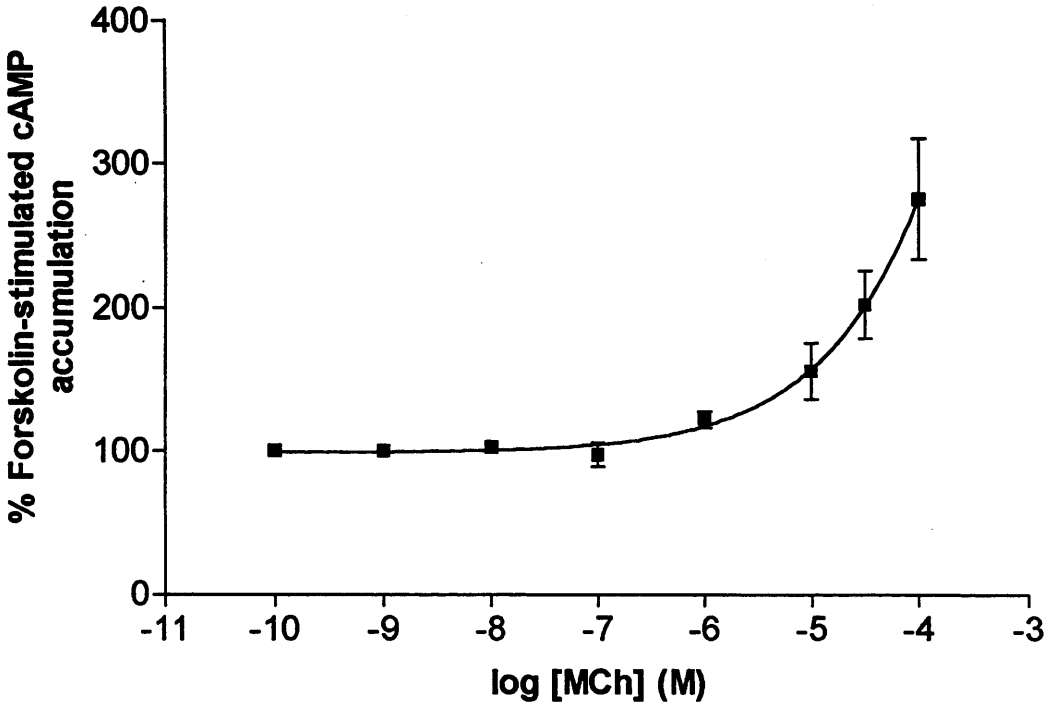


Figure 3.13 - Stimulation of forskolin-stimulated cAMP accumulation in response to methacholine stimulation in CHO-M3 cells

Cells were washed and incubated with KHB for 10 min, before the addition of MCh at various concentrations. After 15 min, cells were stimulated with forskolin (10 μM) for 5 min, and then the reaction was stopped by removal of buffer and addition of 0.5 M TCA. Cyclic AMP levels were then analysed as described in Methods. Basal levels of forskolin-stimulated cAMP were 1619 ± 322 pmol mg protein. Data represent mean ± s.e.m. of three separate experiments performed in duplicate.

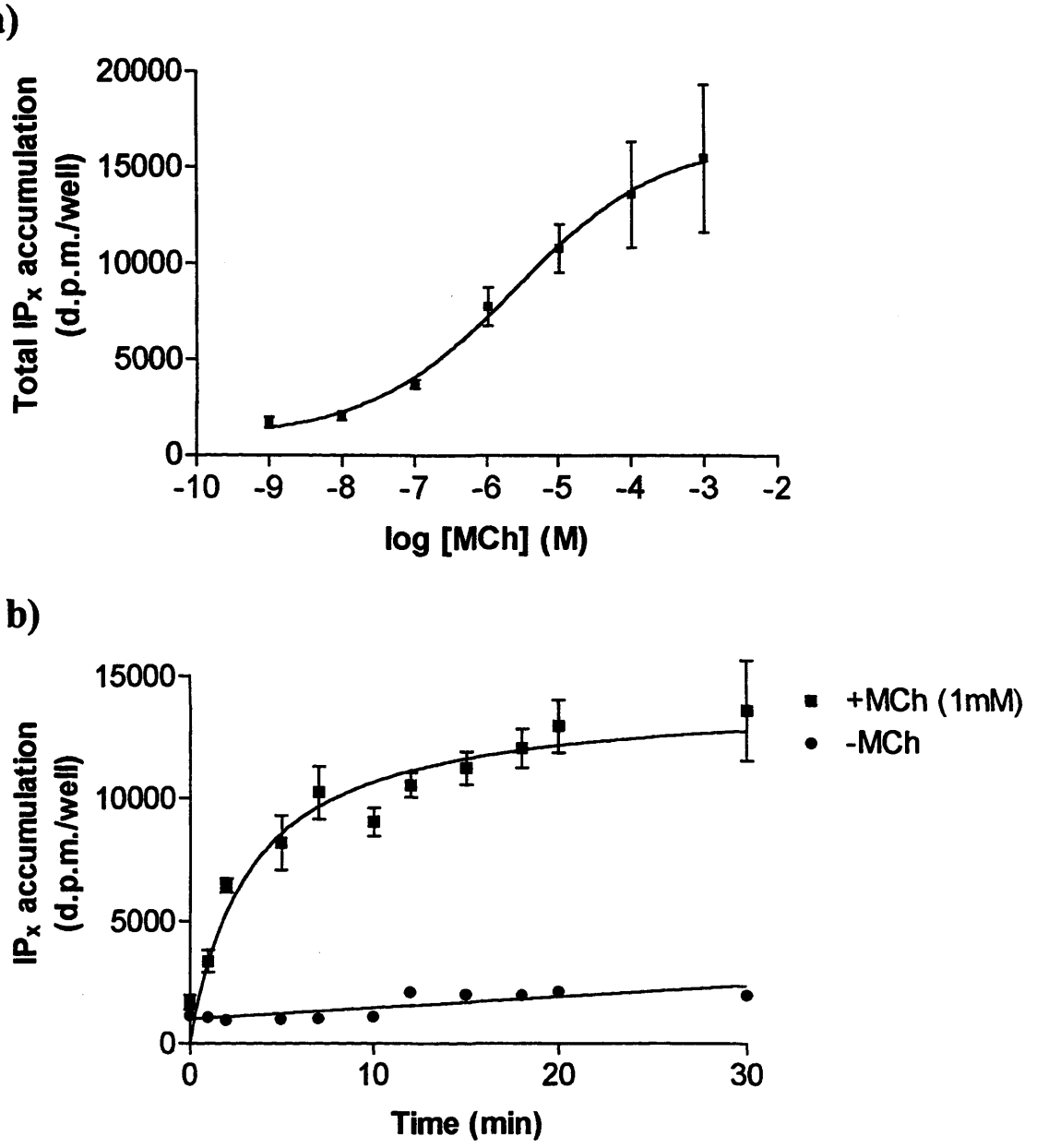


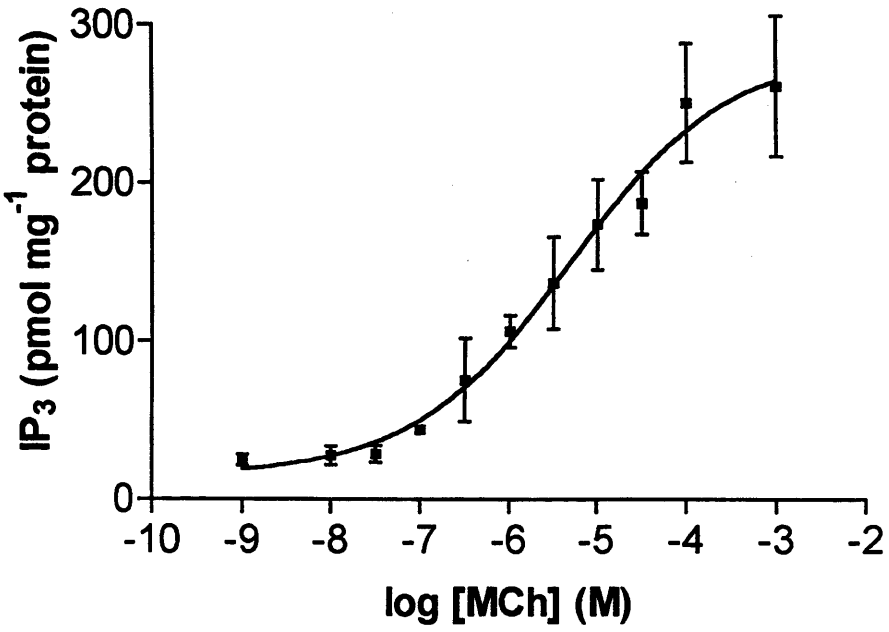
Figure 3.14 - Total $InsP_x$ accumulation a) with varying concentrations of MCh and b) with time after maximal MCh stimulation in CHO-M3 cells.

Cells were labelled with 1 μCi ml^{-1} [^{3}H]-inositol for 48 h. The cells were then washed with KHB, and incubated ± Li^+ (10 mM) for 15 min. MCh was added at various concentrations for 15 min, or at 1mM for various times. Reactions were terminated by the removal of buffer and addition of 0.5 M TCA and total $InsP_x$ were analysed by Dowex column chromatography as described in Methods. Data represent mean ± s.e.m. for three separate experiments performed in duplicate.

3.2.6 Effect of methacholine stimulation on inositol 1,4,5-trisphosphate mass measurements in CHO-M3 and CHO-SLM2 cells

Similar to results seen when measuring total [^{3}H]-inositol phosphate accumulation, an increase in inositol 1,4,5-trisphosphate (IP_3) accumulation which was both concentration- and time-dependent could be observed in CHO-M3 cells stimulated with methacholine (Figures 3.15a and b), whereas no such response was observed after the application of MCh to CHO-SLM2 cells. The application of a maximal concentration of MCh (1 mM) to intact CHO-M3 cells resulted in a biphasic elevation of IP_3 mass, characteristic for this response in this cell type (Tobin *et al.*, 1992). Initially IP_3 levels increased rapidly from basal levels of 30 ± 7 pmol mg^{-1} protein to a peak of 263 ±41 pmol mg^{-1} protein. The peak was observed at about 10s after application of the agonist, after which time the response decreased over a period of about 1 min to a plateau level above basal of 124 ± 20 pmol mg^{-1} protein which was maintained for at least 10 min (Figure 3.15a). Concentration-dependent increases in peak IP_3 accumulation from a basal value of 25 ± 3 pmol mg^{-1} protein to a maximal stimulated level of 262 ± 45 pmol mg^{-1} protein after 10s application of MCh were observed in CHO-M3 cells, with the EC_{50} for this response being 4.9μM (Figure 3.15b). This response was prevented by atropine and is therefore mediated via mACh receptors.

a)



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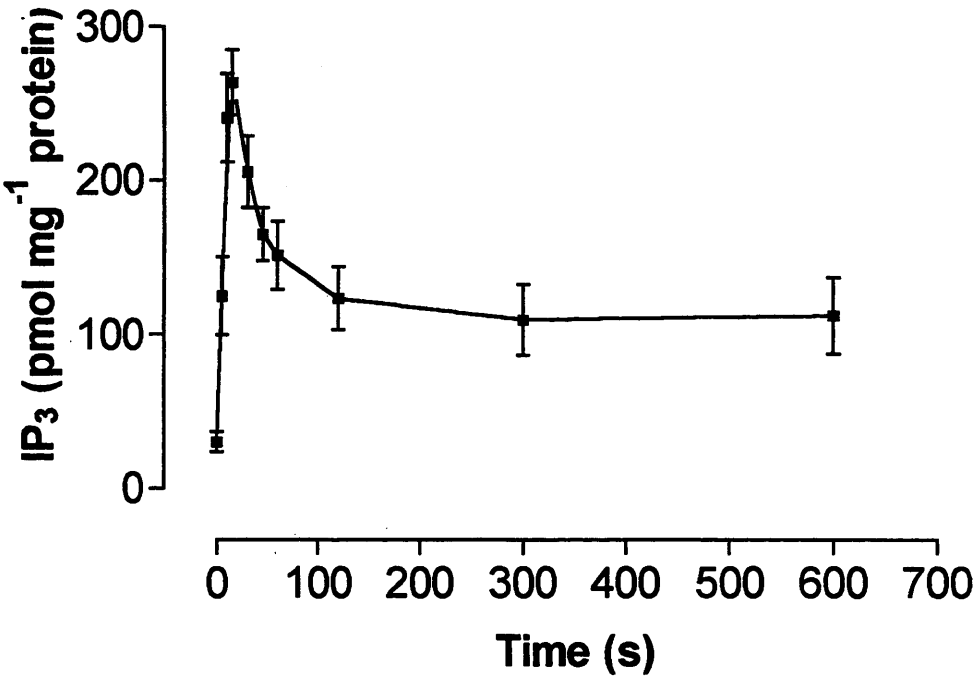


Figure 3.15 - a) Peak Ins(1,4,5)P_3 production with increasing concentrations of MCh and b) Ins(1,4,5)P_3 production with time in reponse to 1mM MCh stimulation, both in CHO-M3 cells.

Cells were washed and incubated with KHB for 10 min, before addition of MCh at varying concentrations or 1mM. After 10 s for concentration-response and vaying time-points for time-courses, the reaction was stopped by addition of an equal volume of 1M TCA. InsP_3 mass levels were then analysed as described in Methods. Data represent mean ± s.e.m. for three separate experiments performed in duplicate.

3.2.7 Effect of methacholine stimulation on [^{3}H]-CMP-phosphatidate accumulation in CHO-M3 and CHO-SLM2 cells

In CHO-M3 cells, methacholine (1 mM) in the presence of LiCl (10 mM) caused a 12-fold increase over basal levels of [^{3}H]-CMP-PA accumulation 20 min after MCh challenge from a basal level of 1304 ± 57 d.p.m./well to a maximum stimulated level of 15405 ± 675 d.p.m./well. In the absence of Li^+, MCh caused a small, but significant increase in [^{3}H]-CMP-PA accumulation (2-fold) in this cell line (Figure 3.16). [^{3}H]-CMP-PA approached a plateau level 5-10 min after agonist application in the absence of Li^+, but [^{3}H]-CMP-PA accumulation in the presence of Li^+ increased in a biphasic manner with the rate of accumulation decreasing at later time-points over 30 min. No such increase was observable in CHO-SLM2 cells, where the maximum increase measured was 2-fold over basal values in the presence of Li^+ (10 mM). This is as expected given the previous evidence showing that the phosphoinositide cycle is not activated by methacholine-stimulation in these cells. It has been shown that an easily detectable increase in CMP-PA accumulation with a longer lag-phase can be seen in CHO cells which express low levels of M_3-mACh receptors (about 100 fmol/mg protein), in response to MCh-plus-Li^+ challenge (CHO-VT9) (Figure 3.17). Measurements of CMP-PA accumulation are much simpler practically and less time-consuming than measurements of [^{3}H]-inositol phosphate or IP_3 mass and therefore could make an ideal tool for screening of M_2/M_3-mACh CHO cell transfects.

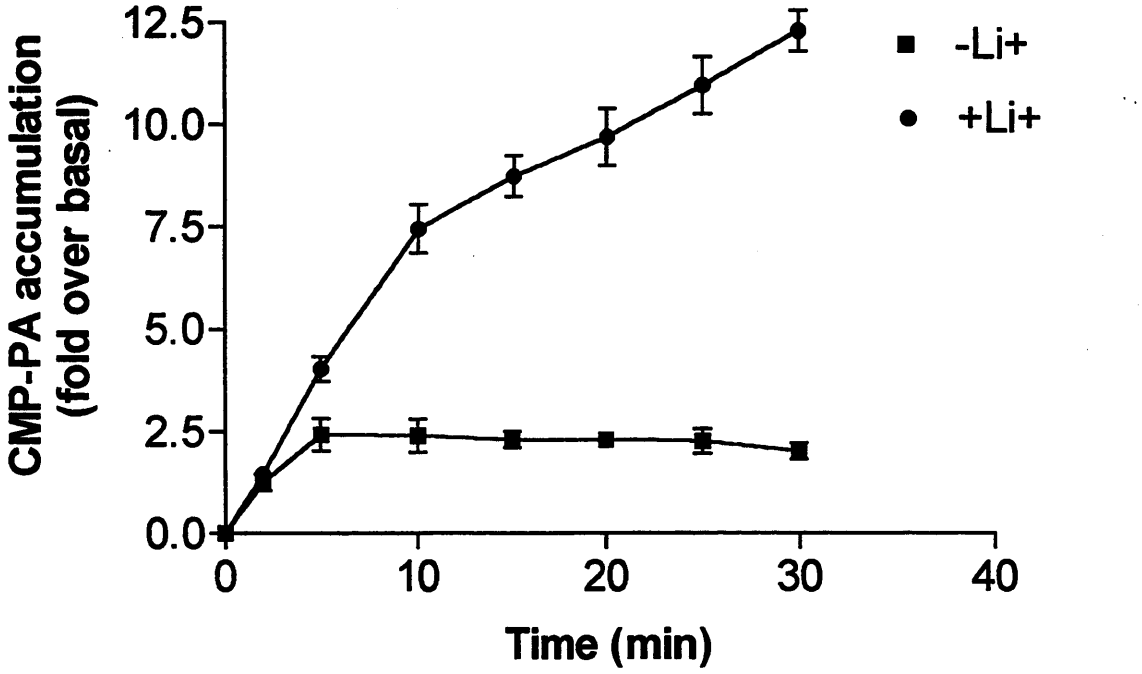


Figure 3.16 - CMP-phosphatidic acid accumulation in the presence and absence of Li^+ (10 mM) in response to methacholine stimulation in CHO-M3 cells

Cells were labelled with 0.3 μCi ml^{-1} for 60 min at 37°C. The cells were washed in KHB and incubated in KHB ± Li^+ (10 mM) for 15 min. The cells were then challenged with 1mM MCh and the reaction stopped after 15 min by the addition of an equal volume of 1M TCA. [^{3}H]-CMP-PA was extracted using acidified chloroform / methanol and aqueous and organic phases resolved as described in Methods. Data represent mean ± s.e.m. for 3 separate experiments performed in triplicate.

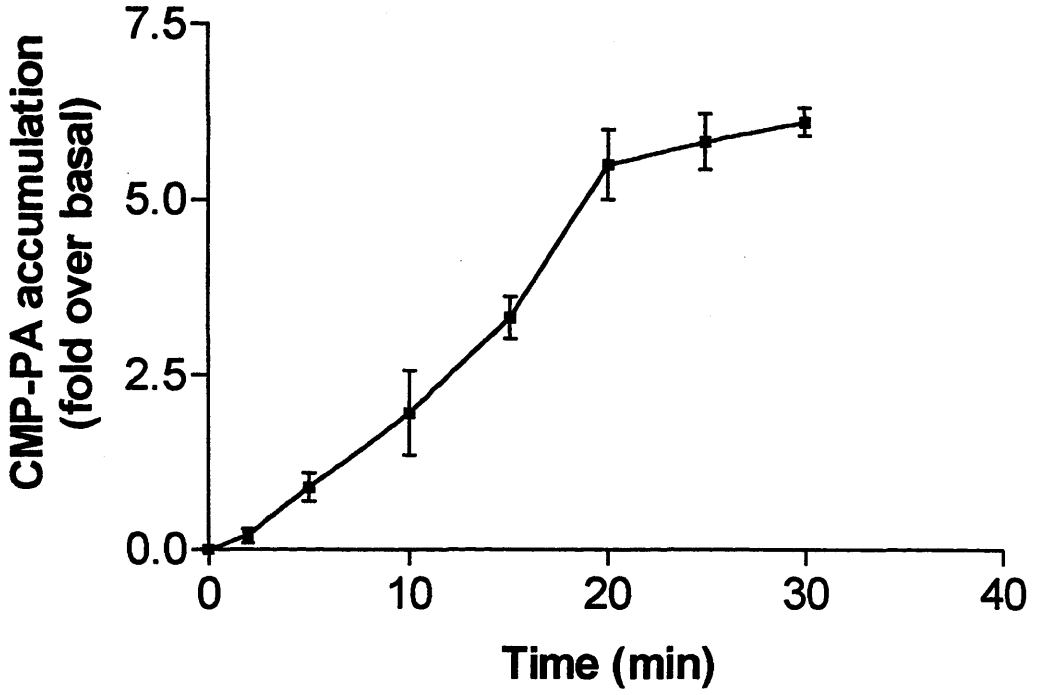


Figure 3.17 - CMP-phosphatidic acid accumulation in the presence and absence of Li^+ (10 mM) in response to methacholine stimulation in CHO-VT9 cells

Cells were labelled with 0.3 μCi ml^{-1} for 60 min at 37°C. The cells were washed in KHB and incubated in KHB ± Li^+ (10 mM) for 15 min. The cells were then challenged with 1mM MCh and the reaction stopped after 15 min by the addition of an equal volume of 1M TCA. [^{3}H]-CMP-PA was extracted using acidified chloroform / methanol and aqueous and organic phases resolved as described in Methods. Data represent mean ± s.e.m. for 3 separate experiments performed in triplicate.

3.3 Discussion

Most studies to determine whether any interaction between M_2- and M_3-mACh receptors occurs have been performed in actual smooth muscle tissue. This approach has not been conclusive, and a role for M_2-mACh receptors in smooth muscle tone has not been satisfactorily proven or disproved. Expression of mACh receptors in a simple cell line not endogenously expressing these receptors creates a model system for the study of the interactions of these receptors. Most of the work presented in this Chapter has been performed in order to develop methods by which new cell lines co-expressing M_2/M_3-mACh receptors can be characterised.

Whilst [^{3}H]-NMS can be used to measure the total number of muscarinic receptors by saturation binding, as shown in CHO-M3 and -SLM2 cell membranes, this radioligand has negligible selectivity between M_2- and M_3-mACh receptor subtypes, since there was no statistically significant difference in K_D values of [^{3}H]-NMS at each of these receptors. Methoctramine may be useful in distinguishing between the two receptor subtypes. It is a mACh receptor antagonist which exhibits approximately 30-fold selectivity for M_2-mACh receptors over M_3-mACh receptors in radioligand binding studies carried out in cell membranes prepared from either CHO-SLM2 or CHO-M3 cells. This agrees with previous studies showing that methoctramine has reasonable selectivity between M_2/M_3-mACh receptors (Michel & Whiting, 1988).

It has been shown that methoctramine displacement studies in known CHO-M3 and CHO-SLM2 membrane mixtures can be best-fitted by a two-site competition curve and the receptor proportions can accurately be predicted by computer-aided analysis of this curve. Affinity estimations for each site agree with those expected for M_2- and M_3- mACh receptors. Theoretical curves, constructed using K_is for methoctramine in homogeneous populations of M_2- and M_3-mACh receptors and known receptor numbers according to the amounts of membranes mixed, resembled closely those curves fitted to real data, giving confidence that

it is possible to distinguish and hence quantify the two receptor subtypes using methoctramine in this way.

Methoctramine displacement analysis has been successfully employed to estimate the proportions of M_2- and M_3-mACh receptors in bovine tracheal smooth muscle membranes giving a minor M_3- and a major M_2-mACh receptor population, as might be expected (Michel & Whiting, 1988). Also, the two affinities predicted for methoctramine are not significantly different from those for homogeneous populations of M_2- and M_3-mACh receptors under the same experimental conditions. Therefore, one approach to quantifying M_2- and M_3-mACh receptors in the new co-expressing cell lines could be to estimate the total mACh receptor population using [^{3}H]-NMS saturation binding and to use methoctramine displacement of [^{3}H]-NMS binding to estimate the proportions of each subtype present.

The use of [^{3}H]-NMS saturation and displacement binding analysis is too complex for initial screening of all hygromycin-resistant, and thus potentially M_3-mACh receptor-expressing clones. Therefore, functional studies were undertaken to see whether these may be used as more direct methods of screening for M_3-mACh receptor expression. It was thought that MCh-stimulated activation of the phosphoinositide cycle could be used as an indication of successful transfection and expression of the M_3-mACh receptor into a cell line previously stably expressing a homogeneous population of M_2-mACh receptors.

Lithium is an uncompetitive inhibitor of inositol monophosphatase activity and therefore prevents inositol recycling, leading to decreased inositol phospholipid resynthesis, decreases in free inositol concentrations and accumulation of inositol phosphates. The enzyme involved in resynthesis of inositol phospholipids is phosphatidylinositol synthase, which recycles free inositol and CMP-phosphatidate (CMP-PA) to form phosphatidylinositol (See Figure 1.1 in Introduction Chapter). The decrease in concentration of inositol caused by lithium blockade therefore causes a concomitant increase in measured CMP-PA concentration due to reduced resynthesis of inositol phospholipids. So theoretically,

increases in total inositol phosphate accumulation and CMP-PA accumulation can be used to indicate activation of phosphoinositide hydrolysis by M_3-mACh receptor stimulation, in the presence of lithium. Accumulation of total inositol phosphates and CMP-PA has been measured in both M_3- and M_2-mACh expressing cells. As was expected, marked increases in the levels of both total inositol phosphates and CMP-PA were seen in methacholine-stimulated CHO-M3 cells, but not in CHO-SLM2 cells. This confirms that a homogeneous population of M_2-mACh receptors does not substantially activate the phosphoinositide cycle when expressed at this level in this cell type, and therefore that the level of M_2-mACh receptor expression in CHO-SLM2 cells is not sufficient to allow promiscuous coupling to G-proteins that can activate this signalling pathway. This is in contrast to M_2-mACh receptor-mediated stimulation of phosphoinositide hydrolysis detected as total inositol phosphate accumulation in some other cell types (Ashkenazi *et al.*, 1987; Richards, 1991).

It is interesting to note that production of total inositol phosphates in CHO-M3 cells is not linear but approaches a plateau after 10 min in the presence of lithium. The non-linearity of the total inositol phosphate response may be due to mACh receptor desensitisation, or depletion of the [^{3}H]-phosphoinositides, in particular PIP_2. Lithium causes depletion of free inositol and so decreases the resynthesis of phosphoinositides, therefore eventually limiting the generation of inositol phosphates by phosphoinositide-specific phospholipases C (PLC). This may be responsible for the non-linearity of this response, however Willars *et al.* (personal communication) have shown that GnRH receptor-mediated PLC activity is sustained despite a marked and persistent depletion in the steady state level of PIP_2 in αT3-1 cells, whilst M_3-mACh receptor-mediated activation of PLC is not sustained despite only a transient reduction in PIP_2 in the same cell type. This would indicate that depletion of phosphoinositide levels may not be sufficient to account for the desensitisation of the PLC response described here. Alternatively, there may be a receptor-desensitisation event occurring which affects the linearity of inositol phosphate response, such as receptor phosphorylation. In fact, Tobin *et al.* (1992) suggest that M_3-mACh receptor desensitisation may be mediated by changes in IP_3 metabolism, PIP_2 availability, or receptor uncoupling,

and have also demonstrated rapid M_3-mACh receptor phosphorylation (Tobin & Nahorski, 1993).

[^{3}H]-CMP-PA accumulation with time upon M_3-mACh receptor stimulation is non-linear in the absence of lithium blockade of inositol monophosphatase, but does not approach a plateau in the presence of lithium. Although [^{3}H]-CMP-PA does accumulate in response to stimulation of the phosphoinositide response, the measured levels of [^{3}H]-CMP-PA are also dependent on other factors. For example, accumulation is dependent on the prevention of recycling of [^{3}H]-CMP-PA with inositol in the resynthesis of phosphoinositides. This can be achieved by limiting the availability of inositol to phosphatidylinositol synthase by the use of lithium, but the effectiveness of this is dependent upon the cellular homeostasis of free inositol (Gray *et al.*, 1994). Therefore, it is not surprising that measured [^{3}H]-CMP-PA accumulation is non-linear in the absence of lithium as it is almost certain that free cellular inositol is not depleted in this case i.e. [^{3}H]-CMP-PA accumulation does not reflect the rate of inositol phospholipid hydrolysis in the absence of lithium, but rather reflects an increase in the steady-state concentration of this intermediate produced by increased flux through the phosphoinositide cycle. Differences in rates of inositol depletion may also explain the longer lag phase for the CHO-VT9 cells as they express fewer M_3-mACh receptors than CHO-M3 cells, and therefore a longer period of receptor activation is necessary to deplete the free inositol in this cell line. The fact that the rate of [^{3}H]-CMP-PA accumulation decreases after 5-10 min in the presence of agonist-plus-lithium in CHO-M3 cells, may be a reflection of the desensitisation events discussed earlier in relation to total inositol phosphate production. Although accumulation depends on continued depletion of inositol and therefore decreased CMP-PA recycling, the accompanying depletion of phospholipids eventually limits phosphoinositide hydrolysis and therefore the reduced rate of accumulation measured for both [^{3}H]-CMP-PA and [^{3}H]-InsPs at later time-points.

Stimulation of CHO-M3 cells with a maximal concentration of MCh results in a biphasic elevation of mass Ins(1,4,5)P_3. The profile of this response is similar to that reported in SH-SY5Y human neuroblastoma cells (Lambert & Nahorski, 1990), and bombesin and

cholecystokinin stimulation of Ins(1,4,5)P_3 production in AR4-2J rat pancreatoma cells (Menniti *et al.*, 1991). The first peak phase of the response, which is sensitive to rapid desensitisation (Tobin *et al.*, 1992), represents initial PLC stimulation via agonist-activation of the receptor, whilst it has been suggested that the second plateau phase of the response, which is insensitive to desensitisation, is maintained by Ca^{2+} entry in the continued presence of the agonist, and is therefore dependent on extracellular Ca^{2+} (Tobin *et al.*, 1992). Again, there is no measurable increase in Ins(1,4,5)P_3 production in CHO-SLM2 cells stimulated in the same way under the same experimental conditions as CHO-M3 cells.

The appraisal of second messenger measurements for screening of phosphoinositide hydrolysis and hence successful transfection and expression of M_3-mACh receptors in new clones, was made according to experimental practicalities as well as the issues discussed above. Near equilibrium labelling of the cytidine pool takes only 60 min, whilst equilibrium labelling of inositol lipid pools can take ≥ 48 h, presumably due to the limited capacity of the Na^+-dependent inositol transporter expressed in Chinese hamster ovary cells (Gray *et al.*, 1994). The extraction and measurement process for [^{3}H]-CMP-PA is also simpler than for [^{3}H]-inositol phosphates and certainly for mass IP_3, so it was decided that measurement of [^{3}H]-CMP-PA accumulation in the presence of lithium might be an appropriate indication of activation of phosphoinositide hydrolysis to use given the simplicity and convenience of the methodology, providing that sufficient time was allowed for the depletion of free inositol such that a low expression level of M_3-mACh receptors (as seen in VT9 cells) could be discerned. The effectiveness of this method of screening is discussed in Chapter 4, where the characterisation of new clones after transfection procedures is discussed in full.

The effect of mACh receptor activation on adenylyl cyclase activity was also studied in CHO-M3 and CHO-SLM2 cells. M_2-mACh receptors have been shown previously to couple to pertussis toxin-sensitive G_i G-proteins to cause inhibition of adenylyl cyclase (Dell'Acqua *et al.*, 1993), and a clear inhibition of forskolin-stimulated cAMP accumulation was observed in methacholine-stimulated CHO-SLM2 cells which would support this

assumption. (Further work showing this response to be pertussis toxin-sensitive in this cell-line is described in Chapter 5). Comparing these data with those for PLC activation suggest that M_2-mACh receptors preferentially couple to G_i G-proteins over other G-proteins in this cell type, and this has been shown to be the case by the use of agonist-stimulated [^{35}S]-guanosine 5'-thiotriphosphate (GTPγS) binding and pertussis toxin modification of carbachol competition-binding curves (Burford *et al.*, 1995). In CHO-M3 cells it was shown that M_3-mACh receptors are able to stimulate accumulation of cAMP above that produced by activation of adenylyl cyclase by forskolin. The EC_{50} for this response was much greater than that for inhibition of forskolin-stimulated cAMP accumulation by M_2-mACh receptor activation in Chinese hamster ovary cells. It has been suggested previously that this stimulation is attributable to coupling of the M_3-mACh receptor to G_s G-proteins (Burford *et al.*, 1995), and this is supported by other studies showing that receptors coupling efficiently to G_i or G_q G-proteins may also couple to G_s at relatively high expression levels and/or at relatively high agonist concentrations (Dittman *et al.*, 1994; Eason *et al.*, 1992; Paulssen *et al.*, 1992; Yamada *et al.*, 1993). Burford *et al.* (1995b) have proposed that the relatively low potency of agonists for stimulation of cAMP accumulation compared to that for PLC activation suggests that the stimulation of adenylyl cyclase was not derived from cross-talk after PLC activation, but may reflect a direct interaction of muscarinic receptors with the G_s family of G-proteins. Issues of cross-talk of effector pathways are again taken up in Chapter 5 of this Thesis. The differential effects of M_2-and M_3-mACh receptors on adenylyl cyclase activity will be important in the characterisation of new co-expressing clones. Not only will it be important to observe if any cross-talk occurs between the two receptor pathways when co-expressed, but also the inhibition of forskolin-stimulated cAMP accumulation will be important in demonstrating the continued expression and coupling of M_2-mACh receptors to G_i G-proteins in these new cell lines.

In summary, the use of a combination of saturation binding using [^{3}H]-NMS and competition binding of [^{3}H]-NMS with selective M_2/M_3-mACh receptor antagonists (exhibiting ≥ 30 fold selectivity) was determined to be the best method to quantify and

characterise the mACh receptor subtype populations in new cell lines co-expressing M_2/M_3-mACh receptors. It is however too complicated an analysis to use in the primary screening of such clones, and for this functional studies of CMP-PA accumulation and adenylyl cyclase activity were determined to be the simplest methods of observing activation of both receptor subtypes in the same cell in the first instance.

Table 3.1 mACh receptor expression and antagonist affinity measurements using saturation and displacement analysis of [^{3}H]-N-methylscopolamine binding in CHO-M3 and CHO-SLM2 cell membranes. Data are expressed as mean ± SEM of number of experiments shown (n), each performed in duplicate. IC_{50} values were converted to K_i values according to Cheng & Prusoff (1973).

	CHO-SLM2 membranes	*CHO-M3 membranes*
[^{3}H]-NMS saturation binding		
K_D (-log M))	9.39 ± 0.04	9.45 ± 0.05
B_{max} (fmol mg^{-1} protein)	687 ± 66	3627 ± 423
nH	0.9 ± 0.1	1.0 ± 0.1
n	5	5
[^{3}H]-NMS displacement binding		
Methoctramine		
K_i (-logM)	7.39 ± 0.03	5.91 ± 0.07
n	6	6
Tripitramine		
K_i (-logM)	8.50 ± 0.07	7.08 ± 0.06
n	3	3
p-f-HHSiD		
K_i (-logM)	6.43 ± 0.08	7.03 ± 0.07
n	3	3
Zamifenacin		
K_i (-logM)	7.36 ± 0.04	8.48 ± 0.07
n	3	3

CHAPTER 4- Characterisation of co-transfected M_2/M_3-mACh receptors in Chinese hamster ovary cells

4.1 Introduction

The use of cloned receptors expressed in cell-lines is becoming a pharmacological tool used more and more frequently in the study of receptors and their properties, such as G-protein coupling, signalling pathways utilised and so on. Untransfected Chinese hamster ovary (CHO) cells are particularly useful in this respect as they do not express native receptors for most neurotransmitters; exceptions include P2Y and P2X purinoceptors (Iredale & Hill, 1993; Michel *et al.*, 1997), cholecystokinin receptors (Dickenson & Hill, 1996) and 5-HT_{1B} receptors (Giles *et al.*, 1996). Care should be taken in assuming the absence of endogenous receptor expression based on binding studies, as in the case of the 5-HT_{1B} receptor significant functional responses in response to 5-HT can be detected in the absence of [^{3}H]-5-HT or [^{125}I]-iodocyanopindolol binding in CHO cells (Giles *et al.*, 1996). However, untransfected CHO cells show no detectable levels of [^{3}H]-NMS binding nor functional responses when stimulated with mACh receptor agonists, and mACh receptors are not detectable using immunological methods, therefore it can be postulated that untransfected CHO cells do not possess native mACh receptors (Buckley *et al.*, 1989; unpublished results from this lab) CHO cells are also particularly convenient to use in this way given the ease of transfection of cDNA compared to some mammalian cell-lines.

The use of transfected cell-lines allows receptors whose function may be difficult to discriminate in their native cells to be studied in perhaps a more simple cellular environment, providing the receptors have been isolated and cloned. In order to study the possible 'cross-talk' between M_2- and M_3-mACh receptors that may occur in smooth muscle cells it therefore seemed appropriate to co-express the two receptor subtypes in CHO cells given the

availability of vectors containing the corresponding human receptor genes and CHO cell-lines expressing each subtype homogeneously, which could be used as control cells. The relationship between different receptors in smooth muscle cells is complex, and especially important is the balance between muscarinic mACh receptor-induced contractile activity and β-adrenoceptor-induced relaxant tone. CHO cells do not have native β-adrenoceptors and therefore the interactions between M_2- and M_3-mACh receptors can be studied in the absence of coupling of adrenoceptors to adenylyl cyclase, which is an important consideration when studying contraction of smooth muscle preparations. The study of interaction between M_2- and M_3-mACh receptor-mediated signalling has consequently been simplified by co-transfection into CHO cells. This may be considered to be a contrived situation, but β-adrenoceptors could be introduced into these cells at a later date, or the findings could be compared to those seen in smooth muscle cell preparations.

Given that M_2- : M_3-mACh ratios differ from species to species e.g. rat colon 61% : 59%; human colon 76% : 24% (Gomez *et al.*, 1992) and from tissue to tissue e.g. guinea-pig ileum 80% : 20%; guinea-pig trachea 65% : 35% (Giraldo *et al.*, 1988; Haddad *et al.*, 1991), it was an essential aim of the transfection to produce several positive clones with varying ratios of M_2- and M_3-mACh receptor subtype expression. Any cross-talk between receptor subtypes must be seen in the context of the size of each receptor-subtype population before any reference can be made to the events possibly occurring in a physiological situation. It was essential to characterise extensively the mACh receptor population of each new cloned cell-line before the relationships between the signalling pathways being utilised by these receptor subtypes were investigated. The methodology for the characterisation has been developed in Chapter 3, and was used in this Chapter to study two different sets of transfected cell-lines. Also assays of second messenger signalling were compared to see whether the profiles of signalling via adenylyl cyclase and phosphoinositide hydrolysis were different in M_2/M_3-mACh receptor-subtype coexpressing cells to those expressing homogeneous populations of the receptors, and to see whether there may be any evidence for cross-talk between the two receptor-subtypes at this level.

4.2 Results

4.2.1 Transfection procedures

To create a model cell system co-expressing both M_2- and M_3-mACh receptors, human m3-mACh cDNA was transfected into CHO-SLM2 cells using a mammalian expression vector (p-CEP-hygro) as described in Methods. As reported in the previous Chapter CHO-SLM2 cells stably express M_2-mACh receptors at a density of 687 ± 66 fmol mg^{-1} protein. The calcium phosphate-DNA co-precipitation method of transfection was used (see Methods and Sambrook *et al.*, 1989) which increases uptake of cDNA into cultured cells either by allowing precipitation of the cDNA onto the cell surface or increasing the binding of the cDNA to the cell surface.The uptake of cDNA accross the cell membrane of CHO cells is good, which increases the probability of successful transfection. In this case the selection marker used to distinguish the cells that had been successfully transfected was a gene conferring hygromycin-resistance i.e. only cells containing the introduced cDNA would be able to form colonies on a petri dish with media containing hygromycin. Prior to the first transfection, a 'kill curve' was not carried out to discern the susceptibility of untransfected CHO cells to hygromycin. This was a mistake as many 'false positives' were identified, subsequent to selection, as described later in this Chapter. This was in spite of the observation of the death of untransfected cells in parallel to selection of the transfected cells, using the same concentration of hygromycin. A study was carried out to observe the most effective concentration of hygromycin to cause cell death in the absence of the resistant gene prior to the second transfection. This produced much clearer colonies (above background), assumed to have originated from a single cell, as the hygromycin had been titrated more successfully. The number of colonies attained from the second transfection was fewer than for the first due to the higher concentration of hygromycin used. However, due to the screening process adopted for cells from this transfection, positive clones were easier to discern from cells which were resistant to hygromycin but did not express the M_3-mACh receptor. The shortcomings of the way in which the selection and screening of the first

transfection was carried out, compared to that of the second transfection are discussed further, later in this Chapter.

4.2.1 Screening of clones produced by the initial transfection procedure

CMP-PA accumulation was measured in clones after 20 min stimulation with a maximal concentration of MCh (1 mM) in the presence of Li^+ (10 mM). The results for several clones in which CMP-PA accumulation was shown to increase upon MCh stimulation are shown in Table 4.1. This time-point was chosen as increases in CMP-PA accumulation could be observed in VT-9 cells (which express relatively low levels M_3-mACh receptors) in the presence of Li^+ after 20 min despite a longer lag phase than more highly M_3-mACh receptor-expressing cells (see Figure 3.17). A maximal concentration of MCh was used in order to produce the largest possible mACh receptor-induced CMP-PA increase observable in the cells. This methodology allowed a large number of clones to be screened quickly and effectively. Cells in which a significant increase in CMP-PA accumulation could be observed upon stimulation with maximal MCh in the presence of Li^+ were investigated further; whilst negatives for this index of activation of phosphoinositide hydrolysis were discarded.

Inhibition of forskolin-stimulated cAMP production was then measured in the positive clones to assess the continued expression and coupling of the M_2-mACh receptors in the cell-lines. Maximal MCh (1 mM) was added for 10 min, after which cells were stimulated with forskolin (10 μM) for 5 min. The level of cAMP measured was compared to that produced by stimulation of adenylyl cyclase by forskolin in the absence of MCh in the same cell-line. The results for inhibition of forskolin-stimulated adenylyl cyclase activity for clones also giving a positive CMP-PA response are shown in Table 4.1.

Table 4.1 Methacholine-stimulated CMP-PA accumulation and inhibition of forskolin-stimulated cAMP accumulation in new putative M_2/M_3-mACh co-expressing cell-lines from initial transfection.

Cells were labelled with [^{3}H]-cytidine (0.3 μCi ml^{-1} ; 60 min), preincubated with Li^+ (10 mM; 15 min) and stimulated with MCh (1 mM; 20 min), before reactions were terminated and [^{3}H]-CMP-PA accumulation was measured as described in Methods. Alternatively, unlabelled cells were incubated with MCh (1 mM; 15 min) before the addition of forskolin (10 μM; 5 min). Reactions were terminated and cAMP mass measurements made according to Methods. Data represent values from a single experiment performed in triplicate.

Identification number of clone (DBx)	*CMP-PA accumulation (Fold increase over basal)*	*% Inhibition of Forskolin-stimulated cAMP*
M3	12	No inhibition
SLM2	< 2	90
2	2.8	70
4	6.6	81
5	7.1	85
7	17.3	50
33	21.7	65
36	29.0	83
39	10.0	81
41	5.0	82
45	8.2	70
49	36.1	56
51	44.1	59
52	34.4	44
54	26.5	52
58	4.8	87

Total number of clones selected = 60

Total number of clones positive for CMP-PA accumulation (1 mM MCh) = 34

Next, three clones showing a range of stimulations of CMP-PA and inhibition of forskolin-stimulated cAMP were chosen randomly, grown in larger flasks and membranes prepared from them. Radioligand binding studies were then performed using both methoctramine and tripitramine, which showed good selectivity for M_2- over M_3-mACh receptors in previous studies (Chapter 3). Saturation binding studies were also carried out using [^{3}H]-NMS and the B_{max} values were found to be larger than that for the parent cell-line CHO-SLM2 (Table 4.2). This was consistent with an increase in total mACh receptor number in these new cell-lines compared to CHO-SLM2 cells, and could indicate the expression of M_3-mACh receptors in addition to the M_2-mACh receptor expression observed in the parent cell-line.

Table 4.2 [^{3}H]-NMS saturation binding parameters for new cloned cell-lines (CHO-DBx) in comparison with those for the parent cell-line CHO-SLM2.
Saturation binding experiments were performed using intact cells as described in Methods. Data represent the mean ± s.e.m. for 3 separate experiments

Identification no. of clone (CHO-DBx)	B_{max} *(fmol mg^{-1} protein)*	K_d *(nM)*
SLM2	692 ± 50	0.5 ± 0.1
4	1194 ± 130	0.4 ± 0.1
7	817 ± 100	0.4 ± 0.1
52	2197 ± 176	0.3 ± 0.1

However, when displacement of [^{3}H]-NMS using methoctramine or tripitramine was measured for these cell-lines, curves were best-fitted by a one-site, and not a two-site analysis (For analysis using Tripitramine see Figure 4.1). Furthermore, Hill coefficients were not significantly different from unity. This indicated that the population of mACh receptors in these clones was likely to be homogeneous and was unlikely to be made up of a mixture of M_2- and M_3-mACh receptors as was expected from the results of functional

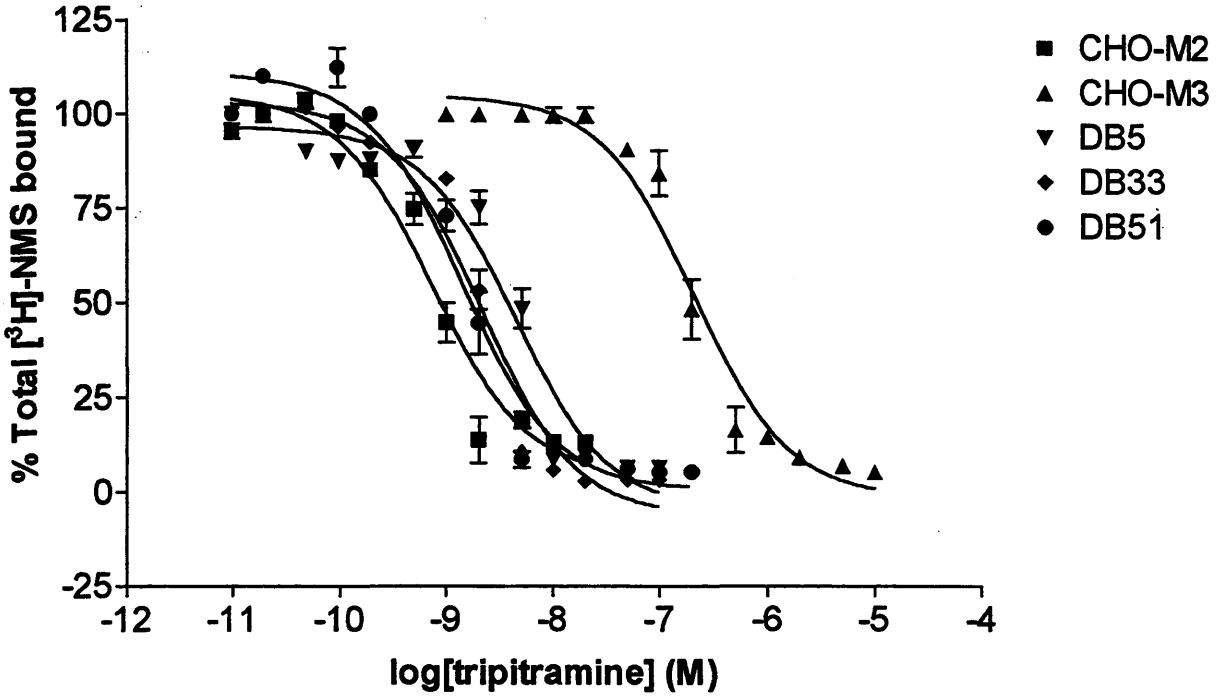


Figure 4.1- Displacement of [^{3}H]-NMS binding from CHO cell membranes by tripitramine

Competition binding using cell membrane preparations was performed as described in Methods. Data represent mean ± s.e.m. for 3 separate experiments, performed in triplicate.

biochemical studies carried out to screen the clones. To resolve these conflicting data, the signalling responses of these cells were examined in more detail.

Full concentration-response curves were constructed for [^{3}H]-CMP-PA accumulation (+ Li^+) in these cell-lines and compared with that for CHO-M3 cells (Figure 4.2). The EC_{50} for this response in these cell-lines was much greater than that for the response in CHO-M3 cells which is 0.65 μM. This might indicate that the increase in CMP-PA accumulation in the new cell-lines is not mediated by activation of M_3-mACh receptors. Studies of the concentration-response relationship for MCh-stimulated inhibition of forskolin-stimulated cAMP production showed that there was little difference in the EC_{50} of the response between the new cell clones and the parent cell-line CHO-SLM2 (Figure 4.3). This would not necessarily be expected in a mixed subtype population of cells, given that M_3-mACh receptors can stimulate cAMP production in this cell-line at high concentrations of agonist and/or receptor expression levels (Chapter 3).

As further confirmation that these new cell-lines did not express detectable levels of M_3-mACh receptors, Western blotting was carried out using simple cell lysates of the newly cloned cell-lines and of CHO-M3 cells and CHO-SLM2 cells, using an antibody specific for M_3-mACh receptors (Tobin & Nahorski, 1993). Figure 4.4a shows a Western blot where decreasing protein concentrations of CHO-M3 cell lysates (i.e. decreasing M_3-mACh receptor number) were separated, transferred to nitrocellulose and then probed with this antibody. It shows that M_3-mACh receptors can be detected in this manner to the lowest concentration of CHO-M3 cell lysate used (5 μg protein loaded, therefore 18 fmol M_3-mACh receptor). This antibody has been shown in previous studies to be highly selective for the M_3-mACh and to have high affinity for it (Tobin & Nahorski, 1993). When it had been established that this antibody could be used to observe low expression levels of M_3-mACh receptors, Western blots were carried out using the cell lysates from the new clones and CHO-SLM2 and -M3 cells (Figure 4.4b). These Western blots showed clearly that M_3-mACh receptor expression occurs in CHO-M3 cells, but not in CHO-SLM2 cells, nor in any

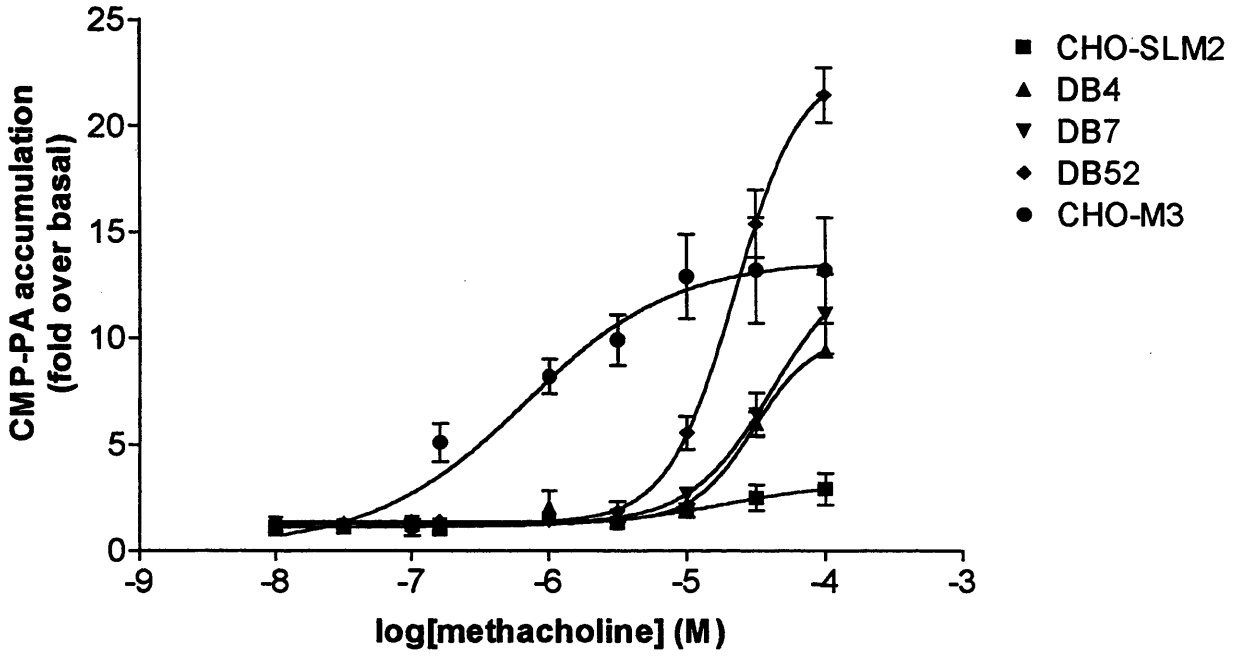


Figure 4.2 - Effect of methacholine-stimulation on CMP-PA accumulation in CHO cell-lines

Cells were washed in KHB and incubated for 1hr at 37°C in KHB containing 0.3 Ci ml^{-1}. Cells were washed with unlabelled buffer and further incubated for 15 min in fresh KHB containing 10mM LiCl. Cells were then incubated with varying concentrations of MCh for 20 min. The reaction was stopped by addition of 500 µl 1M TCA, the plates were left to stand on ice for 15 min, washed with 5% TCA containing 1 mM EDTA, and then CMP-PA extracted from the cell debris as described in Methods. Data represents mean ± s.e.m of 3 separate experiments performed in duplicate.

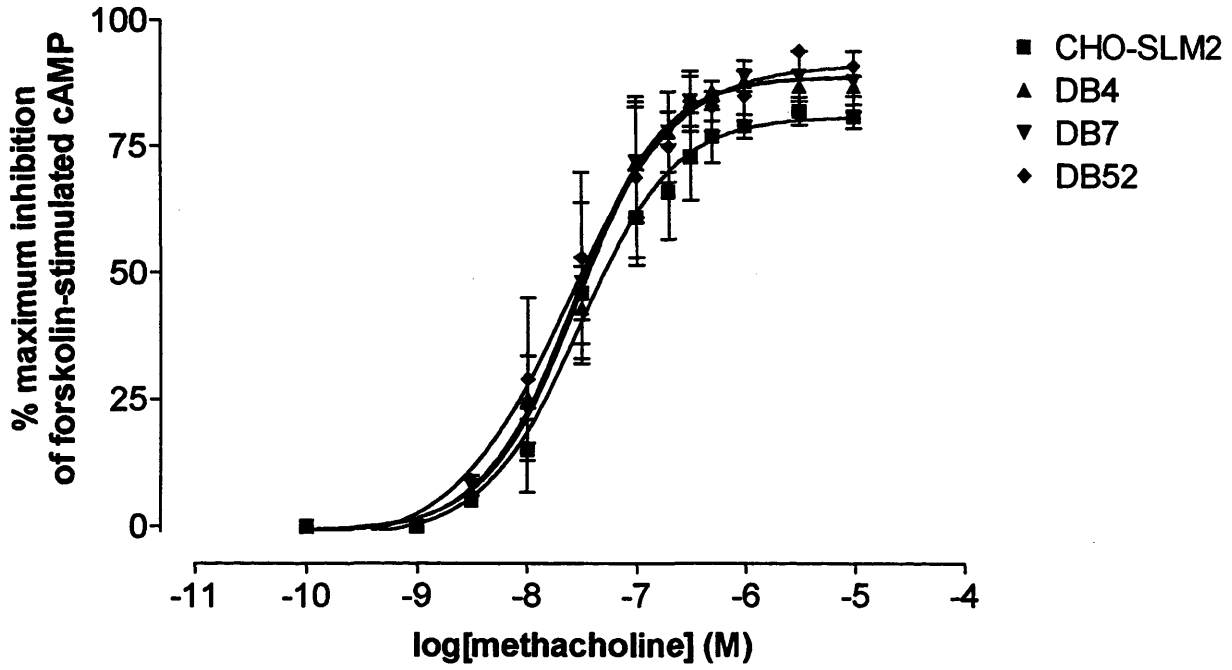


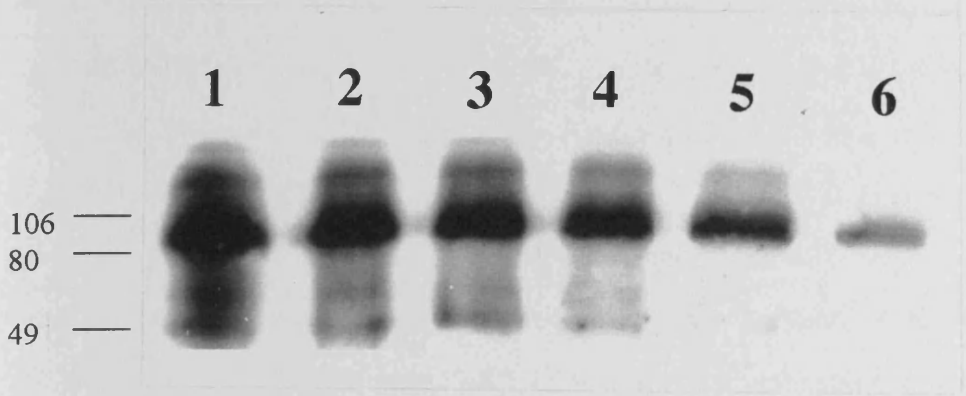
Figure 4.3 - Methacholine-mediated inhibition of forskolin-stimulated cAMP accumulation

Cells were washed and incubated in oxygenated KHB at 37°C for 10 min. Cells were incubated with or without MCh at varying concentrations for 15 min, before being challenged with forskolin (10 μM) for 5 min. Reactions were terminated by the removal of buffer and addition of 0.5 M TCA. Cyclic AMP was then extracted and measured as described in Methods. Data represent mean ± s.e.m. of 3 separate experiments, performed in duplicate. Values are expressed as % maximum inhibition achieved in each cell-line, in order to normalise data to compare EC_{50} values.

Figure 4.4 - Western blots using simple cell lysates, and an antibody specific for M_3-mACh receptors. a) Decreasing protein concentrations of CHO-M3 cell lysates were separated on a SDS-acrylamide (10%) gel, transferred to nitrocellulose and probed with the M_3-mACh receptor-specific antibody as described in Methods. b) Cell lysates (30 μg protein / lane) from new clones, CHO-M3 and CHO-SLM2 cells were separated on a SDS-acrylamide gel, transferred to nitrocellulose and probed as described in Methods. The results shown are after 60 sec exposure to photographic film after ECL processing. Bands are labelled in kDa, according to separation of low molecular weight markers as described in Methods.

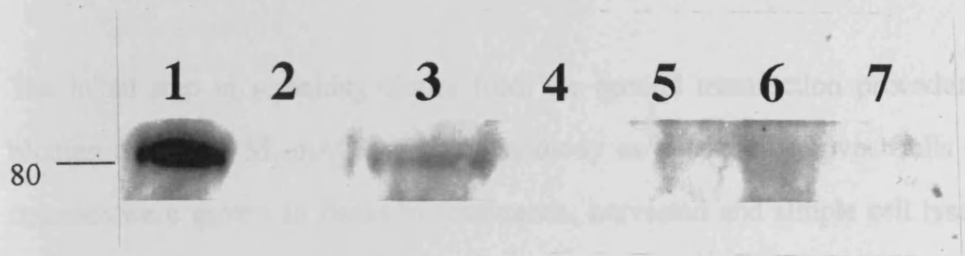
a)

		Protein (μg)	M_3-mACh Receptor no. (fmol)
Lane	1	30	108
	2	25	91
	3	20	73
	4	15	54
	5	10	36
	6	5	18



b) Lane

1 CHO-M3
2 CHO-SLM2
3 CHO-VT9
4 DB4
5 DB5
6 DB33
7 DB51



of the new cell-lines. Therefore, the initial transfection of m3-cDNA into the SLM2 cell-line had not resulted in the selection of cell-lines co-expressing M_2/M_3-mACh receptors, where M_3-mACh receptors were present at anything greater than trace levels.

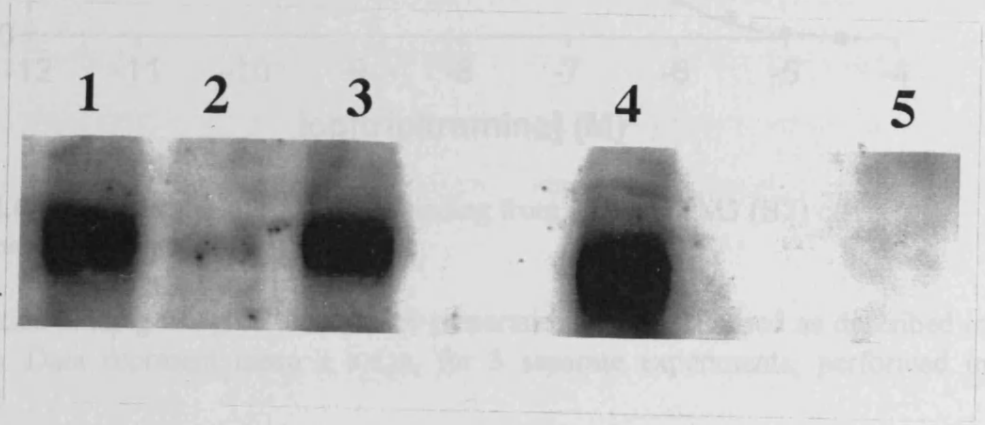
4.2.3 Screening of clones produced by the second transfection procedure

The initial step in screening clones from the second transfection procedure was Western blotting using the M_3-mACh receptor antibody as described above. Cells from individual colonies were grown in flasks to confluence, harvested and simple cell lysates were made. Western blotting was used to screen for M_3-mACh receptor expression. Unfortunately, no suitable M_2-mACh receptor antibody was available for immunoblotting for M_2-mACh receptor expression. This method was more time-consuming than the use of CMP-PA accumulation as a primary screen for M_3-mACh receptor expression, but was a more reliable method for the selection of positive clones. A sample blot illustrating three positive clones and CHO-SLM2 and CHO-M3 cell lysates is shown in Figure 4.5. Varying levels of M_3-mACh receptor expression can be discerned in the three cloned cell-lines. Saturation binding using [^{3}H]-NMS was carried out using the three clones positive for M_3-mACh receptor expression. These data were combined with analysis of tripitramine displacement of [^{3}H]-NMS binding in membrane preparations to estimate actual receptor numbers for each mACh receptor subtype (Table 4.3). Figure 4.6 shows tripitramine displacement of [^{3}H]-NMS binding using membranes of cells from clone B2. The displacement curve is best-fitted by a two-site analysis of the data. This enables estimation of the K_i values of the two affinity sites (after transformation of EC_{50} values for each affinity state using the Cheng and Prusoff equation) and the relative fractions of each receptor subtype. The calculated K_is for the two affinity states in this instance agree with those expected for M_2- and M_3-mACh receptors, giving confidence that clone B2 expresses a mixed population of M_2- and M_3-mACh receptors in approximately equal proportions, a clear improvement on the disappointing results from the first transfection. The levels of M_2-mACh receptors are not significantly

Figure 4.5 - Western blots using simple cell lysates, and an antibody specific for M_3-mACh receptors. Cell lysates (30 μg protein / lane) were separated on a SDS-acrylamide (10%) gel, transferred to nitrocellulose and probed with the M_3-mACh receptor-specific antibody as described in Methods. The results shown are after 60 sec exposure to photographic film after ECL processing. Bands are labelled in kDa, according to separation of low molecular weight markers as described in Methods.

Lane

1 CHO-M2/M3 (B2)
2 CHO-M2/M3 (B7)
3 CHO-M2/M3 (R3)
4 CHO-M3
5 CHO-SLM2



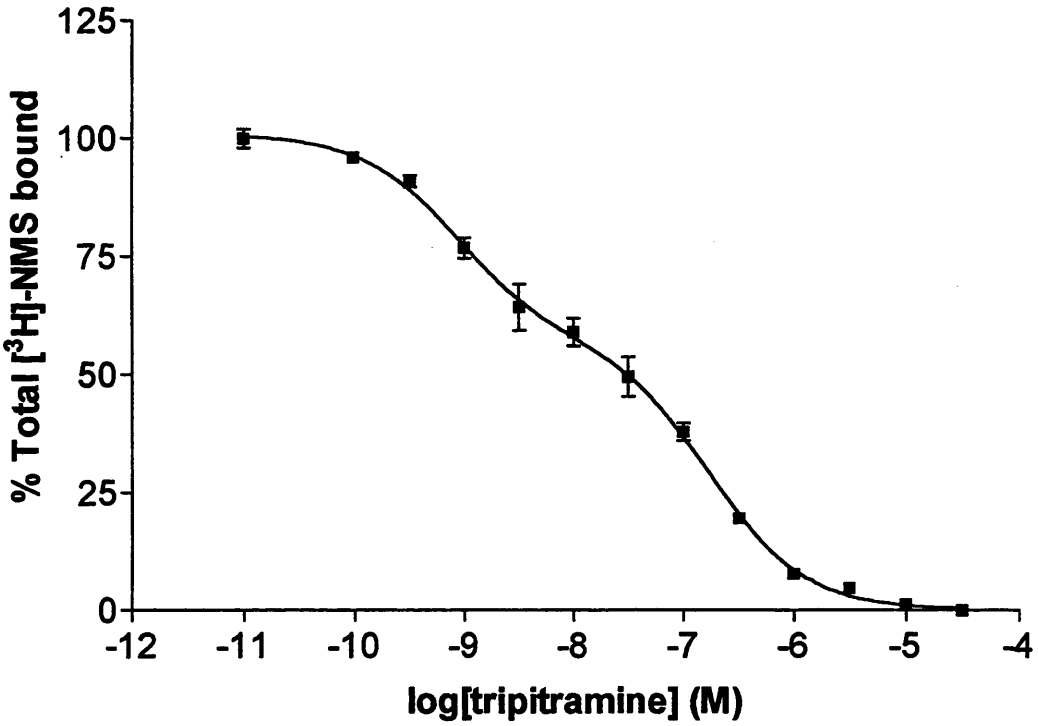


Figure 4.6- Displacement of [^{3}H]-NMS binding from CHO-M2/M3 (B2) cell membranes by tripitramine

Competition binding using cell membrane preparations was performed as described in Methods. Data represent mean ± s.e.m. for 3 separate experiments, performed in triplicate

different between new cell-lines and the parent CHO-SLM2, indicating that introduction of M_3-mACh receptors has not perturbed the expression of M_2-mACh receptors.

Table 4.3 Comparison of total mACh receptor number, M_2- and M_3-mACh receptor numbers and measurements of total inositol phosphate accumulation to stimulation by MCh in various CHO cell-lines.

Saturation binding analysis was carried out using intact cells as described in Methods. Competition binding displacement from cell membrane preparations using tripitramine was carried out as described in Chapter 3. Total inositol phosphates ($[^3H]$-$InsP_x$) were determined in $[^3H]$-inositol labelled cells (1μCi ml^{-1}; 48 h) stimulated with 1mM MCh for 20 min in the presence of 10 mM Li^+. Data represent mean ± s.e.m. for 3 separate experiments or in the case of mACh receptor subtype numbers the values shown are estimates based on the calculation of receptor subtype fractions from 3 separate displacement experiments.

Clone	B_{max} *(fmol mg^{-1}protein)*	*Increase in IP_x (Fold above basal)*	M_2 *(fmol mg^{-1}protein)*	M_3 *(fmol mg^{-1}protein)*
M3	2800 ± 304	6.8 ± 0.2	-	2800 ± 304
SLM2	688 ± 32	<2	688 ± 32	-
B2	2062 ± 316	10.8 ± 1.8	928	1134
B7	1589 ± 215	9.7 ± 0.6	953	636
R3	1261 ± 252	N.D.	927	334

N.D. = not determined

There are clear increases in total $[^3H]$-inositol phosphate accumulation in two of the three clones in response to stimulation with maximal MCh in the presence of Li^+ (Table 4.3). The magnitude of the total inositol phosphate response does appear to be related to the estimated expression levels of M_3-mACh receptors, although differences in coupling efficiencies cannot be ruled out, as the concentration of MCh used is maximal. The relationship between

M_3-mACh receptor number and total [^{3}H]-inositol phosphate accumulation may be more complex as in clone B2 total inositol phosphate accumulation in response to maximal MCh is greater than that in CHO-M3 cells which express a higher concentration of M_3-mACh receptors (Table 4.3). A more definitive study of adenylyl cyclase and inositol phosphate signalling in these receptor subtype co-expressing cells is described below.

4.2.4 Phosphoinositide signalling in M_2/M_3-mACh receptor-subtype co-expressing cells

The relative merits of using inositol-1,4,5-trisphosphate ($InsP_3$) mass compared with total [^{3}H]-inositol phosphate measurements as indicators of phosphoinositide hydrolysis have been discussed previously in Wojcikiewicz *et al.*, (1993). In this study, it was decided that $InsP_3$ measurements may be more useful in indicating important differences in phosphoinositide signalling of the M_3-mACh receptor in the presence or absence of concurrent M_2-mACh receptor expression. In Chapter 3 it was shown that a homogeneous population of M_3-mACh receptors typically stimulates a peak-plateau time-course of $InsP_3$ production in response to maximal MCh. Time-courses for two clones expressing varying levels of M_3-mACh receptors (B2 and B7) were compared with that for CHO-M3 cells (Figure 4.7).

In the cell-line B7, which expresses approximately 1.1 pmol mg^{-1} protein M_3-mACh receptor, the general pattern of the production of $InsP_3$ production with time was similar to that in CHO-M3 cells, but the maximal peak response was less than half the magnitude (102 $\pm$ 5 pmol mg^{-1} protein and 264 $\pm$ 21 pmol mg^{-1} protein respectively).

Although the response in B2 cells was biphasic, the profile of $InsP_3$ production with time was different than that seen in CHO-M3 cells in response to the same concentration of MCh. After the initial peak, there was a decrease in $InsP_3$ production which was lowest at about 60 seconds after agonist application. This was followed by a further later sustained increase in $InsP_3$ production, in contrast to the steady-state plateau level observed for up to 20 min in

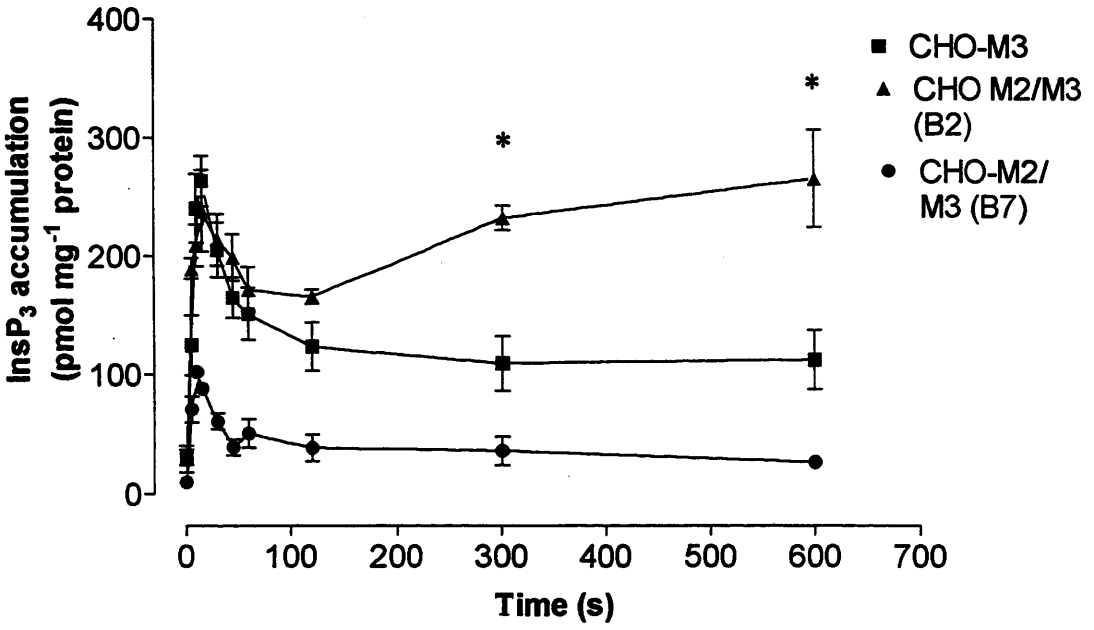


Figure 4.7 - $InsP_3$ production with time in response to methacholine (1mM) in CHO cells

Cells were washed and incubated for 10 min in KHB. Methacholine (1mM) was then added and incubated for varying times. The reactions were stopped by the addition of an equal volume of TCA (1M). InsP3 was extracted and levels determined as described in Methods. Data represents mean ± s.e.m. for 3 to 5 separate experiments performed in duplicate. *$P<0.01$, Student's unpaired *t* test

CHO-M3 cells. The peak value for 1 mM MCh at the 10 s time-point was 238 ± 34 pmol mg^{-1} protein, but $InsP_3$ production was still increasing at 30 min after application of MCh. The possibility that this is an effect of mACh receptor subtype co-expression is investigated in Chapter 5.

The concentration-response relationship of peak agonist-stimulated $InsP_3$ production was studied by measuring $InsP_3$ mass concentration in cells after 10 s application of varying concentrations of MCh (Figure 4.8). The basal value of $InsP_3$ of 25 ± 3 pmol mg^{-1} protein increased to a value of 262 ± 44 pmol mg^{-1} protein in the presence of a maximal concentration of agonist. There was no statistically significant difference in the EC_{50}s of the concentration-response relationships of peak $InsP_3$ production between CHO-M3 cells and the clone B2 co-expressing mACh receptor subtypes. This experiment showed considerable variability, leading to curves which were shallower than would be expected, because the peak of IP_3 production does not always fall exactly 10 s after agonist application in all cell populations, and the short time-point is difficult to perform accurately.

4.2.5 Adenylyl cyclase signalling in M_2/M_3-mACh receptor-subtype co-expressing cells

Levels of cAMP mass were measured in response to concurrent forskolin-stimulation (10 μM) of adenylyl cyclase activity and agonist stimulation of mACh receptors. In CHO-SLM2 cells there is a clear inhibition of the forskolin-mediated stimulation of cAMP production by MCh, with the EC_{50} for this response of 0.01 μM. In CHO-M3 cells there is a stimulation of cAMP production above that of forskolin at higher concentrations of MCh (see also Chapter 3). The response in clone B2 was biphasic, made up of an inhibitory response at low concentrations of MCh and a stimulatory response at higher concentrations of MCh (Figure 4.9). Maximum inhibition for this cell-line was 78% of forskolin-stimulated levels of cAMP production. Stimulation of cAMP production was still increasing at 1 mM MCh. This biphasic response would therefore appear to be consistent with possible predictions for the

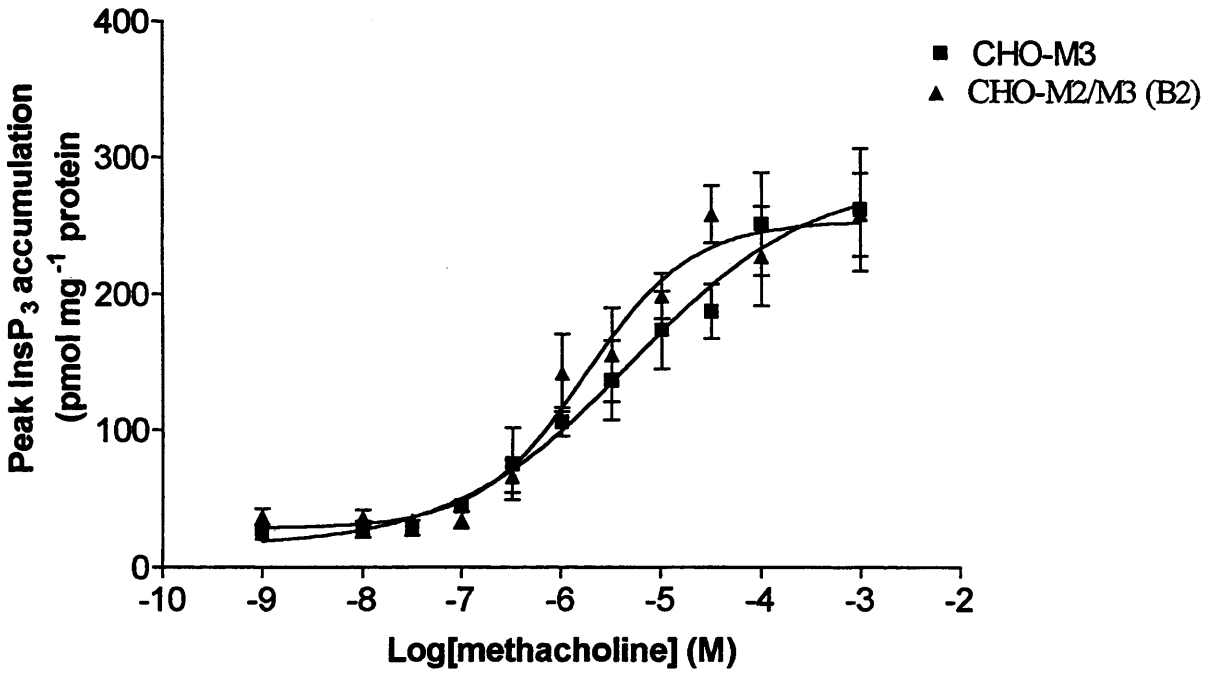


Figure 4.8 - Peak InsP3 production with increasing concentrations of methacholine in CHO-M3 versus CHO-M2/M3 (B2) cells

Cells were washed and incubated with KHB for 10 min, before the addition of MCh at various concentrations. After 10s, the reaction was stopped by addition of an equal volume of TCA (1M). $InsP_3$ was extracted and measured as described in Methods. Data represents mean ± s.e.m. for 3 separate experiments performed in duplicate. There was no statistically significant difference between the responses in the two cell-types as established by Student's unpaired *t*-test.

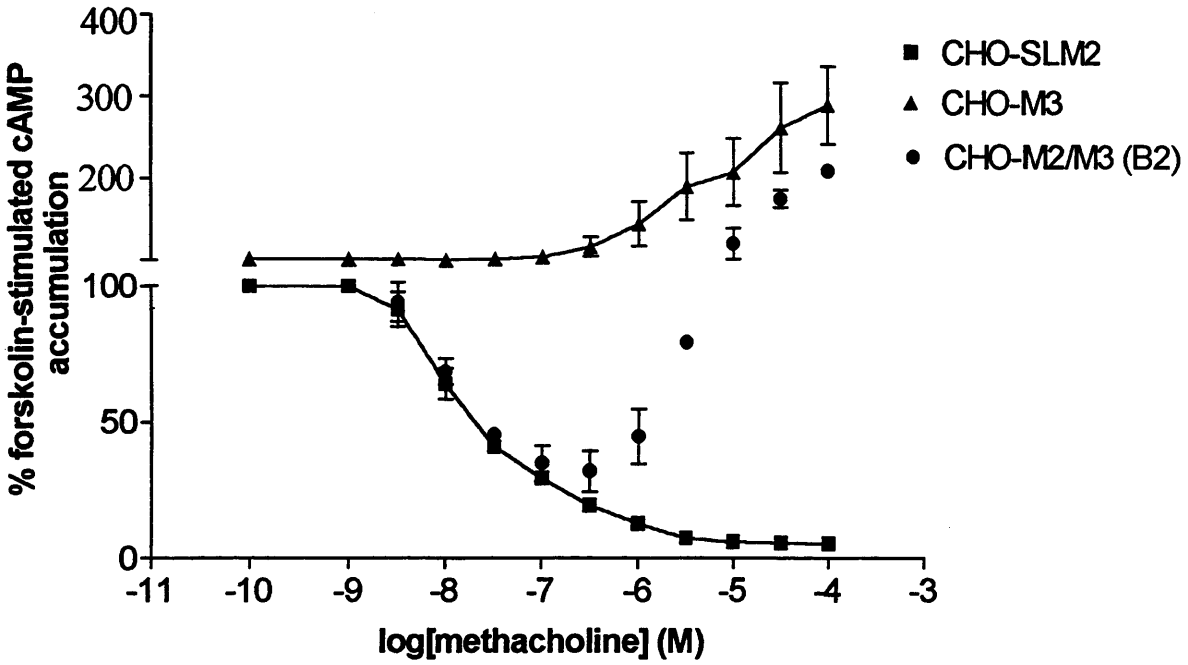


Figure 4.9 - Inhibition of forskolin-stimulated cAMP accumulation in response to increasing concentrations of methacholine in CHO cells

Cells were washed and incubated with KHB for 10 min, before the addition of MCh at various concentrations. After 15 min, cells were stimulated by the addition of forskolin (10 μM) for 5 min, and then the reaction was stopped by the removal of buffer and addition of TCA (0.5 M). Cyclic AMP levels were then analysed as described in Methods. Data represent mean ± s.e.m. of 3 separate experiments performed in duplicate.

adenylyl cyclase signalling of cells exhibiting co-expression of M_2- and M_3-mACh receptors. Further assessment of the components of this response are described in Chapter 5.

4.3 Discussion

In Chapter 3 it was shown that although [^{3}H]-NMS saturation and displacement binding analysis is an accurate way to demonstrate M_2/M_3-mACh receptor-subtype co-expression it is too complex for initial screening of all hygromycin-resistant clones. It was proposed that activation of the phosphoinositide cycle could be used to display M_3-mACh receptor expression in new clones. However, a number of problems were identified with the initial approach to selection and screening.

Firstly, no attempt was made initially to optimise the concentration of hygromycin necessary to cause the death of 100% of those cells without the transfected gene encoding resistance, whilst allowing the growth of successfully transfected clones. The 'kill' curve constructed for untransfected CHO cells prior to the second transfection procedure demonstrated that the 'window' for selection was actually small, with many false positives being selected at lower concentrations of hygromycin (i.e. untransfected cells could grow in media containing hygromycin at low concentrations), and total cell death being observed at higher concentrations despite possible successful transfection. This contrasts with the situation where neomycin has been used for selection where positive clones 'tolerate' much higher concentrations of the antibiotic than negative cells. In other words, transfected cells expressing the gene conferring resistance to hygromycin are actually only slightly less likely to die than untransfected cells in the presence of a higher than optimal concentration of hygromycin.

Also contributing to the selection of false positives from the first set of clones was the use of a maximal concentration of MCh to stimulate the phosphoinositide cycle in a single point assay for rapid screening of the large number of selected clones. It was shown that no significant increase in [^{3}H]-CMP-PA accumulation or total [^{3}H]-inositol phosphate accumulation was measured in CHO-SLM2 cells in the presence of lithium (Chapter 3), even when stimulated with maximal MCh and therefore it was assumed that no [^{3}H]-CMP-PA

accumulation would be measured in transfected cells that did not express M_3-mACh receptors. This turned out not to be the case, with [^{3}H]-CMP-PA accumulation being stimulated at high concentrations of agonist (Figure 4.2) and at high concentrations of total mACh receptor expression (Table 4.2), consistent with results expected from promiscuous coupling of a homogeneous population of M_2-mACh receptors. The results from inhibition of forskolin-stimulated cAMP also concurred with this conclusion, with all cells exhibiting almost identical EC_{50} values for this response to the parent cell-line, suggesting a homogeneous population of mACh receptors negatively coupled to adenylyl cyclase (Figure 4.3). Displacement binding analysis using methoctramine or tripitramine was best-fitted by a one-site curve (Figure 4.1) and M_3-mACh receptors could not be detected in Western blot analysis using a high affinity specific M_3-mACh antibody (Figure 4.4). It is possible that there was M_3-mACh receptor expression at very low densities, but with efficient receptor-effector coupling such that a response could be seen in the absence of low affinity displacement of [^{3}H]-NMS binding by methoctramine and no immunological detection. This may also explain why there was no effect on adenylyl cyclase activity, if M_3-mACh receptor-mediated stimulation of adenylyl cyclase only occurs at higher expression levels. For M_3-mACh receptors to be undetectable by immunoblotting it can be assumed that this receptor-subtype population makes up < 5% of the total mACh receptor population in these clones and cannot therefore account for the increases in saturation binding of [^{3}H]-NMS in new clones compared to the parent cell-line. Also, M_3-mACh receptor expression at this low density is not considered to be viable for our experiments 'modelling' the situation in most smooth muscle tissues.

Another explanation is that the original CHO-SLM2 cell-line is not derived from a single parent cell and is made up of a mixed population of cells expressing different densities of M_2-mACh receptor. The screening of the first set of clones using a maximal concentration of MCh merely led to selection of a higher M_2-mACh receptor expressing cell-line which was capable of activation of the phosphoinositide cycle at high concentrations of agonist. This would be consistent with the finding that no CMP-PA accumulation is observed under the

same conditions in the CHO-SLM2 cell-line, since the effect was measured in a mixed population of cells, with varying M_2-mACh receptor expression and therefore different abilities to activate this signalling pathway. The mean effect in this cell-line is therefore no measurable response; further subcloning leads to cell-lines with a greater ability to activate a phosphoinositide hydrolysis response (but see below).

Irrespective of the underlying cause for the data generated, these clones were not good model cell-lines for the study of cross-talk between M_2- and M_3-mACh receptors. Either they did not express M_3-mACh receptors at all, or they expressed them at too low density to model the situation in many smooth muscle tissues (Eglen *et al.*, 1997), or to give robust second messenger responses necessary for these studies. The transfection procedure was therefore repeated using a different mode of screening. One other interesting point to note from the results from the first set of clones (Table 4.1) was the different level of inhibition of forskolin-stimulated cAMP between the cell-lines despite the probable homogeneity of the mACh receptor population. The differences in total mACh receptor number (Table 4.2) are not sufficient to explain the variation in measured ability of the receptors to inhibit the forskolin-stimulated cAMP response. One possible explanation is that the variation in forskolin stimulation of adenylyl cyclase between cells is sufficiently great to lead to differences in the ability of mACh receptor activation to oppose it. Alternatively, the M_2-mACh receptor-effector coupling efficiency may differ between the cloned cell-lines.

The selection and screening of transfected cells from the second transfection procedure differed from that for cells from the initial transfection in that a higher concentration of hygromycin was used for selection of resistant clones, and Western blotting using cell lysates was the first step in the screening process. This was a more complex and time-consuming method of screening the clones, but produced no false positives i.e. those cells in which M_3-mACh receptor could be detected using immunological methods also showed increased phosphoinositide hydrolysis upon agonist stimulation, with EC_{50}s for that response not significantly different from the EC_{50} for the same response in CHO-M3 control

cells (Figure 4.8). Also, it was assumed that due to the high affinity of the M_3-mACh receptor antibody used, only cells with very low expression of M_3-mACh receptors would be mistakenly discarded. This approach was successful, with some hygromycin-resistant clones also exhibiting detectable levels of expressed M_3-mACh receptors (Figure 4.5).

Tripitramine was used for displacement binding studies, due to its excellent selectivity between M_2- and M_3-mACh receptors. Tripitramine displacement was performed in some cell-lines shown to express M_3-mACh receptors by immunological techniques (Figure 4.6). Displacement isotherms were best-fitted by two-site analysis with K_i values for each site agreeing with those expected for M_2- and M_3-mACh receptor affinities. In combination with saturation binding analysis it was possible to estimate to receptor subtype densities in these transfected cell-lines. In some clones, not only was the expression level of M_3-mACh receptor 'physiological' in terms of some smooth muscle tissue (e.g. rat colon), but also robust second messenger responses could be measured. The displacement binding procedure first described in Chapter 3 has played an invaluable role in distinguishing and quantifying M_2- and M_3-mACh receptor subtypes in these co-transfected cell-lines. The number of binding sites displayed by this method concurs with functional and immunological evidence using clones from each transfection procedure. Interestingly, the numbers of M_2-mACh receptors appears by these methods to be reasonably constant between the different new cell-lines. This would suggest that the original parent (CHO-SLM2) was a homogeneous population of cells in relation to M_2-mACh receptor expression. Therefore, it is not possible to explain the increase in total mACh receptor expression seen in cell-lines after the first transfection in terms of differing M_2-mACh receptor expression in parent cells prior to the transfection procedure. Tripitramine has been shown to bind to M_2/M_3-mACh receptors with sufficiently different affinities (>100 fold) that it may perhaps be used to selectively block M_2-mACh receptors without occupying M_3-mACh receptors even when expressed in the same cell. This property of tripitramine is applied to studies of cross-talk in Chapter 5.

The measurements of muscarinic agonist-stimulated effects on forskolin-stimulated cAMP also suggest co-expression of M_2- and M_3-mACh receptors, given the data for the measurement of this response in CHO cells expressing homogeneous populations of these receptor subtypes (Chapter 3 and Figure 4.9). The response in the co-expressing cell-line (B2) is biphasic. Although the extent of inhibition of forskolin-mediated increases in cAMP accumulation in co-expressing cells is not as great as that demonstrated in CHO-SLM2 cells, it still occurs over the same range of MCh concentrations, and the stimulatory phase of the response occurs over the same concentrations as that seen in CHO-M3 cells. This suggests that M_2- and M_3-mACh receptor co-expression does not affect the coupling of each receptor to adenylyl cyclase, that is M_2-mACh receptor activation remains inhibitory and M_3-mACh receptor activation remains stimulatory (at high agonist concentrations) to the production of the second messenger cAMP, and the end response is a sum of these two different effects. This is investigated further in Chapter 5. Although the stimulation of adenylyl cyclase activity by G_s G-protein activation has been extensively studied, receptor-mediated inhibition of adenylyl cyclase in smooth muscle tissues has been disregarded by comparison. There is evidence to show that mACh receptor activation causes inhibition of adenylyl cyclase via M_2-mACh receptor activation of pertussis toxin-sensitive G-proteins in smooth muscle (Griffin & Ehlert, 1992; Jones *et al.*, 1987; Yang *et al.*, 1991) but there is no reference to any stimulation of cAMP accumulation at higher concentrations of agonist. There is however evidence in CHO cells that cAMP accumulation via activation of β_2-adrenoceptors is enhanced when M_3-mACh receptors are coexpressed (Ellis *et al.*, 1996) possibly via a protein kinase C-independent mechanism (Stanford *et al.*, 1996), indicating that M_3-mACh receptor-induced effects on cAMP accumulation may have a profound influence on other receptor signalling pathways in CHO cells. The concentration-response profile for cAMP accumulation in M_2/M_3-mACh receptor co-expressing cells, although perhaps very useful in demonstrating the concomitant activation of the two receptor subtypes, may be misleading with regard to effects on adenylyl cyclase activity in actual smooth muscle tissues.

The profile of the time-course of $InsP_3$ production is different in M_2/M_3-mACh receptor-expressing cells compared to that seen in CHO cells expressing M_3-mACh receptors alone. The second phase of $InsP_3$ production, which is maintained for up to 2 h in CHO-M3 expressing cells (Tobin *et al.*, 1992), is statistically significantly different in M_2/M_3-mACh receptor-coexpressing CHO cells (Figure 4.7). The early peak response appears to be unaffected in contrast, with the concentration-response relationships for peak $InsP_3$ production being not statistically different between CHO-M3 cells and CHO-M2/M3 cells (Figure 4.8). It has been stated that this early peak response represents an activation and desensitisation event (Tobin *et al.*, 1992), and the sustained phase is due to the ability of the influx of extracellular Ca^{2+} to maintain the activation of phosphoinositidase C. Thus this second phase of $InsP_3$ production has been shown in the same study in CHO-M3 cells to be dependent on extracellular Ca^{2+} concentration (Tobin *et al.*, 1992). Therefore, it is interesting to note that this phase is different in this particular co-expressing cell-line (B2). In Chapter 5, studies are performed to determine whether it is cross-talk between M_2/M_3-mACh receptor-subtypes which is the cause of the increase during this phase of $InsP_3$ production, and the possible mediators of such cross-talk will be discussed.

In summary, several cell-lines expressing both M_2- and M_3-mACh receptors at varying densities have been cloned. The most sucessful method of screening for M_3-mACh receptor expression was by immunlogical detection. Co-expression of M_2- and M_3-mACh receptors does alter the profiles of signalling via adenylyl cyclase and $InsP_3$ from that seen in homogeneous populations of these receptor subtypes in the same cell-line, and the possibility of cross-talk between these receptor subtypes will be investigated further in Chapter 5.

CHAPTER 5 - Studies of 'cross-talk' between mACh receptor subtypes in M_2/M_3-mACh receptor co-transfected CHO cell lines

5.1 Introduction

Although some studies in smooth muscle have shown that M_2-mACh receptor activation contributes to the relative resistance of mACh receptor-induced tone to β-adrenenoceptor-evoked relaxation (Fernandes *et al.*, 1992; Mitchell *et al.*, 1993; Reddy *et al.*, 1995; Thomas & Ehlert, 1994; Watson & Eglen, 1994), other studies have found no apparent contribution of M_2-mACh receptors to smooth muscle contraction (Roffel *et al.*, 1993; Roffel *et al.*, 1995; Watson *et al.*, 1995; Watson *et al.*, 1995). M_2-mACh receptor expression is conserved in smooth muscles with few exceptions, and in most cases forms the predominant mACh receptor population (Eglen *et al.*, 1994). Therefore it is perhaps surprising that no definitive role for M_2-mACh receptors in smooth muscle has as yet been conclusively elucidated. It is also interesting to note that the development of selective M_3-mACh receptor antagonists, such as zamifenacin, has not been an entirely successful strategy in the clinical treatment of disorders such as irritable bowel syndrome and urinary bladder incontinence, where the function of M_2-mACh receptors in smooth muscle contraction has largely been disregarded. The studies described in this Chapter have used a model cell system to investigate the possible modulatory role of M_2-mACh receptors on the intracellular signalling of M_3-mACh receptors, as this could explain the presence of M_2-mACh receptors in smooth muscle cells in the absence of a direct role for this receptor subtype in the direct mediation of contraction.

In Chapter 4 it was reported that an increase in the second phase of $Ins(1,4,5)P_3$ production with time was observed in cells co-expressing M_2/M_3-mACh receptors compared to that in cells expressing a homogeneous population of M_3-mACh receptors. In CHO cells expressing a homogeneous population of M_2-mACh receptors at a similar density to that

observed in the co-expressing cell-line, no increase in $InsP_3$ production is detected on stimulation with muscarinic agonists. This suggests that whilst M_2-mACh receptors do not directly activate phosphoinositide hydrolysis in this cell type when expressed at this density, they may modulate signalling via this pathway when it is activated by concurrent M_3-mACh stimulation. This Chapter describes experiments used to ascertain whether the observed increase in the second phase of $InsP_3$ production in response to MCh challenge is brought about due to co-activation of the M_2-mACh receptors and not by a mechanism secondary to co-expression of M_2/M_3-mACh receptor subtypes, bearing in mind the enormous diversity of cross-talk.

Since smooth muscle is often innervated by the parasympathetic nervous system (cholinergic) to cause contraction, and by the sympathetic nervous system (adrenergic) to cause relaxation, the interaction between phosphoinositide hydrolysis and cAMP production may be central to the control of smooth muscle tone. Much work has been done to investigate the interaction of these pathways in order to discover a biochemical correlate for the physiological antagonism of the two systems. There is a body of work that shows that agents which cause elevation of cAMP levels such as β_2-adrenoceptor agonists, phosphodiesterase inhibitors, and some prostaglandins (PGEs), cause inhibition of the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) into $InsP_3$ and DAG (Abdel-Latif, 1996; Hall & Hill, 1988; Laglia *et al.*, 1996; Peakman & Hill, 1994; Prestwich & Bolton, 1995), and hence can inhibit contraction by decreasing Ca^{2+} mobilisation (Camello *et al.*, 1996; Hoiting *et al.*, 1996). This may occur via activation of protein kinase A (PKA) (Liu & Simon, 1996; Song *et al.*, 1997; Zhang & Buxton, 1993), and in addition to direct modulation of myosin light chain kinase (MLCK), as was reported previously (De Lanerolle *et al.*, 1984). Therefore, it can be suggested that M_2-mACh receptors could act synergistically with M_3-mACh receptors to increase IP_3 and DAG production by inhibiting the production of cAMP and hence this pathway. However, there is also evidence that in some cells, increased cAMP production can cause increases in signalling via IP_3 and DAG

(Mau *et al.*, 1997), therefore the effect of cAMP on phosphoinositide hydrolysis may be cell-specific.

Another, perhaps simpler, way in which activation of M_2-mACh receptors may influence signalling of M_3-mACh receptors is via release of βγ-subunits of the G_i G-protein which has been reported to modulate isoforms of PLC (Camps *et al.*, 1992; Carozzi *et al.*, 1993) and possibly the inhibitory feedback onto the phosphoinositide pathway mediated via PKC (Huwiler *et al.*, 1997; Mau *et al.*, 1997; Watanabe *et al.*, 1996). Other possible pathways which may be involved are arachadonic acid production by PLA_2 (Conklin *et al.*, 1988; Dennis, 1997; Dickenson, 1996), stimulation of PLD (Sandmann *et al.*, 1991), direct activation of channels e.g. non-specific cation channels (Benham *et al.*, 1985) and indirect channel activation via Ca^{2+} mobilisation (Nahorski *et al.*, 1994). Also involved may be the small GTPases and the extracellular signal-regulated kinase (ERK) family or the mitogen-activated protein kinases (MAPKs), which can be activated by G-protein linked receptors as well as receptors with tyrosine kinase activity (Malarkey *et al.*, 1995). This is not an exhaustive description of possible modes of cross-talk between the signalling pathways activated by these two mACh receptor subtypes studied here, but does give an indication of the potential complexity of cell signalling in general.

If M_3-mACh receptor signalling is modified by cross-talk with M_2-mACh receptors then an effect might also be expected at the level of Ca^{2+} signalling within the cells. As described in the Introduction to this Thesis, $Ins(1,4,5)P_3$ causes release of Ca^{2+} from intracellular stores via activation of $InsP_3$ receptors. If the $InsP_3$ signal mediated by M_3-mACh receptors is modified by cross-talk with an M_2-mACh activated pathway, then it is possible that M_2/M_3-mACh receptor co-expression would also have an effect on the Ca^{2+} signalling of the cell. Also, by using a single cell imaging method to measure $[Ca^{2+}]_i$, the heterogeneity or homogeneity of cell populations can be studied, in terms of Ca^{2+} signalling at least, where other more simple fluorimetric methods can only supply information on the average changes in $[Ca^{2+}]_i$ of a population of cells. Therefore, single cell Ca^{2+} imaging (using the fura-2

fluorescent dye method) was used to study Ca^{2+} signalling of M_2/M_3-mACh co-expressing cells and CHO cells expressing a homogeneous population of each receptor subtype, in order to investigate not only the effect of co-expression of receptor-subtypes on Ca^{2+} signalling, but also to evaluate the probability that each cell-line has been derived from a single cell (i.e. the cells are identical within each cell-line and do not represent a mixed clonal population).

These studies will ascertain whether cross-talk does occur between M_2- and M_3-mACh receptors at the level of phosphoinositide hydrolysis and adenylyl cyclase activity in this model cell system, and will allow the planning of further work to discern at which point in the signalling pathways the interaction occurs, what mediates this cross-talk and ultimately whether this actually occurs in smooth muscle cells.

5.2 Results

5.2.1 Effect of pertussis toxin pre-treatment on cAMP and $InsP_3$ measurements in control cells

Before pertussis toxin could be used as a tool in the study of M_2/M_3-mACh receptor cross-talk, it was important to study its effects on mACh receptor signalling in CHO cells expressing homogeneous populations of mACh receptor-subtypes. The effects on MCh-induced cAMP inhibition or stimulation in CHO-SLM2 and CHO-M3 cells respectively, and phosphoinositide signalling in CHO-M3 cells were investigated.

Since it has been reported that M_2-mACh receptors signal via the G_i family of G-proteins to cause inhibition of adenylyl cyclase (Dell'Acqua *et al.*, 1993), and that pertussis toxin uncouples G_i G-proteins from their activating receptors by ADP-ribosylating the α subunit of the G-protein (Caulfield, 1993), it was expected that pertussis toxin pre-treatment of CHO-SLM2 cells would result in abolition of the M_2-mACh receptor-mediated inhibitory response (see also Chapter 3). It can be seen in Figure 5.1a that although MCh-induced inhibition of forskolin-stimulated cAMP levels was significantly less after pertussis toxin pre-treatment compared to that in untreated cells ($P < 0.01$), the inhibitory response was not completely abolished (35 ± 15 % inhibition with 0.1 mM MCh). It was assumed that the pre-treatment of CHO cells with 100 ng ml^{-1} for 24 h was sufficient to allow pertussis toxin to completely ADP-ribosylate sensitive G-protein α subunits, since this protocol has been shown previously to reduce carbachol-stimulated levels of [^{35}S]GTPγS binding by 96 %, compared to untreated cells, and to abolish high-affinity agonist binding and shifts in agonist binding caused by GTP addition, in CHO-M2 cells (Burford *et al.*, 1995). Pertussis toxin pre-treatment of CHO-M3 cells causes a small leftward shift of the stimulation of adenylyl cyclase activity induced by methacholine stimulation (Figure 5.1b) but this is not statistically significant ($P > 0.05$) suggesting that this response is caused by M_3-mACh receptor coupling to PTx-insensitive G-proteins.

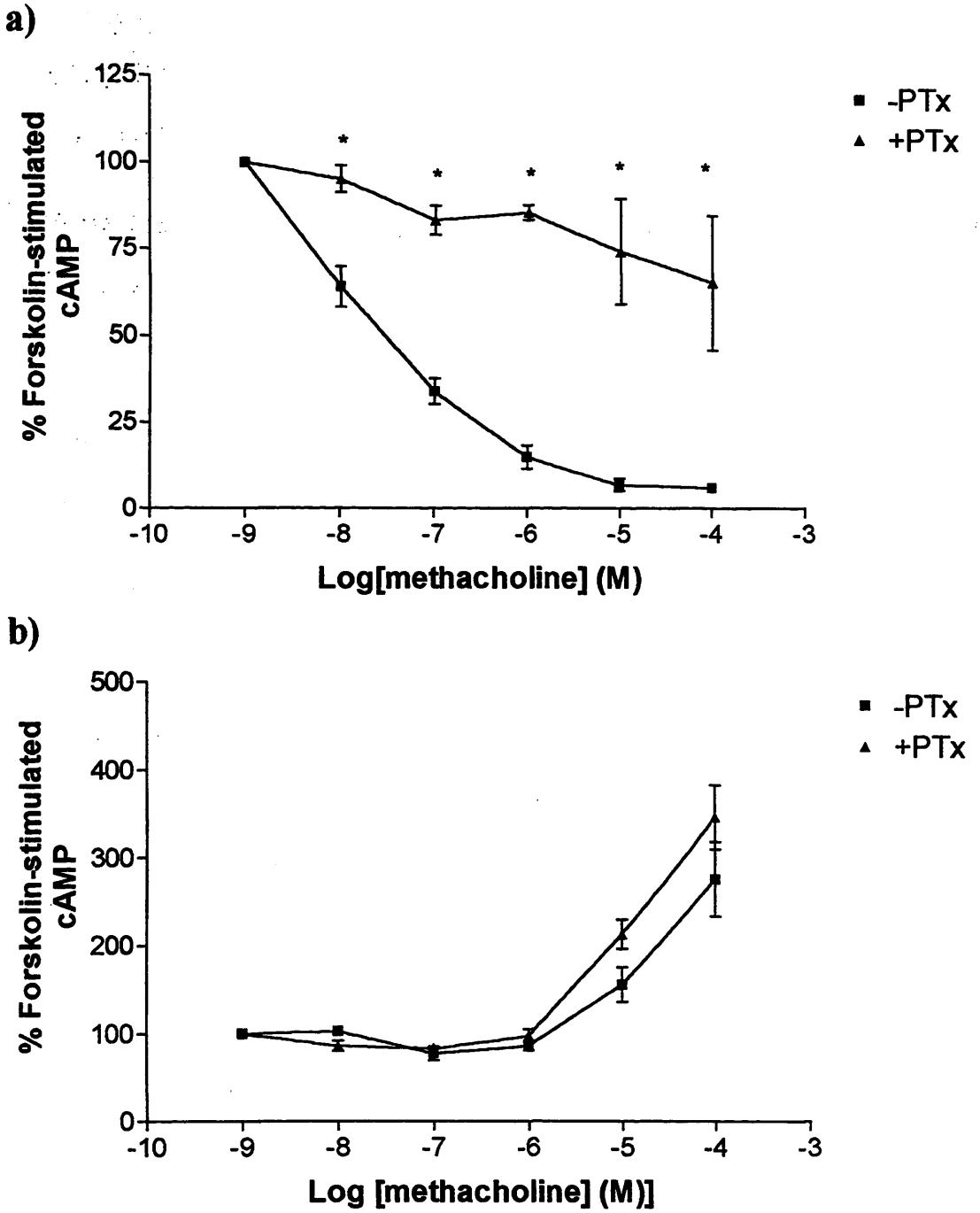


Figure 5.1 - The effect of pertussis toxin pre-treatment on the response to increasing concentrations of methacholine on forskolin-stimulated cAMP accumulation in a) CHO-SLM2 cells and b) CHO-M3 cells.

Cells were incubated for 24 h with 100 ng ml^{-1} pertussis toxin or vehicle in growth medium at 37°C in 95% O_2/5% CO_2. Cells were then washed and incubated with KHB for 10 min, before the addition of MCh at various concentrations. After 15 min, cells were stimulated with the addition of forskolin (10 μM) for 5 min, and then the reaction was stopped by removal of buffer and addition of 0.5 M TCA. Cyclic AMP levels were then analysed as described in Methods. Basal levels of forskolin-stimulated cAMP were 1619 ± 322 pmol mg^{-1} protein. Data represents mean ± s.e.m of 3 separate experiments performed in duplicate. *P<0.01, Student's unpaired *t* test.

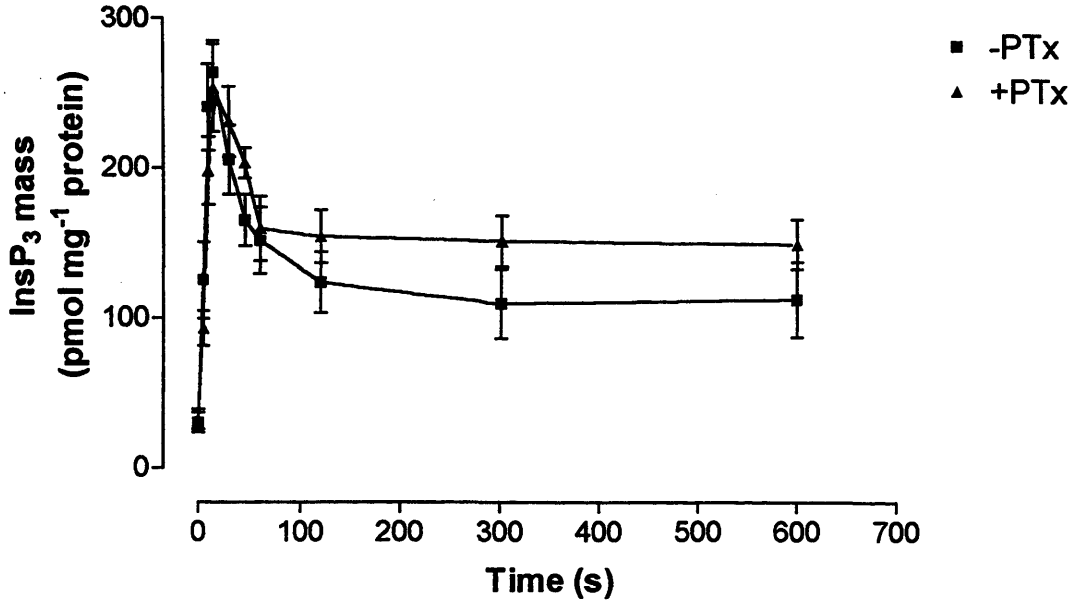
There was no statistically significant difference in the time-course of $InsP_3$ production in response to maximal MCh stimulation between pertussis toxin pre-treated and untreated CHO-M3 cells (Figure 5.2a).The effect of pertussis toxin treatment on the concentration-response relationship of peak $InsP_3$ production and methacholine stimulation in CHO-M3 cells is shown in Figure 5.2b. Pertussis toxin had no statistically significant effect on the EC_{50} or maximum stimulated value of this response ($P > 0.05$), although the inherent variability of this measurement has been mentioned previously in Chapter 4.

5.2.2 Effect of tripitramine on cAMP and $InsP_3$ measurements in control cells

Tripitramine has been shown to be highly selective for M_2-mACh receptors in previous Chapters of this Thesis, giving a clear two-site displacement of [^{3}H]-NMS binding in co-expressing cells. Using previous binding data, a concentration of tripitramine (0.03 μM) that would theoretically occupy > 95% M_2-mACh receptors and <10% M_3-mACh receptors was chosen and used in studies of signalling in cells expressing a homogeneous population of mACh-receptors to demonstrate selective blockade of M_2-mACh receptors at this concentration.

As previously described, activation of M_2-mACh receptors results in inhibition of forskolin-stimulated cAMP accumulation. This response in CHO-SLM2 cells is completely abolished by prior equilibration with tripitramine at 0.03 μM (Figure 5.3a). This inhibition appears not to be overcome at higher concentrations of methacholine, suggesting that tripitramine may not be acting competitively at the mACh receptor. Tripitramine (0.03 μM) had no effect on M_3-mACh receptor-mediated stimulation of cAMP accumulation in CHO-M3 cells (Figure 5.3b).

a)



b)

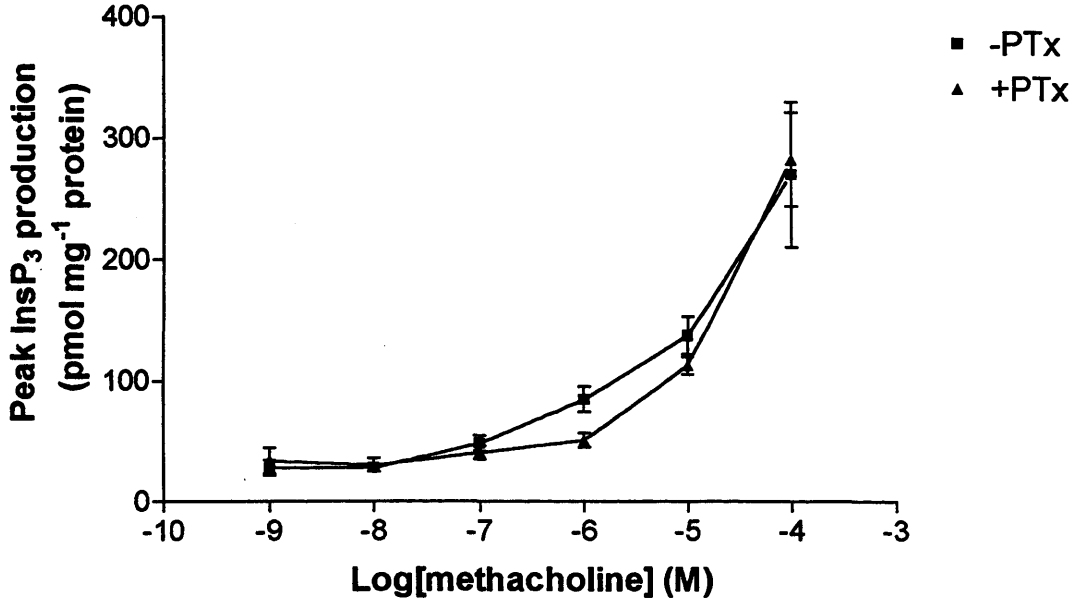
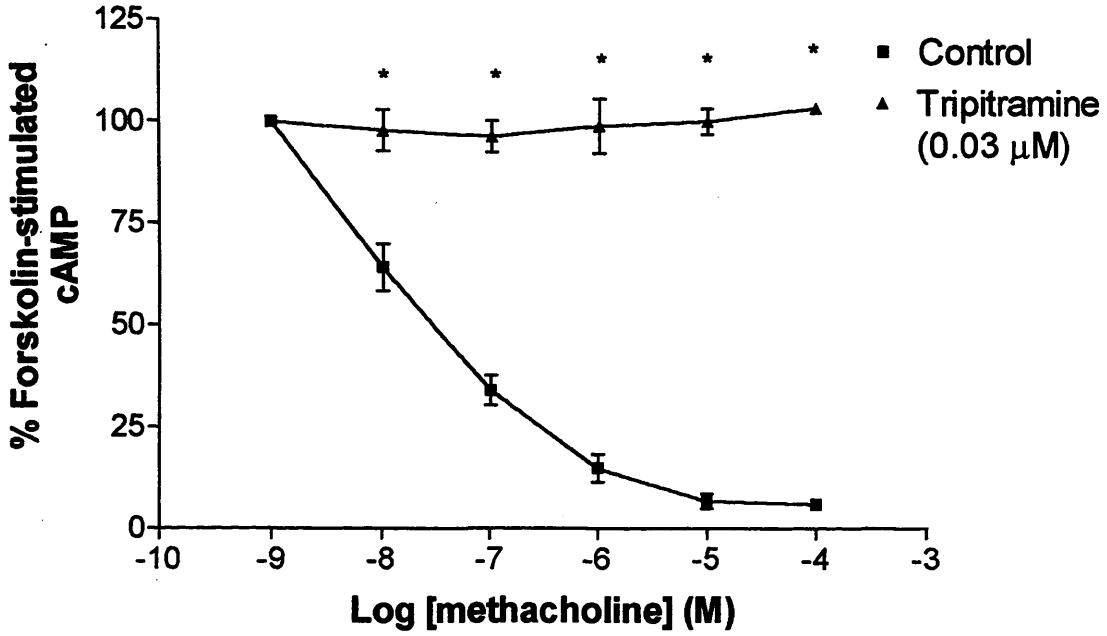


Figure 5.2 - Effect of pertussis toxin pre-treatment on a) $InsP_3$ production with time in response to stimulation with methacholine (1 mM) and b) on peak $InsP_3$ production with increasing concentrations of methacholine, both in CHO-M3 cells.

Cells were incubated for 24 h with 100 ng ml^{-1} pertussis toxin or vehicle in growth medium at 37°C in 95% O_2/5% CO_2. Cells were then washed and incubated with KHB for 10 min, before the addition of MCh at various concentrations or 1mM MCh for varying times. After 10 s for concentration-response and varying time points for time-course, the reaction was stopped by addition of an equal volume of 1 M TCA. $InsP_3$ mass levels were then analysed as described in Methods. Data represents mean ± s.e.m of 3 separate experiments performed in duplicate. There was no statistically significant difference between the responses in treated and untreated cells as established by Student's unpaired *t*-test.

a)



b)

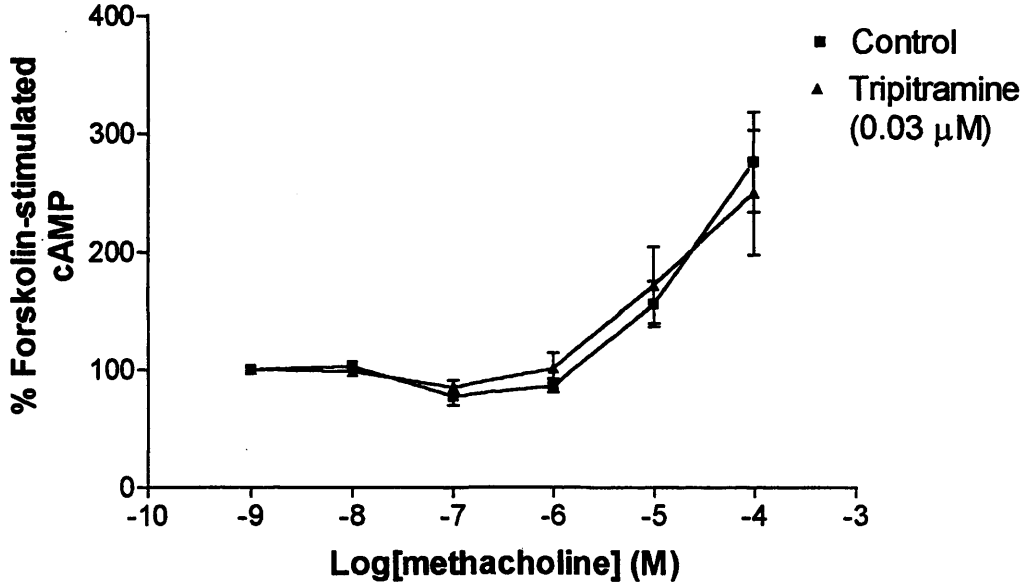


Figure 5.3 - The effect of tripitramine (0.03 μM) on the response to increasing concentrations of methacholine on forskolin-stimulated cAMP accumulation in a) CHO-SLM2 cells and b) CHO-M3 cells.

Cells were washed and incubated with KHB for 10 min, before the addition of tripitramine (0.03 μM) which was incubated for a further 30 min. MCh at various concentrations were then added and after 15 min, cells were stimulated with the addition of forskolin (10 μM) for 5 min, and then the reaction was stopped by removal of buffer and addition of 0.5 M TCA. Cyclic AMP levels were then analysed as described in Methods. Basal levels of forskolin-stimulated cAMP were 1619 ± 322 pmol mg^{-1} protein. Data represents mean ± s.e.m of 3 separate experiments performed in duplicate. *P<0.01, Student's unpaired *t* test.

Tripitramine (0.03 μM) had no statistically significant effect on the time-course of $InsP_3$ production in response to maximal MCh stimulation in CHO-M3 cells (Figure 5.4a). The effect of tripitramine (0.03 μM) treatment on the concentration-response relationship of peak $InsP_3$ production and methacholine stimulation in CHO-M3 cells is shown in Figure 5.4b. Tripitramine (0.03 μM) had no statistically significant effect on the EC_{50} or maximum stimulated value of this response ($P > 0.05$).

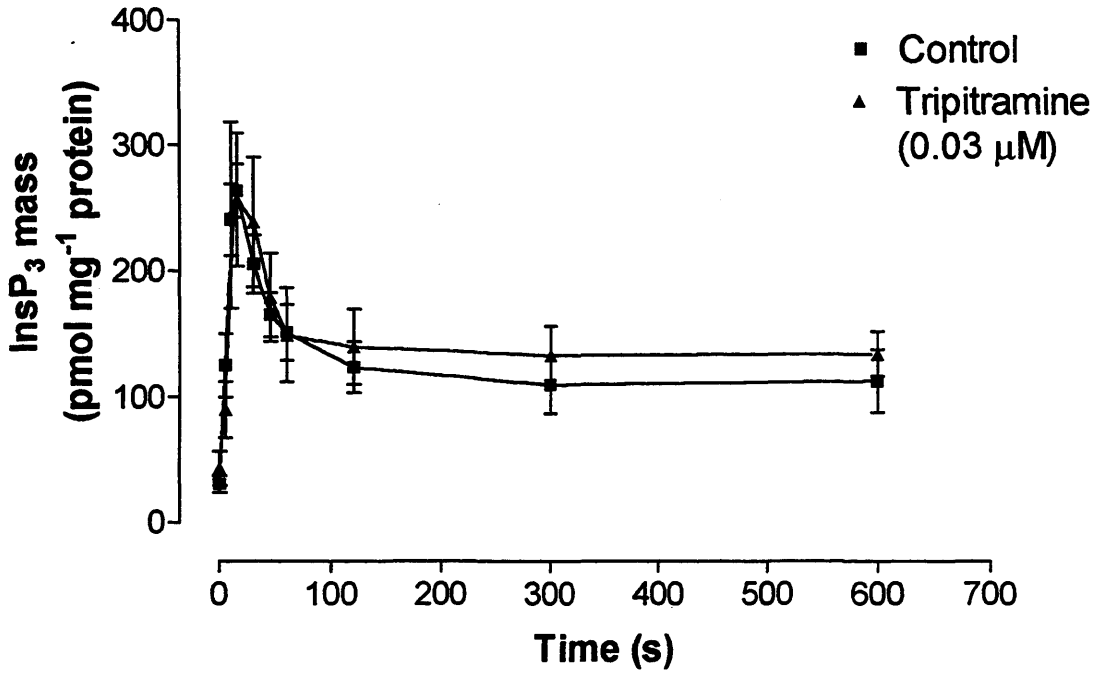
Collectively, these data would suggest that at this concentration (0.03 μM), tripitramine can antagonise methacholine-stimulated signalling via M_2-mACh receptors, but not via M_3-mACh receptors i.e. tripitramine binds to M_2-mACh receptors but not to M_3-mACh receptors at this concentration.

5.2.3 Effect of pertussis toxin pre-treatment on cAMP and $InsP_3$ measurements in M_2/M_3-mACh receptor co-expressing cells (B2)

The effect of pertussis toxin pre-treatment on adenylyl cyclase activity in M_2/M_3-mACh receptor co-expressing CHO cells (B2) is shown in Figure 5.5. The inhibition of forskolin-stimulated cAMP levels observed at lower concentrations of methacholine was reduced by 41 % to 21 ± 4 % compared to untreated cells. At higher concentrations of MCh, stimulation of adenylyl cyclase activity was observed in both treated and untreated cells. Although higher concentrations of cAMP were measured in pertussis toxin pre-treated cells at higher concentrations of MCh due to the reduction of the inhibitory phase of the response, the stimulatory phase in pre-treated cells occurred over similar concentrations of MCh as in untreated cells.

Pertussis toxin pre-treatment had no effect on the peak $InsP_3$ response over a full range of MCh concentrations (Figure 5.6). However, pertussis toxin pre-treatment does abolish the increase in the second phase of $InsP_3$ accumulation in response to a maximal concentration of MCh (Figure 5.7). The response now resembles that seen for a homogeneous population

a)



b)

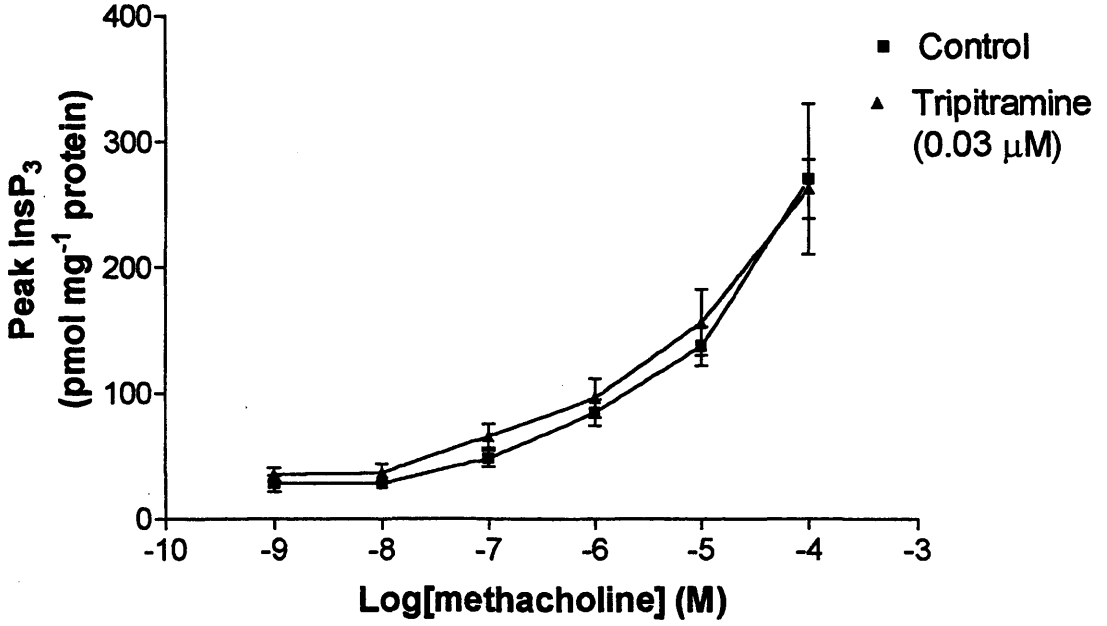


Figure 5.4 - Effect of tripitramine (0.03 μM) on a) $InsP_3$ production with time in response to stimulation with methacholine (1 mM) and b) on peak $InsP_3$ production with increasing concentrations of methacholine, both in CHO-M3 cells.

Cells were washed and incubated with KHB for 10 min, and tripitramine (0.03 μM) was incubated for 30 min before the addition of MCh at various concentrations or 1mM MCh for varying times. After 10 s for concentration-response and varying time points for time-course, the reaction was stopped by addition of an equal volume of 1 M TCA. $InsP_3$ mass levels were then analysed as described in Methods. Data represents mean ± s.e.m of 3 separate experiments performed in duplicate.

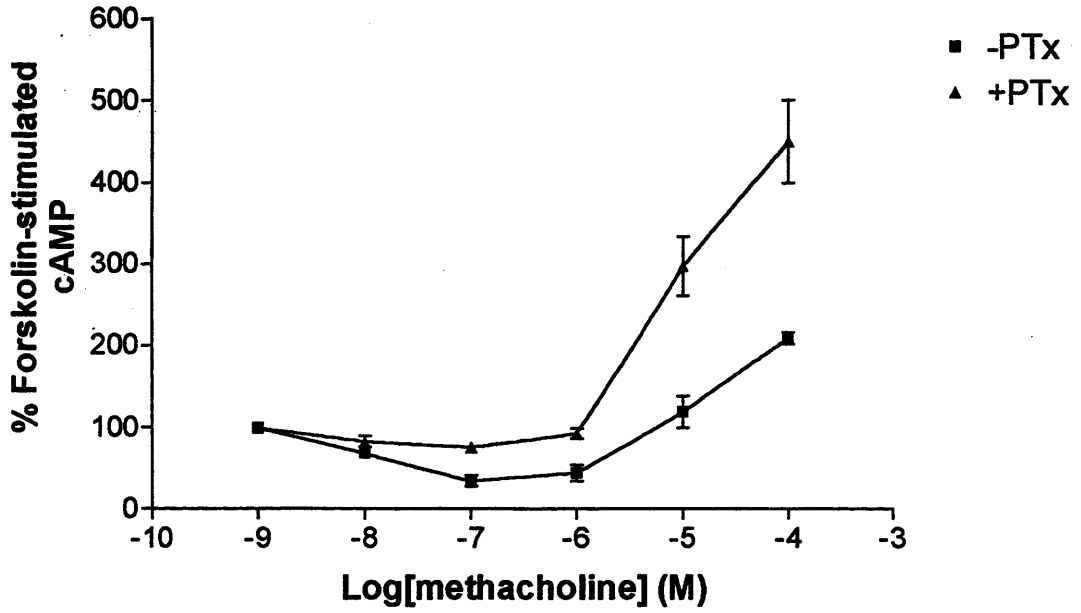


Figure 5.5 - The effect of pertussis toxin pre-treatment on inhibition of forskolin-stimulated cAMP accumulation with increasing concentrations of methacholine in CHO-M2/M3 (B2) cells.

Cells were incubated for 24 h with 100 ng ml^{-1} pertussis toxin or vehicle in growth medium at 37°C in 95% O_2/5% CO_2. Cells were then washed and incubated with KHB for 10 min, before the addition of MCh at various concentrations. After 15 min, cells were stimulated with the addition of forskolin (10 μM) for 5 min, and then the reaction was stopped by removal of buffer and addition of 0.5 M TCA. Cyclic AMP levels were then analysed as described in Methods. Data represents mean ± s.e.m of 3 separate experiments performed in duplicate.

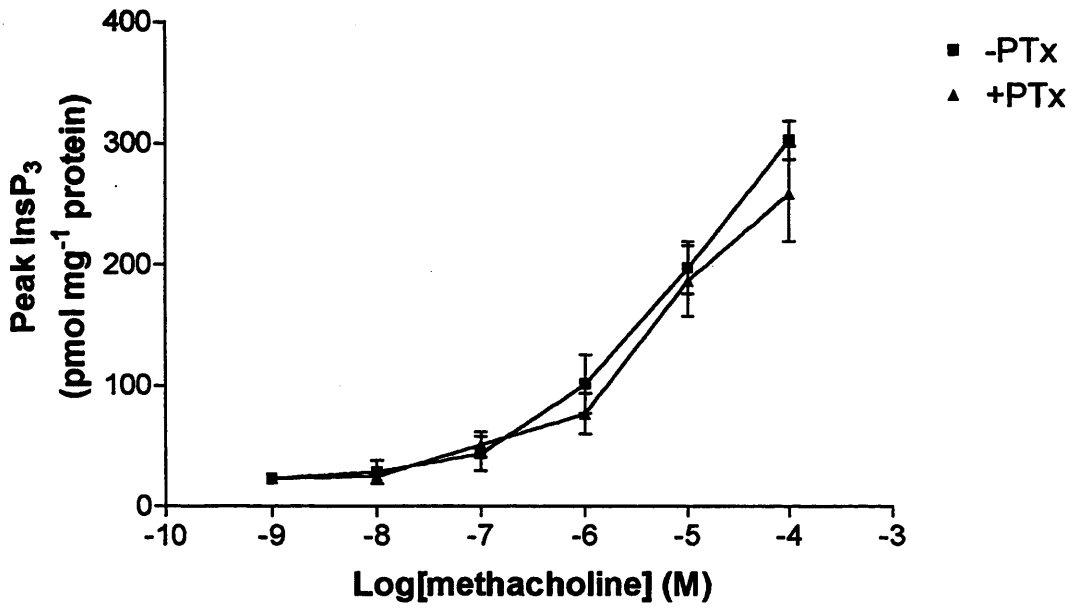


Figure 5.6 - Effect of pertussis toxin pre-treatment on peak $InsP_3$ production with increasing concentrations of methacholine in CHO-M2/M3 (B2) cells.

Cells were incubated for 24 h with 100 ng ml^{-1} pertussis toxin or vehicle in growth medium at 37°C in 95% O_2/5% CO_2. Cells were then washed and incubated with KHB for 10 min, before the addition of MCh at various concentrations. After 10 s, the reaction was stopped by addition of an equal volume of 1 M TCA. $InsP_3$ mass levels were then analysed as described in Methods. Data represents mean ± s.e.m of 3 separate experiments performed in duplicate.

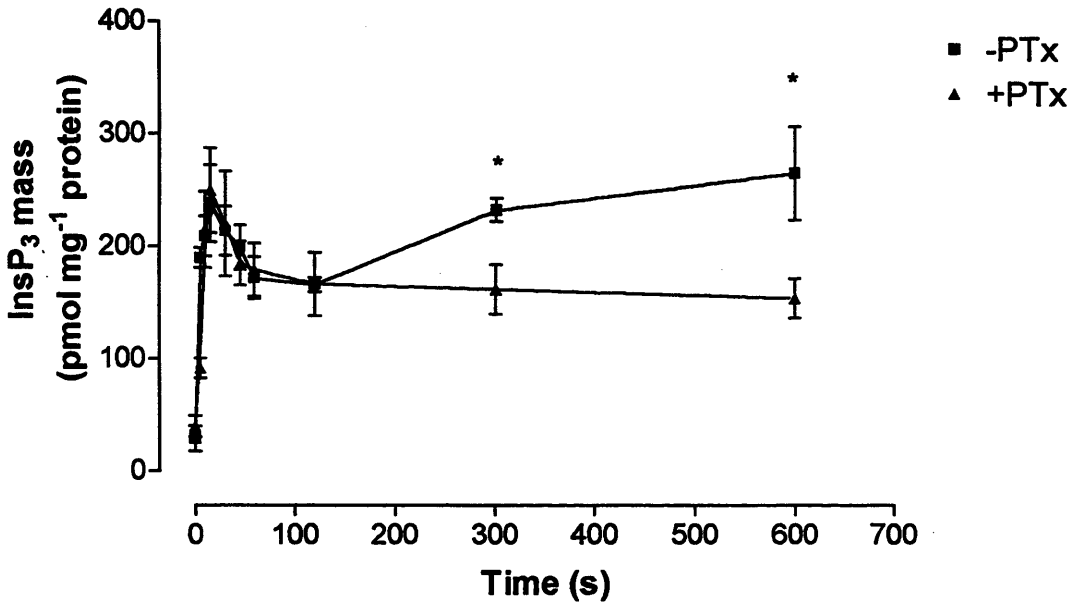


Figure 5.7 - Effect of pertussis toxin pre-treatment on $InsP_3$ production with time in response to stimulation with methacholine (1 mM) in CHO-M2/M3 (B2) cells.

Cells were incubated for 24 h with 100 ng ml^{-1} pertussis toxin or vehicle in growth medium at 37°C in 95% O_2/5% CO_2. Cells were then washed and incubated with KHB for 10 min, before the addition of MCh at 1 mM. After varying times, the reaction was stopped by addition of an equal volume of 1 M TCA. $InsP_3$ mass levels were then analysed as described in Methods. Data represents mean ± s.e.m of 3 separate experiments performed in duplicate. *P < 0.01, Student's unpaired *t*-test.

of M_3-mACh receptors (c.f. Figure 4.7). After the initial peak response, a steady-state plateau level of $InsP_3$ production was observed over 10 min in pre-treated cells.

5.2.4 The effect of tripitramine on $InsP_3$ measurements in M_2/M_3-mACh receptor co-expressing cells (B2)

The effect of 0.03 μM tripitramine on the time-course of $InsP_3$ production in the M_2/M_3-mACh receptor co-expressing cell-line (B2) is shown in Figure 5.8. As with pertussis toxin, the increase in $InsP_3$ production over plateau levels seen in cells expressing a homogeneous population of M_3-mACh receptors was abolished by the addition of tripitramine (0.03 μM), and the response resembled that seen in CHO-M3 cells (c.f. Figure 4.7). After the initial peak response, a steady-state plateau level of $InsP_3$ production was observed over 10 min, and this plateau level of 170 ± 27 pmol mg^{-1} protein (after 10 min stimulation) was not statistically significantly different to that measured in pertussis toxin pre-treated M_2/M_3-mACh receptor expressing cells (B2; 154 ± 17 pmol mg^{-1} protein), or in CHO-M3 cells (123 ± 25 pmol mg^{-1} protein) ($P > 0.05$).

5.2.5 Single cell Ca^{2+} imaging in CHO-M3, -SLM2 and M_2/M_3-mACh receptor co-expressing (B2) cells

The effect of maximal MCh (1 mM) on $[Ca^{2+}]_i$ against time was studied in CHO-M3 (Figure 5.9), -SLM2 (Figure 5.10) and-M_2/M_3 (B2; Figure 5.11) cells. In Figure 5.9a it can be seen that almost all cells in the field of view responded to MCh (1 mM) and an increase in $[Ca^{2+}]_i$ was measured. This was true for > 80 cells (Mean peak response = 341 ± 11 nM) from three different experiments, as might be expected in cells expressing M_3-mACh receptors, which couple to phosphoinositide hydrolysis. Only 5 cells observed did not respond, and were probably not viable. The same effect was seen in cells expressing both M_2- and M_3-mACh receptors (Figure 5.11a). On stimulation of the cells with 1 mM MCh, an increase in $[Ca^{2+}]_i$ in all cells in the field of view was seen, and this again was true for > 80 cells (mean peak

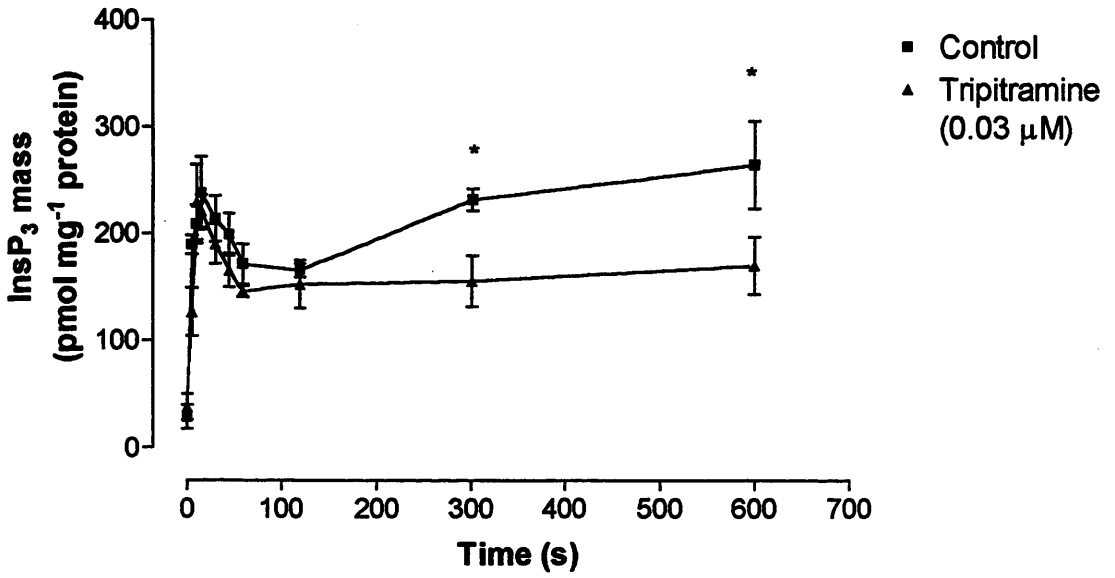
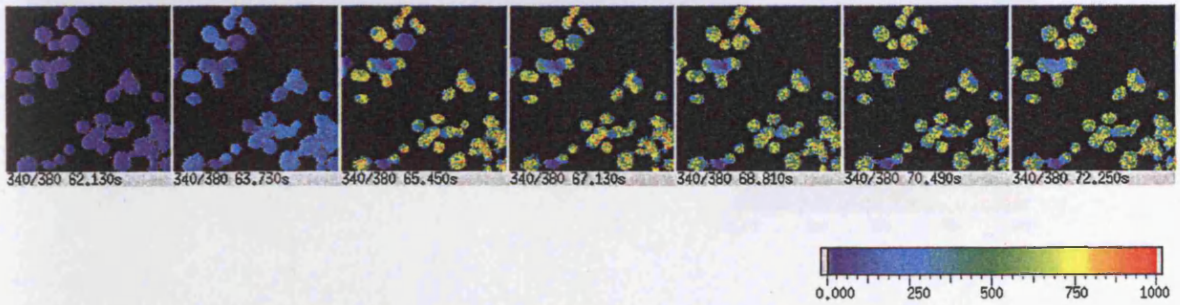


Figure 5.8 - Effect of tripitramine (0.03 μM) on $InsP_3$ production with time in response to stimulation with methacholine (1 mM) in CHO-M2/M3 (B2) cells.

Cells were washed and incubated with KHB for 10 min, and tripitramine (0.03 μM) was incubated for 30 min before the addition of MCh at 1 mM. After varying times, the reaction was stopped by addition of an equal volume of 1 M TCA. $InsP_3$ mass levels were then analysed as described in Methods. Data represents mean ± s.e.m of 3 separate experiments performed in duplicate. *P < 0.01, Student's unpaired *t*-test.

a)



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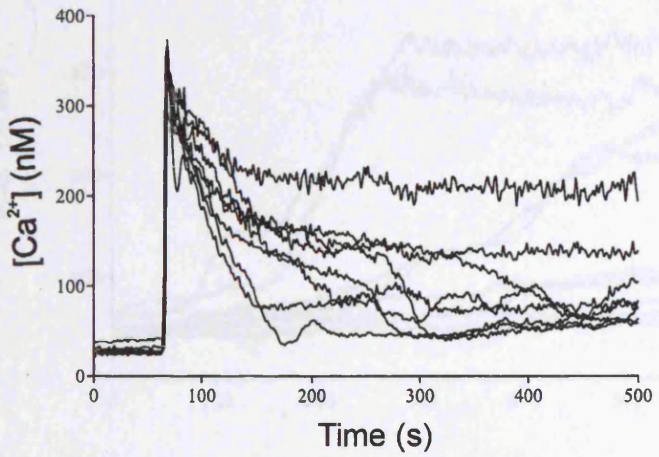
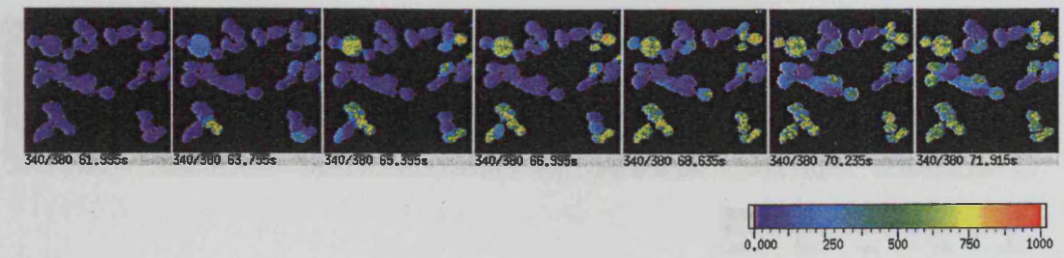


Figure 5.9 - Effect of methacholine (1mM) stimulation on $[Ca^{2+}]_i$ measured using single cell imaging at room temperature in CHO-M3 cells

Cells were loaded with Fura-2AM (in 1 mg ml^{-1}BSA) for 30 min,washed with KHB and left for 30 min in KHB at room temperature. KHB was continuously perfused over the cells, and agonist was added via the perfusion buffer. After subtraction of background fluorescence, images at wavelengths above 510 nm, were collected using an ICCD camera, and ratiometric values converted to $[Ca^{2+}]_i$as described in Methods.
a) Image from field of view from one typical experiment - selected frames
b) Representative cells from one experiment illustrating changes in $[Ca^{2+}]_i$ with time

a)



b)

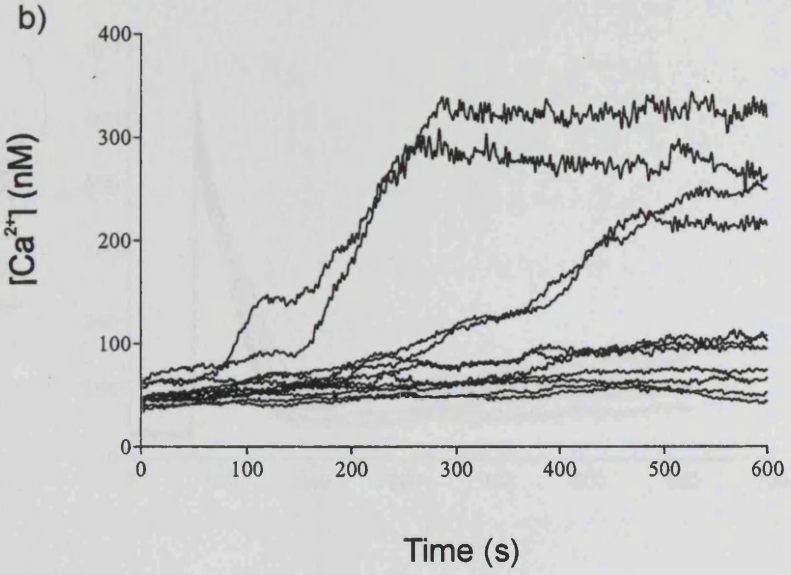


Figure 5.10 - Effect of methacholine (1mM) stimulation on $[Ca^{2+}]_i$ measured using single cell imaging at room temperature in CHO-SLM2 cells

Cells were loaded with Fura-2AM (in 1 mg ml^{-1}BSA) for 30 min,washed with KHB and left for 30 min in KHB at room temperature. KHB was continuously perfused over the cells, and agonist was added via the perfusion buffer. After subtraction of background fluorescence, images at wavelengths above 510 nm, were collected using an ICCD camera, and ratiometric values converted to $[Ca^{2+}]_i$as described in Methods.
a) Image from field of view from one typical experiment - selected frames
b) Representative cells from one experiment illustrating changes in $[Ca^{2+}]_i$ with time

response = 498 ± 10 nM) from 3 separate experiments. It was expected that no such increase would be observed in CHO-SLM2 cells as no $InsP_3$ response or total inositol phosphate accumulation had been measured in these cells (see Chapter 3). However, approximately 40% of the cells studied did show an increase in $[Ca^{2+}]_i$ (Figure 5.10a) in response to 1 mM MCh, although the response was smaller in magnitude than that for CHO-M3 or CHO-M_2/M_3 cells, and the increase was more gradual.

No increase above basal $[Ca^{2+}]_i$ was seen in CHO-SLM2 cells if 1μM MCh was used to stimulate them (in 66 out of 66 cells measured in three separate experiments), although a response was measured in CHO-M3 (Mean peak response = 291 ± 12 nM) and CHO-M_2/M_3 cells (Mean peak response =214 ± 33 nM). This would suggest that in CHO-SLM2 cells, a Ca^{2+} response is only observed at very high concentrations of agonist. The response seen in some CHO-SLM2 cells when stimulating with maximal MCh (1 mM) was also sensitive to pertussis toxin pre-treatment (no response in 33 out of 33 pre-treated cells measured from 2 different experiments), whilst responses in the other two cell types were not.

There are interesting differences in the time-courses of the Ca^{2+} response between cell-lines. In CHO-M3 cells the peak response declined to a variable plateau or to basal levels, with this decrease occuring at variable time-points (Figure 5.9b). However, this contrasts with CHO-M_2/M_3 cells, where the response declines more constistently to basal levels in > 80 % of cells measured, within 1-2 min after agonist addition (Figure 5.11b). For this effect to be truly significant, more cells would need to be studied, due to the variability of responses measured between CHO-M3 cells, however it is an interesting observation which requires further scrutiny.

5.3 Discussion

Receptor cross-talk between signalling pathways is complex, even in a model cell system, and can occur at various different points in a signalling cascade. As has been discussed previously, there are no completely selective mACh receptor subtype ligands, and therefore pertussis toxin and tripitramine, at a carefully chosen concentration, must be used initially to dissect the signalling pathways of the two co-expressed mACh receptor subtypes. The selective blockade produced by tripitramine at 0.03 μM is of paramount importance, as the use of pertussis toxin to block the 'M_2-mACh receptor signalling pathway' requires the assumption that all M_2-mACh receptors and no M_3-mACh receptors in the co-expressing cell-line are coupled to G-proteins sensitive to this toxin. This has been shown to not always be the case, for example, pertussis toxin pretreatment results in a 62% reduction in [^{35}S]GTPγS binding in CHO-M3 cells compared to untreated cells (Burford *et al.*, 1995). So, pertussis toxin pre-treatment can only impart information about the G-proteins involved.

In fact, by pre-treating CHO-SLM2 cells with pertussis toxin it was shown that not all of the mACh receptor-mediated cAMP inhibitory response was mediated via pertussis toxin-sensitive G-proteins, especially at high concentrations of agonist (Figure 5.1a). This is assuming that the pre-treatment protocol used is sufficient to allow ADP-ribosylation of all sensitive G-proteins. This concentration and incubation time has been used by Burford *et al.* (1995) in CHO-M2 cells, and has been shown to abolish all M_2-mACh receptor-mediated responses, but the expression level of M_2-mACh receptors was lower than that in CHO-SLM2 cells used in this study. It is possible that the higher expression levels of M_2-mACh receptors leads to promiscuous coupling. Although it seems unlikely that βγ-subunits from pertussis toxin-insensitive G-proteins contribute to the response, it has been shown that βγ-subunits can inhibit Type 1 adenylyl cyclase (Iyengar, 1993), and it is also possible that activation of pertussis toxin-insensitive G-proteins may cause inhibition of adenylyl cyclase indirectly by some other pathway e.g. by Ca^{2+} mobilisation, as Ca^{2+} can inhibit Type 2 adenylyl cyclase at low concentrations (Iyengar, 1993). However, tripitramine (0.03 μM)

abolished M_2-mACh inhibition of forskolin-stimulated adenylyl cyclase activity in CHO-SLM2 cells (Figure 5.3a), and therefore acts as a more direct method of blocking M_2-mACh receptor signalling without making any assumptions about the G-protein coupling of these receptors.

Neither of the two treatments had any effect on the stimulation of adenylyl cyclase by M_3-mACh receptor activation in CHO-M3 cells (Figures 5.1b and 5.3b). This is in contrast to previous experiments where pertussis toxin pretreatment of CHO-M3 cells increased this response (from a maximum of 71± 14 to 117± 22 pmol mg^{-1} protein), though not as markedly as a similar response seen in CHO cells expressing M_1-mACh receptors (Burford *et al.*, 1995). Both these and the current data would indicate that this response is via activation of M_3-mACh receptors coupled to pertussis toxin-insensitive G-proteins, and the results of Burford *et al.* (1995b) may suggest that removal of G_i G-proteins by ADP ribosylation enhances stimulation of adenylyl cyclase by increasing the likelihood of these other receptor-G-protein interactions. This interaction may simply be promiscuous coupling of M_3-mACh receptors to G_s G-proteins. Alternatively, the stimulation of adenylyl cyclase could be via βγ-subunits from G_q G-proteins, although stimulation of types 2 and 4 adenylyl cyclases by βγ-subunits also requires concomitant activation by $G_s\alpha$ subunits (Iyengar, 1993). M_3-mACh receptor-mediated stimulation of adenylyl cyclase by Gβγ-subunits released from G_q in smooth muscle has been reported recently (Murthy & Makhlouf, 1997). Alternatively Ca^{2+} mobilisation by pertussis toxin-insensitive G-proteins could result in activation of Ca^{2+}-dependent calmodulin, which in turn can activate types 1 and 3 adenylyl cyclase. Protein kinase C, which is activated by diacylglycerol produced by PLC activation by pertussis toxin-insensitive G_q, can also stimulate certain isoforms of adenylyl cyclase (Iyengar, 1993). However, these latter options are unlikely given the reported difference in stimulation of adenylyl cyclase in CHO-M1 and CHO-M3 cells, contrasting to the similar agonist activation of PLC and Ca^{2+} mobilisation in both cell-lines (Burford *et al.*, 1995). Also, in further studies, Burford and Nahorski (1996) showed that M_1-mACh receptor-mediated stimulation of cAMP accumulation was not sensitive to pertussis toxin, was not

affected by phorbol ester addition, and, most importantly, was attenuated by addition of a C-terminal-directed anti-$G_s\alpha$ antiserum. It can be concluded, therefore, that it is likely that M_3-mACh receptor–mediated stimulation of adenylyl cyclase is due to promiscuous coupling of the receptors to G_s G-proteins, and not an event related to M_3-mACh receptor-mediated activation of phosphoinositide hydrolysis.

The observation that tripitramine (0.03 μM) had no effect on the stimulation of cAMP production by M_3-mACh receptors in CHO-M3 cells gives confidence that at this concentration it selectively binds to M_2-mACh receptors, does not antagonise M_3-mACh receptor-mediated responses, and can therefore be used in studies of cross-talk in a cell-line expressing both receptor subtypes.

As shown in Chapter 4, the adenylyl cyclase response to MCh stimulation was made up of superimposed inhibitory and stimulatory components in a M_2/M_3-mACh receptor subtype co-expressing cell-line (B2). After pre-treatment with pertussis toxin, the inhibitory phase was attenuated, but not abolished, and the concentration range over which stimulation occurs was unaffected (Figure 5.5). This indicates that the inhibition of forskolin-stimulated cAMP production in this cell-line is mediated mostly by pertussis toxin-sensitive G-proteins. This correlates with that described above for CHO-SLM2 cells. The fraction of the inhibitory response that was attenuated by pertussis toxin pre-treatment was similar to that in CHO-SLM2 cells, which express M_2-mACh receptors at a similar density. There was however, no effect of pertussis toxin pre-treatment, and hence removal of the greater part of the M_2-mACh receptor-mediated response, on adenylyl cyclase stimulation which is presumably mediated by M_3-mACh receptors. This would suggest that M_2- and M_3-mACh receptors have opposing effects on cAMP production in CHO cells with different concentration dependencies when expressed at these expression levels, and do not act in a synergistic manner in signalling via cAMP.

Both pertussis toxin and tripitramine (0.03 μM) abolished the increase in the second phase of $InsP_3$ production with time after stimulation with 1 mM MCh in coexpressing cells (B2) (Figures 5.7 and 5.8), despite having no effect on $InsP_3$ time-courses in CHO-M3 cells (Figures 5.2a and 5.4a), nor on peak $InsP_3$ responses in either cell types (Figures 5.2b, 5.4b and 5.6). It has been shown previously in this Chapter that tripitramine at this concentration can block activation of M_2-mACh receptors but not of M_3-mACh receptors. It can therefore be used to study whether the additional increase in $InsP_3$ with time in the co-expressing clone B2 compared to CHO-M3 cells was mediated by M_2-mACh receptors coupled to $G_{i/o}$ G-proteins. Obviously, pertussis toxin, whilst showing that this effect is mediated by $G_{i/o}$ G-proteins, cannot impart any information about which receptor is activating these G-proteins in the presence of MCh. Therefore, using both treatments it can be concluded that the increase in the second phase of $InsP_3$ production in co-expressing cells is mediated by the activation of M_2-mACh receptors and their subsequent coupling to pertussis toxin-sensitive G-proteins.

As was described in the Introduction of this Chapter, it has been shown that an increase in cAMP inhibits hydrolysis of phosphoinositides to decrease formation of $InsP_3$ and DAG, and hence Ca^{2+} mobilisation in many cell types (Abdel-Latif, 1996; Hall & Hill, 1988; Laglia *et al.*, 1996; Peakman & Hill, 1994; Prestwich & Bolton, 1995). Liu & Simon (1996) have suggested that receptors coupling to $G\alpha_s$ activate adenylyl cyclases, increasing intracellular cAMP levels and activating PKA, which phosphorylates PLC-β2 and prevents its activation. On the other hand, they suggest that receptors coupled to $G\alpha_i$ inhibit adenylyl cyclase, lowering adenylyl cyclase and PKA activity, thereby increasing activation of PLC by βγ-subunits derived from G_i G-proteins, perhaps thus explaining the pertussis toxin sensitivity of potentiation of PLC activity (see Figure 5.12). However, in the experiments presented in Chapter 4, it can be seen that at 1 mM, MCh causes an increase in the cAMP level above that stimulated by forskolin in the co-expressing cells (B2) due to due to M_3-mACh receptor-mediated stimulation of adenylyl cyclase, and also an increase in the second phase of IP_3 production with time. Also, it is unlikely that PLC-β2 isoforms are expressed in CHO cells

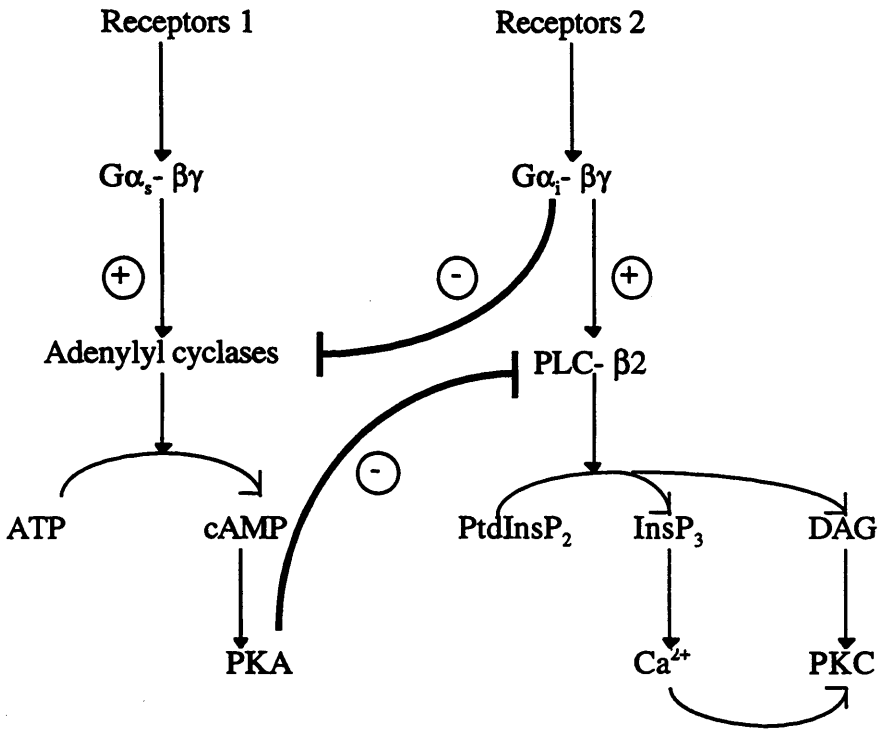


Figure 5.12 - Proposed model for cross-talk between G-protein-coupled cAMP and inositol phosphate signalling pathways via PKA (After (Liu & Simon, 1996)).

as they appear not to be ubiquitously expressed but are generally only found in haematopoetic cells.

This theory is also in marked contrast to evidence reported by Watkins *et al.* (1994) that in stem cells (F_9 carcinoma) or rat osteosarcoma 1712.8 cells in which $G_i\alpha2$ expression is abolished using an antisense strategy there is markedly elevated basal $InsP_3$ accumulation and a potentiated PLC response to stimulatory hormones. Expression of a constitutively active form of $G_i\alpha2$ was found to block stimulated PLC activity, suggesting that $G_i\alpha2$ is an inhibitory regulator of PLC. This interpretation has also been used to explain why pertussis toxin pretreatment increases phosphoinositide hydrolysis stimulated by activation of metabotropic glutamate receptors (mGluR1α) in baby hamster kidney cells (Carruthers *et al.*, 1997). However, this explanation cannot account for data presented here, where activation

of G_i G-proteins by M_2-mACh receptors increased phosphoinositide hydrolysis stimulated by M_3-mACh receptors in the same cell.

It seems very likely that M_3-mACh receptor-mediated stimulation of PLC enzymes via G_q G-proteins can be potentiated by concommitant stimulation of PLC enzymes by βγ-subunits. These may be derived from pertussis toxin-sensitive G-proteins, which are activated by M_2-mACh receptor stimulation. It has been shown that βγ-subunits can activate certain isoforms of PLC-β (Camps *et al.*, 1992; Carozzi *et al.*, 1993; Katz *et al.*, 1992), and it is possible that signalling via βγ-subunits may be a universal cross-talk mechanism mediated by a PTx-sensitive G-protein between adenylyl cyclase-inhibitory and PLC-stimulatory mechanisms, resulting in the enhancement of PLC activation (Gerwins and Fredholm, 1992 ; Gerwins and Fredholm, 1992b; Connor and Henderson ,1996; Tomura *et al.*, 1997). Although it may be a simple synergism between different G-protein subunits at the level of activation of PLC that results in elevation in the amount of measurable phosphoinositide hydrolysis or Ca^{2+} response, Tomura *et al.*, (1997) suggest that the interaction occurs at the level of the G-proteins themselves, as overexpression of βγ-subunits did not increase the adenosine enhancement of PLC stimulation by thyroid stimulating hormone receptors in a model cell-line. They submit that βγ-subunits released by the activation of G_i G-proteins could enhance or inhibit the activity of PLC depending on their stoichiometry with the α-subunits of the G_q G-proteins. If there is an excess of $G_q\alpha$.GDP over the βγ-subunits, then formation of a heterotrimer would be favoured and the G-protein could interact with the activated receptor again, thus increasing the flux through the pathway and hence increasing PLC activation. If the βγ-subunits were in excess, then they could also increase formation of the heterotrimer by binding to the active form of the G-protein $G_q\alpha$.GTP, but would have an inhibitory effect. Similar action of βγ-subunits has been demonstrated by Boyer *et al.* (1989) in a cell-free reconstitution system, and pertussis toxin-sensitive synergy of PLC activation between various receptors has been shown in CHO and COS-7 cells (Dickenson, 1996; Dickenson & Hill, 1996; Megson *et al.*, 1995; Okajima *et al.*, 1995). In COS-7 cells, Koch *et al.* (1994) have demonstrated that expression of the carboxyl βγ-binding domain of β-adrenergic

receptor kinase can attenuate the activation of PLC by M_2-mACh receptors and α_2-adrenoceptors linked to G_i G-proteins, again indicating the probable significance of $\beta\gamma$-subunit signalling. The interpretation of the results of this Chapter that $\beta\gamma$-subunits, resulting from M_2-mACh receptor-stimulated disassociation of G_i G-protein heterotrimers, may potentiate the activation of PLC by G_q G-proteins coupled to M_3-mACh receptors, seems valid, but does not explain why the potentiation occurs only in the sustained phase of $InsP_3$ production, and why there is no observed effect on peak $InsP_3$ production.

Another potential site for regulation of the phosphoinositide signalling pathway is at the level of $InsP_3$ inactivation. The actions of $InsP_3$ at its receptor are terminated by the breakdown of $InsP_3$ catalysed by a series of phosphatases and kinases which convert it into a number of inositol phosphates (see Introduction). In most cells, $InsP_3$ is dephosphorylated by $InsP_3$ 5-phosphatase to $InsP_2$, which is then sequentially dephosphorylated by specific phosphatases to allow recycling of inositol into the phosphoinositides. $InsP_3$ can also be phosphorylated by $InsP_3$ 3-kinase to $InsP_4$, itself purported to be an important signalling molecule involved in refilling of intracellular Ca^{2+} stores (Nahorski *et al.*, 1994). So, increases in $InsP_3$ levels could also be via inhibition of either dephosphorylation by $InsP_3$ 5-phosphatase or phosphorylation by $InsP_3$ 3-kinase. Protein phosphorylation by PKA or by PKC could change the activities of these enzymes, however this does not appear to be the case for phosphatases either *in vitro* or in intact smooth muscle (Abdel-Latif, 1996). However, PKA does seem to be able to phosphorylate and thereby stimulate $InsP_3$ 3-kinase (Abdel-Latif, 1996). However, although M_2-mACh receptors could theoretically increase phosphoinositide hydrolysis activated by M_3-mACh by decreasing levels of cAMP, decreasing activation of PKA, and therefore preventing stimulation of $InsP_3$ 3-kinase, this cannot be the case here as the levels of cAMP were increased during stimulation with 1 mM MCh and activation of PKA would therefore be increased, as discussed previously. It is nevertheless possible, although not probable, that $InsP_3$ kinases or phosphatases are important in the observed cross-talk between M_2- and M_3-mACh via a pathway not involving the stimulation or inhibition of PKA activity.

Phospholipase A_2 is a rapidly expanding effector superfamily (Dennis, 1997), which can be modulated by Ca^{2+} (Dennis, 1994), and has been shown to be activated by receptors which couple to pertussis toxin-sensitive mechanisms, including M_2-mACh receptors (Conklin *et al.*, 1988; Dickerson & Weiss, 1995; Felder *et al.*, 1991; Piomelli *et al.*, 1991; Winitz *et al.*, 1994), and also in a pertussis toxin-insensitive manner (Axelrod, 1990; Dickenson & Hill, 1997). Also of note is a unique group of Ca^{2+}-insensitive phospholipases A_2, which has only been observed in CHO cells (Dennis, 1997). Therefore, phospholipase A_2 may be an important pathway which could be modulated by both M_2- and M_3-mACh receptors and / or downstream Ca^{2+} mobilisation. Synergism of the two receptor subtypes at this effector must not be overlooked, as there may be downstream effects of cytoplasmic arachidonic acid release e.g. on the PLC-activated pathway. Future experiments must be designed to observe release of arachidonic acid in this co-expressing cell-line (B2), however care must be taken in the interpretation of any such experiments as activation of phospholipase A_2 by either mACh receptor subtype may not be direct, but may occur as a 'downstream' event e.g. secondary to DAG production. The likelihood that cross-talk between the two co-expressed mACh receptor-subtypes at the level of PLA_2 activation results in the potentiation of the PLC recponse seems remote, but the fact that this potentiation is observed at the sustained phase alone, i.e. at later time-points, may suggest that it occurs downstream of another effector pathway.

Activation of phospholipase D (PLD) by G-protein coupled receptors, including mACh receptors, has been reported in many cell types (Sandmann *et al.*, 1991), and the G-proteins involved have been shown to be both pertussis toxin-sensitive and -insensitive depending on the cell and / or receptor studied (Challiss & Blank, 1997). Also, PLD can be regulated by many other intracellular components such as protein kinase C, tyrosine kinases, small molecular weight G-proteins, particularly Arf and Rho, and PIP_2 (Challiss & Blank, 1997). The exact mechanisms linking cell-surface receptors to PLD activation are unknown, and as such cannot be discounted in the search for modes of cross-talk between M_2- and M_3-mACh

receptor signalling, although no reports have been made that PLD activation can potentiate or inhibit PLC activation in a way which may explain the results of the present study.

The activation of extracellular signal-regulated kinases (ERK) has been implicated in growth and proliferation of smooth muscle cells and has been observed upon stimulation of mACh receptors, and although these processes are implicated in chronic stimulation, pathways leading to mitogenesis may also occur on a time-scale that overlaps and interacts with more rapidly activated signalling pathways (Challiss & Blank, 1997). Receptors which preferentially couple to G_i and G_q G-proteins have been shown to lead to ERK activation, though not necessarily in a direct manner, and this pathway may also interact with ERK pathways activated by tyrosine kinases (Malarkey *et al.*, 1995). Recent studies using Rho inactivating agents, such as toxin B from *Clostridium difficile* and C3 exo-enzyme from *Clostridium botulinum*, have indicated that signalling of M_3-mACh receptors through both PLD and PLC enzymes may be regulated by Rho proteins (Schmidt *et al.*, 1996; Schmidt *et al.*, 1997). These toxins inactivate Rho family proteins in a highly specific manner by covalent modification, and cause an inhibition of PLC activity stimulated by M_3-mACh receptor activation in HEK cells. So, it has been concluded that small G-proteins may increase stimulation of PLC by M_3-mACh receptors, possibly by stimulating PtdIns(4)P 5-kinase activity and hence increasing PIP_2 formation i.e. not by direct stimulation of the PLC enzyme itself (Schmidt *et al.*, 1996; Schmidt *et al.*, 1997). Therefore, if M_2-mACh receptors were also able to regulate small G-proteins in a pertussis toxin-sensitive manner, or alter the way in which M_3-mACh receptors interacted with Rho proteins, then this may be another level at which cross-talk between M_2- and M_3-mACh receptors occurs which might ultimately modulate the activation of PLC by M_3-mACh receptor activation. Activation of a MAP kinase cascade may occur downstream of PKC activation by DAG which occurs due to G_q activation of PLC. MAPK activation in response to receptor stimulation has been shown to be dependent on PKC in smooth muscle in some cases (Challiss & Blank, 1997). Therefore, there may be more than one point at which ERK activation and modulation is important in M_2- and M_3-mACh receptor signalling, and further investigation of the patterns

of ERK activation in CHO-M2, CHO-M3 and CHO-M2/M3 cells needs to be done before the relative importance of these pathways in the observed potentiation of the PLC response can be assessed.

As has been discussed previously, $InsP_3$ production upon M_3-mACh receptor stimulation is biphasic, with the sustained phase being dependent on extracellular Ca^{2+} (Wojcikiewicz *et al.*, 1994). Increases in measured $[Ca^{2+}]_i$ are also biphasic, with the peak phase being due to Ca^{2+} release from intracellular stores and the sustained phase being due to Ca^{2+} entry through plasma membrane channels (Berridge, 1993). It has been shown that by inhibiting the entry of Ca^{2+} during receptor stimulation, increases in $[Ca^{2+}]_i$ during the plateau phase are attenuated, and the rates of $InsP_3$ and total inositol phosphate accumulation (the latter in the presence of Li^+) are reduced (Lambert *et al.*, 1991). Therefore, one obvious point to make is that if Ca^{2+} entry stimulates PLC activation during concurrent agonist stimulation, then if Ca^{2+} entry were to increase, the second phase of $InsP_3$ production might also be seen to increase, as in the co-expressing cells. This effect may be due to a second family of PLC enzymes, which are co-activated by increases in $[Ca^{2+}]_i$ and stimulation via G-protein linked receptors, being active in the later phase of $InsP_3$ production in response to agonist application. This co-activation by increases in $[Ca^{2+}]_i$ may be due to direct sensitivity of the PLC enzymes to Ca^{2+}, or due to phosphorylation of the enzyme causing activation of the PLC isoform, initiated by an increase in $[Ca^{2+}]_i$. Therefore, is it possible for M_2-mACh receptors coupling via pertussis toxin sensitive G-proteins to increase Ca^{2+} entry?

It is known that M_2-mACh receptors couple to non-specific membrane cation channels (Benham *et al.*, 1985) via pertussis toxin-sensitive G-proteins (Inoue & Isenberg, 1990; Komori *et al.*, 1992), which modulate voltage-dependence as well as gating of these channels (Zholos & Bolton, 1994). The activation of these channels mostly causes Na^+ entry in smooth muscle, but this brings about depolarisation of smooth muscle membrane, hence activating voltage-sensitive Ca^{2+} channels and allowing Ca^{2+} entry (Inoue *et al.*, 1987). The release of Ca^{2+} from intracellular stores can potentiate this response (Pacaud & Bolton,

1991), and it has been suggested that co-stimulation of the M_3-mACh receptor may potentiate the 'channel-opening' effect of M_2-mACh receptor stimulation (Zholos & Bolton, 1997). Therefore, it is possible that M_2-mACh receptor stimulation of Ca^{2+} entry through cell membrane receptors may cause the potentiation of PLC activation by M_3-mACh receptors when co-expressed in the same cell, if these particular non-specific cation channels exist in CHO cells. Some electrophysiological studies would be useful to determine whether the mACh receptor-mediated non-specific cation current can be measured in this cell-line, alongside studies into the effect of varying $[Ca^{2+}]_e$ on $InsP_3$ production in these cells, compared to that in CHO-M3 cells. This may be particularly important to assess given the evidence of Singer-Lahat *et al.* (1996), where Ca^{2+} influx could not be measured in CHO cells expressing M_2-mACh receptors. This data suggests that non-specific ion channels and/or voltage sensitive Ca^{2+} channels may not be expressed in the membrane of CHO cells. However, this remains a very interesting avenue of further investigation, and M_2-mACh mediated Ca^{2+}-entry is still a plausible explanation for the reported increases in PLC signalling in CHO-M2/M3 cells, given the increases in $[Ca^{2+}]_i$ seen in CHO-SLM2 cells in this study in single cell imaging experiments.

It is possible that cross-talk occurs at the level of the receptors themselves. Studies indicate that specific receptor phosphorylation is involved in rapid desensitisation of receptors linked to the phosphoinositide response. Human M_3-mACh receptors are phosphorylated in an agonist-dependent manner by a protein serine/threonine kinase distinct from PKA, Ca^{2+}-calmodulin-dependent protein kinase and PKC (Tobin *et al.*, 1996; Tobin & Nahorski, 1993; Wojcikiewicz *et al.*, 1993), which has been shown to be casein kinase Iα (Tobin *et al.*, 1997). This phosphorylation may perhaps cause uncoupling of the receptor from its G-protein to cause rapid, partial desensitisation of phosphoinoside hydrolysis mediated by this receptor. In CHO-M3 cells, receptor internalisation does not occur to any significant extent for at least the first 10 min of agonist stimulation and is therefore not responsible for rapid desensitisation (Tobin *et al.*, 1992). Perhaps the presence of M_2-mACh receptors alters M_3-mACh receptor phosphorylation, and hence alters the pattern of desensitisation of the

phosphoinositide response, thereby increasing $InsP_3$ production. However, the profile of the $InsP_3$ production with time in response to stimulation with 1 mM MCh was not as expected, if this was the case. As has been stated, it is the peak phase of $InsP_3$ production which represents the desensitising phase, whilst the plateau phase is resistant to desensitisation by prior receptor stimulation. Even though it is unlikely that changes in receptor phosphorylation are responsible for the increase observed in the plateau phase of $InsP_3$ production in response to MCh stimulation, there may be important consequences of mACh receptor subtype co-expression on receptor phosphorylation and desensitisation, which should be investigated.

The most important of these possible methods of cross-talk between M_2- and M_3-mACh receptors when coexpressed in the same cell are illustrated in Figure 5.13.

It was important to this work to illustrate further that this cell-line does in fact represent the co-expression of M_2- and M_3-mACh receptors within the same cell, and that it is not a mixed population of cells where each cell in the population expresses M_2-mACh receptors or M_3-mACh receptors alone. This could be possible if the picking and diluting of colonies was not sufficient to select a clonal cell-line derived from a single cell. For this reason, single cell Ca^{2+} imaging was performed. It was assumed that in cells expressing solely M_2-mACh receptors no increase in $[Ca^{2+}]_i$ would be measured upon agonist stimulation, but that in all CHO-M3 cells an increase would be observed. Taking these two observations, it was then assumed that if *all* M_2/M_3-coexpressors (B2) also exhibited a Ca^{2+} response, this would indicate M_3-mACh receptor expression in *all* cells, and hence suggest that this was a homogenous cell population. Although $[Ca^{2+}]_i$ increases were observed in all CHO-M3 and CHO-M2/M3 (B2) cells as expected when stimulated with maximal MCh, there were also a proportion of SLM2 cells in which a response was measured. However, using 1 μM MCh there was no response observed in SLM2 cells, but an, albeit smaller, response was measured in both CHO-M3 and CHO-M2/M3 cells, and only the response in SLM2 cells was sensitive to PTx pre-treatment. This would suggest that in SLM2 cells the increase in

$[Ca^{2+}]_i$ was due to coupling of M_2-mACh receptors to G_i G-proteins either to cause Ca^{2+} influx via channels or to cause stimulation of PLC via βγ-subunits, although this is unlikely given the lack of agonist-stimulated $InsP_3$ response or increase in $InsP_x$ accumulation above basal levels. Perhaps, in this case, the Ca^{2+} response is mediated via small G-proteins, and not heterotrimeric G-proteins. There must be a pertussis toxin-insensitive signalling pathway in CHO-M2/M3 cells which causes increases in $[Ca^{2+}]_i$, and it is possible to conclude that this is via M_3-mACh receptors, given the evidence for their expression (particularly radioligand binding and immunological detection) presented in Chapter 4. Therefore, it can be concluded that in this cell-line (B2), all cells express both M_2- and M_3-mACh receptors at their surface. M_2-mACh receptors can cause increases in $[Ca^{2+}]_i$ at high concentrations, but this response is not seen at lower concentrations of agonist and is PTx-sensitive. M_3-mACh receptors increase $[Ca^{2+}]_i$ at lower concentrations of agonist, and this response is insensitive to PTx, as expected.

An interesting point to note, is that PTx pre-treatment has no significant effect on the increase in $[Ca^{2+}]_i$ measured upon stimulation with 1mM MCh in co-expressing cells (B2), despite the fact that it has been shown earlier in this Chapter that this pretreatment changes the profile of $InsP_3$ production with time in these cells. This would indicate that perhaps there is an excess of $InsP_3$ produced, in this case, over what is needed to produce a maximal Ca^{2+} response. Perhaps the increase in $InsP_3$ produced by M_2-mACh receptor co-expression is not after all the most important factor in modulation of M_3-mACh receptor signalling by M_2-mACh receptors, but it may be the expected concommitant increase in DAG production caused by increased activation of PLC, or other pathways, as described above, which may be more important. Connor and Henderson (1996) have observed that in SHSY-5Y neuroblastoma cells, co-activation of opioid receptors increases the Ca^{2+} response observed by stimulation of muscarinic receptors in a pertussis toxin-sensitive manner, whilst opioid receptors themselves have no effect on inositide signalling pathways and were unable to activate a Ca^{2+} response when opioid agonists alone were applied. This represented a real cross-talk event, as merely elevating $[Ca^{2+}]_i$ was not sufficient to reveal an opioid-mediated

response. They suggest that the potentiaion could be due to inhibiting the acute desensitisation of the muscarinic receptor, by altering the regulation of PLC, the $InsP_3$ receptor or enzymes involved in resynthesis of PIP_2, and may be mediated by $\beta\gamma$ subunits from pertussis toxin-sensitive G-proteins. These are explanations that could be applied to our study, except that there may be differences in the nature of the cross-talk between M_2- and M_3-mACh receptors, and opioid and muscarinic receptors. In the present study, the potentiation of the $InsP_3$ response is pertussis toxin sensitive, whilst the Ca^{2+} response is not, in contrast to Connor and Henderson's study. Further work could therefore include studies of Ca^{2+} in co-expressing cells with removal of the M_2-mACh receptor population by tripitramine, to see whether the receptor synergy observed at the level of phosphoinositide hydrolysisis is also apparent at the level of Ca^{2+} signalling.

The profile of acute desensitisation may be different in co-expressing cells from that seen in cells expressing a homogeneous population of M_3-mACh receptors. It can be seen from this preliminary study that in cells expressing M_2- as well as M_3-mACh receptors, the Ca^{2+} response decreased over time more quickly than that in CHO-M3 cells. This could be due to a number of factors arising at the $InsP_3$ receptor, Ca^{2+} influx, or Ca^{2+} stores. Further investigations using greater numbers of cells, as well as detailed studies of the desensitisation of the Ca^{2+} response, $InsP_3$ receptor sensitivity (not only to $InsP_3$ but also to Ca^{2+} feedback mechanisms) , and Ca^{2+} store refilling should be carried out.

So, overall it is possible to see that co-expression of M_2-mACh receptors with M_3-mACh receptors does have an effect on phosphoinositide signalling and possibly Ca^{2+} signalling in these cells. The increase in the second phase of M_3-mACh stimulated $InsP_3$ signalling is caused by concomitant M_2-mACh receptor activation and consequential signal 'cross-talk' via, or downstream of, a pertussis toxin-sensitive G-protein. The cause and effect of this observed cross-talk may be many-fold and needs further investigation. The most likely rationale, and therefore most important to study next, is the synergistic stimulation of PLC by $G_q\alpha$ from M_3-mACh receptor stimulation, and by $\beta\gamma$ subunits from pertussis toxin-

sensitive G-proteins activated by M_2-mACh receptors. Also very important and relatively easy to evaluate is the contribution of Ca^{2+} influx to the potentiation of the $InsP_3$ response, perhaps mediated by M_2-mACh receptor stimulation of non-specific cation channels. Studies similar to those reported in this Chapter could be carried out in other co-expressing clones which express different levels of M_2- and M_3-mACh receptors to the clone used in these studies, to see whether this cross-talk occurs at other receptor expression levels. Further suggestions of possible future experiments are detailed in the final Chapter.

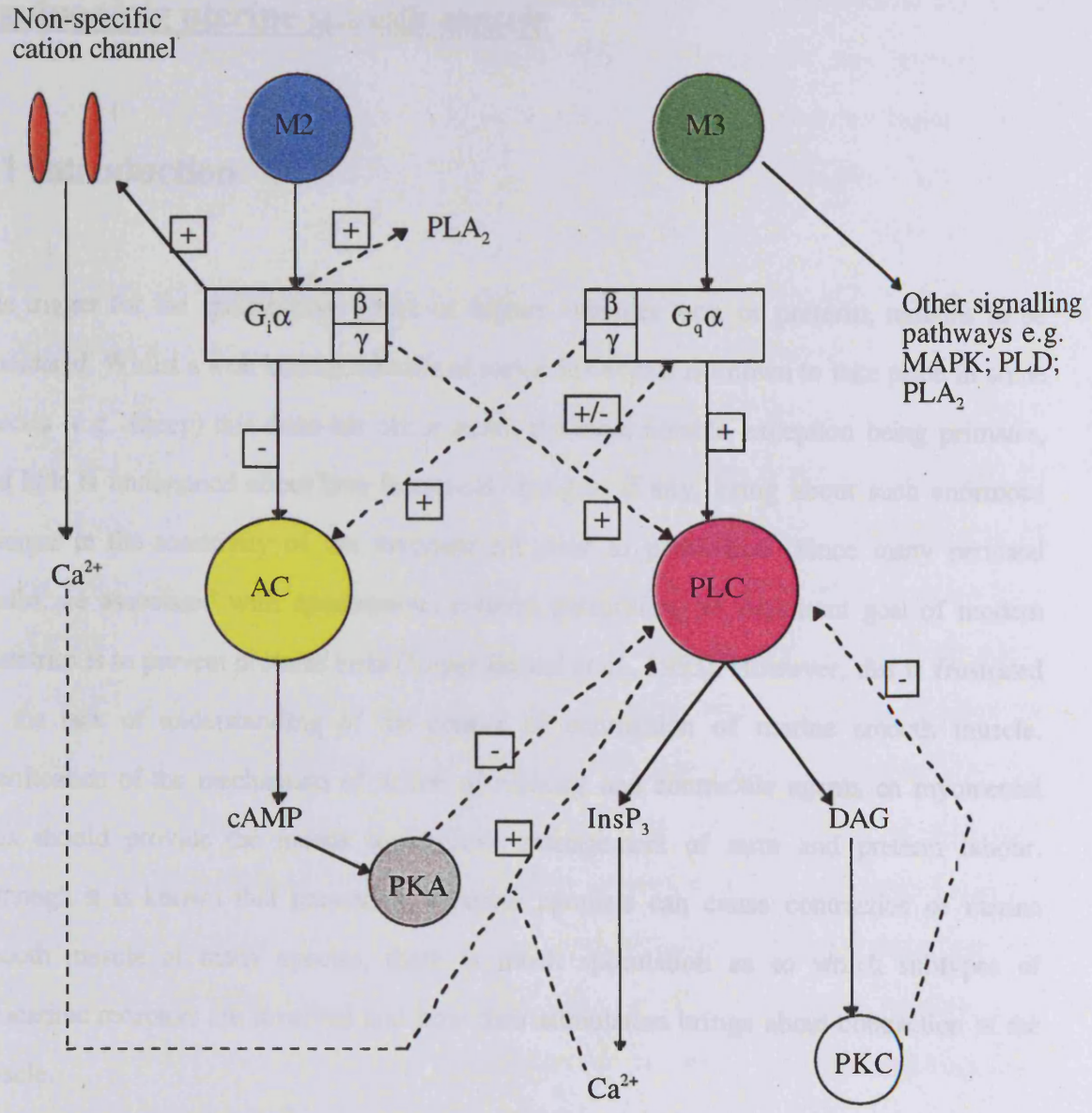


Figure 5.13 - Scheme showing possible modes of cross-talk between adenylyl cyclase and phosphoinositide signalling pathways regulated by M_2- and M_3-mACh receptor subtypes. Ca^{2+} is also a regulator of other effector enzymes such as AC, Ca^{2+} dependent-calmodulin, PLA_2 and others, and these interactions are not shown for clarity.

Abbreviations: PLC- phospholipase C; $InsP_3$- inositol 1,4,5-trisphosphate; DAG- diacylglycerol; AC- adenylyl cyclase; cAMP- adenosine cyclic 3',5'-monophosphate; cGMP- guanosine cyclic 3',5'-monophosphate; PKC - protein kinase C; MAPK - mitogen-activated protein kinase; PLA_2 - phospholipase A_2; PLD - phospholipase D

CHAPTER 6 - Muscarinic ACh receptor-mediated contraction of guinea-pig uterine smooth muscle

6.1 Introduction

The trigger for the spontaneous onset of labour, whether term or preterm, remains to be elucidated. Whilst a well known cascade of endocrine events is known to take place in some species (e.g. sheep) this does not occur in all, the most notable exception being primates, and little is understood about how hormonal changes, if any, bring about such enormous changes in the sensitivity of the myometrium prior to parturition. Since many perinatal deaths are associated with spontaneous preterm parturition, an important goal of modern obstetrics is to prevent preterm birth (Lopez Bernal *et al.*, 1993). However, this is frustrated by the lack of understanding of the control of contraction of uterine smooth muscle. Clarification of the mechanism of action of relaxant and contractile agents on myometrial cells should provide the means to improve management of term and preterm labour. Although it is known that muscarinic receptor agonists can cause contraction of uterine smooth muscle of many species, there is much speculation as to which subtypes of muscarinic receptors are involved and how their stimulation brings about contraction of the muscle.

Uterine muscle contracts rhythmically both *in vivo* and *in vitro*, and these contractions are intrinsic to the tissue, requiring no hormonal or neural inputs to maintain them (Wray, 1993). This spontaneous contractility can be seen in non-pregnant and pregnant uterus, although the uterus is necessarily more quiescent during pregnancy. Spontaneous contractions are preceded by action potentials, and pacemaker and electrical activity differs depending on species, gestational states and muscle bundles (Mironneau, 1990). Nevertheless, smooth muscle contractility can be modulated directly by a variety of neurotransmitters and hormones. The uterine body of many species including human, is

extensively innervated by cholinergic neurones of the autonomic nervous system (Tetsuro *et al.*, 1994; Traurig & Papka, 1993) and this probably allows for co-ordinate control of contraction and blood flow (Sato *et al.*, 1996), although the precise role of cholinergic stimulation in uterine contraction is not known since denervation does not impair normal parturition (e.g. in paraplegic women).

Evidence has been presented to show that the populations of mACh receptors and adrenoceptors may change in response to hormonal changes, particularly in oestrogen levels, during the oestrus cycle (Arkinstall & Jones, 1990; Matucci *et al.*, 1996; Riemer *et al.*, 1987; Varol *et al.*, 1989). In the guinea-pig there is a rhythmical hormonal cycle, despite the additional role of copulation in ovulation (Stockard & Papanicolaou, 1917). A peak of oestrogen production occurs in response to follicle stimulating hormone (FSH) release from the pituitary. A plateau phase of oestrogen production persists (a 'heat' phase), until mating occurs which helps to induce ovulation. Although copulation is not a prerequisite to ovulation there is a greater influence of external factors than in primates, for example. At this stage there is little involvement of progesterone, and this continues until 1-2 days after ovulation. It is the plateau or 'heat' phase which is referred to as the 'oestrogen-dominant' component of the oestrus cycle of the guinea pig, and proliferation of the endometrium and keritinisation of the vaginal epithelium occurs at this time. The fact that the populations of mACh receptors and their sensitivity to stimulation can be altered by circulating hormones such as oestrogen suggest that these mACh receptors may have an important part to play in the normal physiology of uterine contraction.

In most smooth muscle tissues, M_2- and M_3-mACh receptor subtypes form the postjunctional mACh receptor population, and in the majority of these tissues it can be shown that M_3-mACh receptors initiate contraction, presumably via their coupling to the stimulation of phosphoinositide hydrolysis (Mahesh *et al.*, 1992; Nahorski *et al.*, 1994; Roffel *et al.*, 1990; Yang *et al.*, 1991). However, this may not be the case for uterine smooth muscle tissue. In contrast to most smooth muscles, myometrial contraction may be

mediated by a subtype other than the M_3-mACh receptor. Initial studies in guinea-pig isolated uterus suggesting that M_2-mACh receptors are responsible for contraction (Eglen *et al.*, 1989) were supported by subsequent functional studies using an extensive series of mACh receptor antagonists (Bognar *et al.*, 1992; Doods *et al.*, 1993). Alternative suggestions that M_2- and M_3-mACh receptors may be jointly responsible (Leiber *et al.*, 1990), or M_4-mACh receptors may mediate this response (Dorje *et al.*, 1990) have also been made. Although immunological methods have demonstrated the presence of M_2- and M_4-mACh receptors in rabbit uterus (Dorje *et al.*, 1991) and northern blot analysis of guinea-pig uterine tissue revealed evidence for m2 and m4 mRNA transcripts (Eglen *et al.*, 1992) it is unclear whether such receptors can mediate a contractile response in myometrial tissue. Inhibition of adenylyl cyclase and stimulation of phosphoinositide-specific phospholipase C by mACh receptor agonists both can occur in guinea-pig isolated uterus, with the former being attributed to M_2- and the latter to M_3-mACh receptor activation (Leiber *et al.*, 1990; Marc *et al.*, 1988). However, promiscuous coupling of M_2-mACh receptors to both G_q and G_i G-proteins reminiscent of that seen in guinea-pig atria (Kenakin & Boselli, 1991) has not been ruled out.

Due to the low receptor subtype selectivity of many of the agents used to characterise the uterine mACh receptor(s) in previous studies, and a lack of any clear conclusions, an extensive characterisation of the mACh receptor(s) subtype responsible for contraction of the guinea-pig isolated uterine smooth muscle has been carried out. Guinea-pigs pre-treated with a synthetic oestrogen have been used in order to synchronise all animals to the oestrogen-dominant phase of their reproductive cycle. The possibility was addressed that the contraction of uterus is mediated by M_2- and M_3-mACh receptors either alone or in a synergistic fashion by using novel M_2/M_3 selective antagonists in conjunction with selective alkylation procedures, *in vitro*. Also, the G-protein coupling of the contractile mACh receptor(s) was investigated using pertussis toxin pre-treatment, and second messenger studies were performed.

6.2 Methods

6.2.1 Animals and tissue preparation

Adult, female Dunkin Hartley guinea pigs were treated with diethylstilboestrol 0.1 mg kg^{-1} i.p. which was dissolved in peanut oil to allow good penetration into tissues, and killed by CO_2 asphyxiation 24 h later or 48 h later if also treated with pertussis toxin (see below). Uterine horns were each cut into four longitudinal strips after removal of surface connective tissue and mounted for contractile studies. Alternatively, tissue was chopped for biochemical studies, or membranes prepared for radioligand binding studies as described below.

6.2.2 Contractile studies

General

Uterine strips were suspended in 10 ml organ baths at an initial tension of 1g in a modified Sund's solution (pH 7.4) containing 154 mM NaCl, 5.63 mM KCl, 0.98 mM $MgCl_2$, 5.95 mM $NaHCO_3$, 0.48 mM $CaCl_2$, 2.78 mM glucose, 3 μM indomethacin, 1 μM tetrodotoxin, 30 μM cocaine and 30 μM corticosterone, at 32°C. The solution was constantly aerated with 95% O_2/ 5% CO_2, and contractile activity was measured using an isometric force transducer, connected to the MacLab system (AD Instruments, U.K.). The reduced temperature and low extracellular Ca^{2+} concentration served to reduce spontaneous contractility, above which agonist-induced contraction could be measured. The addition of tetrodotoxin prevented neuronal release of acetylcholine from intact nerve terminals by blocking neuronal Na^+ channels thereby preventing depolarisation. Thus the involvement of neuronal nicotinic and muscarinic acetylcholine receptors was excluded. The use of indomethacin blocks cyclooxygenase activity and therefore synthesis of contractile eicosanoids. Cocaine and

:orticosterone are used to prevent re-uptake of adrenergic agonists into nerve terminals and other cells.

Determination of antagonist affinities

Agonist additions were cumulative and increased in 0.5 log molar steps. Antagonist affinities were obtained by Schild regression analysis (Arunlakshana & Schild, 1959) of carbachol-induced contractions. For Schild regression analysis to be valid the agonist-concentration curves must be shifted in a parallel manner and there must be no decrease in the maximum response. For this reason carbachol and not oxotremorine-M for example was used, as carbachol concentration-response curves could be shifted using a larger range of antagonist concentrations before a decrease in maximal response was observed, despite its lower affinity for muscarinic receptors.

After set-up in the tissue baths the uterine strips were allowed to equilibrate for 1 h. A preliminary concentration-response curve was then carried out to carbachol. The tissues were washed every 5 min until basal tension was reached, and then at least 5 concentrations of antagonists were incubated with the tissue for 1 h, alongside time-matched controls. The tissues were washed every 20 min and antagonists replaced during this incubation time. This period was sufficient in each case to allow equilibration of the antagonist, as no further antagonism was seen when the incubation was increased to 2 h for each antagonist. Each uterine strip was exposed only to a single concentration of the antagonist. Schild regression analysis was used to determine antagonist affinities.

Selective alkylation procedures

Selective alkylation procedures were carried out in a similar manner to Thomas *et al.*, (1993). This stratagem enables a single population of receptors to be removed using a non-selective alkylating agent by using a more selective reversible competitive antagonist to

protect' another subtype of the same receptor class. This has been used successfully in airway, ileal and other smooth muscles where M_2- and M_3- mACh receptors co-exist (Eglen & Harris, 1993; Eglen *et al.*, 1994). This process is particularly important for muscarinic receptors because although the subtypes can be distinguished by primary sequence or differential rank orders of antagonist affinities no single ligand, either agonist or antagonist, is totally selective for one subtype over another (Caulfield, 1993).

Tissues were treated with 3 μM phenoxybenzamine for 20 min with or without prior 60 min equilibration with 0.1 μM methoctramine. The tissues were washed every 20 min in Sund's solution and antagonists replaced where necessary. Those tissues not treated with methoctramine acted as controls to observe alkylation of 'unprotected' receptors. Tissues were then washed every 5 min for 90 min to ensure that all antagonist was washed from the tissues. Responses to carbachol were not significantly different after incubation with methoctramine alone followed by this washout protocol from time-matched controls (Figure 6.1). Tripitramine, although it is more selective than methoctramine for M_2- over M_3-mACh receptors was not used as the 'protecting' agent as it could not be effectively washed from the tissues within 90 min unlike methoctramine, presumably due to greater lipophilicity. Therefore, methoctramine was used as the protecting agent due to its greater practicality and adequate selectivity. After washout of phenoxybenzamine and methoctramine a concentration curve to carbachol was then constructed which was further characterised by a single concentration of antagonist after a period of 60 min, and pA_2 values calculated at these 'protected' receptors.

Partial receptor inactivation

Agonist affinities were estimated using partial receptor inactivation procedures according to the operational model of agonism (Black & Leff, 1983). This model is based on traditional receptor theory developed from the law of mass action. Although its validity is constantly being questioned as further knowledge emerges of receptor-G-protein interactions and other

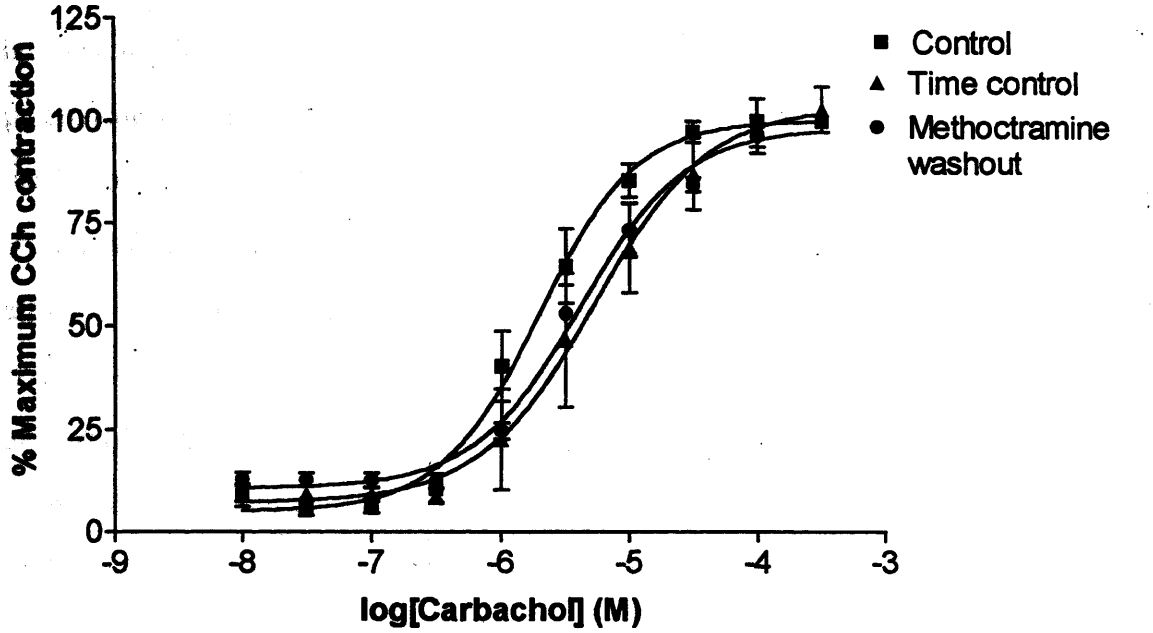


Figure 6.1 - Effect of methoctramine incubation and washout on subsequent carbachol-induced contraction

Concentration-response curves to carbachol were constructed. Methoctramine (0.1 μM) was then equilibrated with the tissue for 1h. Time control tissues were incubated without methoctramine for an equal incubation time. All tissues were then washed every 5 min for 90 min. Another concentration-response curve was then constructed to carbachol. Data are shown as % maximum carbachol-induced contraction prior to incubation with antagonist and are expressed as means ± s.e.m. of 3 independent experiments.

receptor properties, such as multiple receptor states which may effect receptor-ligand interactions (Keen, 1991; Leff, 1995), this method is still a useful tool in quantitative pharmacology. Agonist concentration-response curves were constructed, followed by incubation with 0.1 μM phenoxybenzamine for 20 min. The tissues were washed with Sund's solution and left for 1 h. Concentration-response curves were repeated and agonist affinities estimated. The agonist L-660,863 (3-(3-amino-1,2,4-oxadiazol-5-yl)-quinuclidine) is a non-quaternary oxadiazole-based tertiary amine which is a potent muscarinic receptor agonist (Freedman *et al.*, 1990). It displays in functional studies a 13- and 25-fold selectivity for M_2-mACh receptors ($-\log K_A = 7.6 \pm 0.05$) versus M_3- ($-\log K_A = 6.2 \pm 0.09$) and M_1- ($-\log K_A = 6.5 \pm 0.24$) mACh receptors respectively (Harris *et al.*, 1991). Carbachol is non-selective for muscarinic receptor subtypes.

Effect of propranolol on carbachol-induced contraction

The effect of the β-adrenoceptor antagonist propranolol on carbachol-induced contraction was studied. After carrying out one concentration-response curve after 1 h equilibration period as before, propranolol (0.1, 0.3, 1 μM) was incubated with the tissues for 30 min. The tissues were washed every 15 min and propranolol replaced. A second concentration-curve to carbachol was then constructed. These responses were compared to time-matched control tissues.

Pertussis toxin pre-treatment

To assess the effect of pertussis toxin pre-treatment, 24 h after diethylstilboestrol was injected, pertussis toxin (50 μg kg^{-1}) or vehicle (saline) was injected via the external jugular vein of the guinea pig. Female guinea-pigs have no easily accessible major veins e.g. ear, tail or penile veins therefore ether anaesthesia was used to allow cut-down to the external jugular vein followed by cannulation, injection and suture closure. Guinea-pigs were allowed to recover under supervision. There were no gross adverse side-effects of pertussis

toxin pre-treatment on the guinea pigs during the time between administration, recovery and sacrifice. Only mild tachycardia was observed, which was expected due to systemic administration of the toxin and implied that the toxin was well distributed via the circulatory system of the animals. The guinea-pigs were killed in the same manner as before, 24 h after pertussis toxin or vehicle administration. Some uterine tissue was used for biochemical studies to measure the effects of pertussis toxin on inhibition of adenylyl cyclase (see below) while the rest was used for contractile studies. A single concentration-response curve to carbachol was constructed and compared to that in tissues from vehicle controls.

6.2.3 Measurement of adenylyl cyclase activity

Uteri from pre-treated animals were dissected into inositol-free Ham's F12 medium at 37°C and strips were chopped using a McIlwain chopper (300 × 300 μm) into cubes. The cubes were washed three times with fresh medium and spun at 500 × *g* for 5 min. The uterus tissue was then incubated with collagenase (1 mg ml^{-1}) at 37°C for 1 h with occasional shaking. The treated preparation was then passed through a cell sieve, washed thoroughly with medium and centrifuged as before. The medium was removed and the cells resuspended carefully in a known volume of fresh medium. The ATP pools of the cells were then labelled with 5 μCi ml^{-1} [^{3}H]-adenine for 1 h at 37°C. Cells were then washed and centrifuged as before. Antagonist or buffer was preincubated with 200 μl of [^{3}H]-adenine-labelled cell suspension (approximately 25 μg protein) for 20 min at 37°C and the following were added simultaneously: carbachol (at various concentrations) or buffer, forskolin (10 μM) or buffer and 3-isobutyl-1-methylxanthine (IBMX; 0.5 mM). IBMX prevents the breakdown of cyclic AMP produced during the incubation period by blocking the action of phosphodiesterases. After 30 min incubation at 37°C the reaction was stopped by the addition of 2.2 M HCl followed by mixing and placing in an ice water bath. [^{3}H]-cyclic AMP was separated by column chromatographic methods as described by Daniels & Alvarez, (1992). Acidic alumina was dispensed into disposable columns and the samples carefully decanted into them. The columns were washed with 5 mM HCl and the effluent discarded. Adenosine,

adenine, xanthine and hypoxanthine are rapidly eluted at this stage while cyclic AMP remains adsorbed to the alumina. [^{3}H]-cAMP (> 90%) was eluted with the addition of 3.5 ml of 0.1 M ammonium acetate, collected into scintillation vials and mixed with 15 ml of scintillation fluid. The vials were counted by liquid spectrophotometry for 5 min per vial.

6.2.4 Measurement of inositol phosphate accumulation

Uteri from animals pre-treated with diethylstilboestrol were dissected into Sund's solution at 37°C and roughly chopped using scissors. The tissue was cross-chopped (300 × 300 μm) using a McIlwain chopper and then shaken in Sund's solution in a waterbath at 37°C. The tissue was washed with 8 changes of buffer by allowing the slices to sediment and removing buffer and cell debris by suction. Slices were packed under gravity into a repeating pipette tip and 50 μl of slices were dispensed into insert vials. Throughout the experiment the vials were purged with 95% O_2/ 5% CO_2 every 15 min and capped. Phospholipid pools were labelled using 5 μCi ml^{-1} [^{3}H]-inositol for 3 h at 37°C. Accumulation of label into the phospholipid pool was linear with time over 3 h and could be stimulated by agonist. Lithium chloride (10 mM) was added to the slices 15 min prior to stimulation for 30 min with varying concentrations of carbachol. The reactions were stopped with trichloroacetic acid (1 M) and the vials left on ice for 20 min before centrifugation at 1000 × *g* for 10 min. The supernatant was extracted with 3 × 5 ml water-saturated diethylether. Total [^{3}H]-inositol phosphates were separated using column chromatographic methods using Dowex (Cl^--form) columns as described by Challiss *et al.*, (1992) (see General Methods).

6.2.5 Preparation of uterus membranes

For membrane preparations, guinea pigs were pre-treated with diethylstilboestrol and killed as described above, the uterine horns removed and roughly chopped with scissors into 20 ml ice-cold 10 mM HEPES, 10 mM EDTA, pH 7.4 and homogenised using a Polytron homogeniser for 10 bursts of 3 s at maximal speed on ice. The membranes were then

centrifuged at 200 × *g* for 5 min to remove large tissue fragments. The supernatant was spun at 40 000 × *g* for 15 min at 4°C. The pellet was resuspended in a HEPES buffer (10 mM HEPES, 0.1 mM EDTA pH 7.4) and homogenised and spun as before. The final membrane preparation was resuspended in HEPES buffer with 0.1 mM EDTA, protein concentration measured using the Lowry method (as described in General Methods) and diluted to 1 mg ml^{-1}. Aliquots of membranes were snap frozen in liquid N_2 and stored at -80°C for later use.

6.2.6 Radioligand binding studies

Saturation and displacement binding assays were carried out using 25 µg of uterus membranes and 0.5 nM [^{3}H]-N-methylscopolomine for displacement assays in a final assay volume of 200 µl in HEPES buffer A, as described in General Methods. Incubations of 90 min were performed at 37°C in duplicate and the non-specific binding described by 1 µM atropine.

6.2.7 Data Analysis

All data are expressed as means ± s.e. mean for the number of experiments indicated, each performed on separate days with fresh solutions and drug dilutions. Where Schild slopes were not significantly different from 1, slopes were constrained to 1 to calculate pK_B values. Agonist affinity values were calculated using simultaneous operational curve fitting methods using SigmaPlot (Jandel Scientific, Corte Madera, CA) as described by Wiener & Thalody, (1993). In radioligand binding experiments, the equilibrium constant (K_D) for [^{3}H]-NMS and the B_{max} were estimated from curves generated by non-linear regression of saturation isotherms using GraphPad Prism (San Diego, CA). Competition curves from three independent experiments for each antagonist were analysed separately using GraphPad Prism and curves for 1 and 2 binding sites were fitted to the data. The curve of best fit was used to estimate the IC_{50} for the antagonist, from which the association constant (K_i) could

be derived according to (Cheng & Prusoff, 1973) and the Hill coefficient (Dahlquist, 1978). Statistical significance was assessed by Student's *t*-test for unpaired observations.

6.3 Results

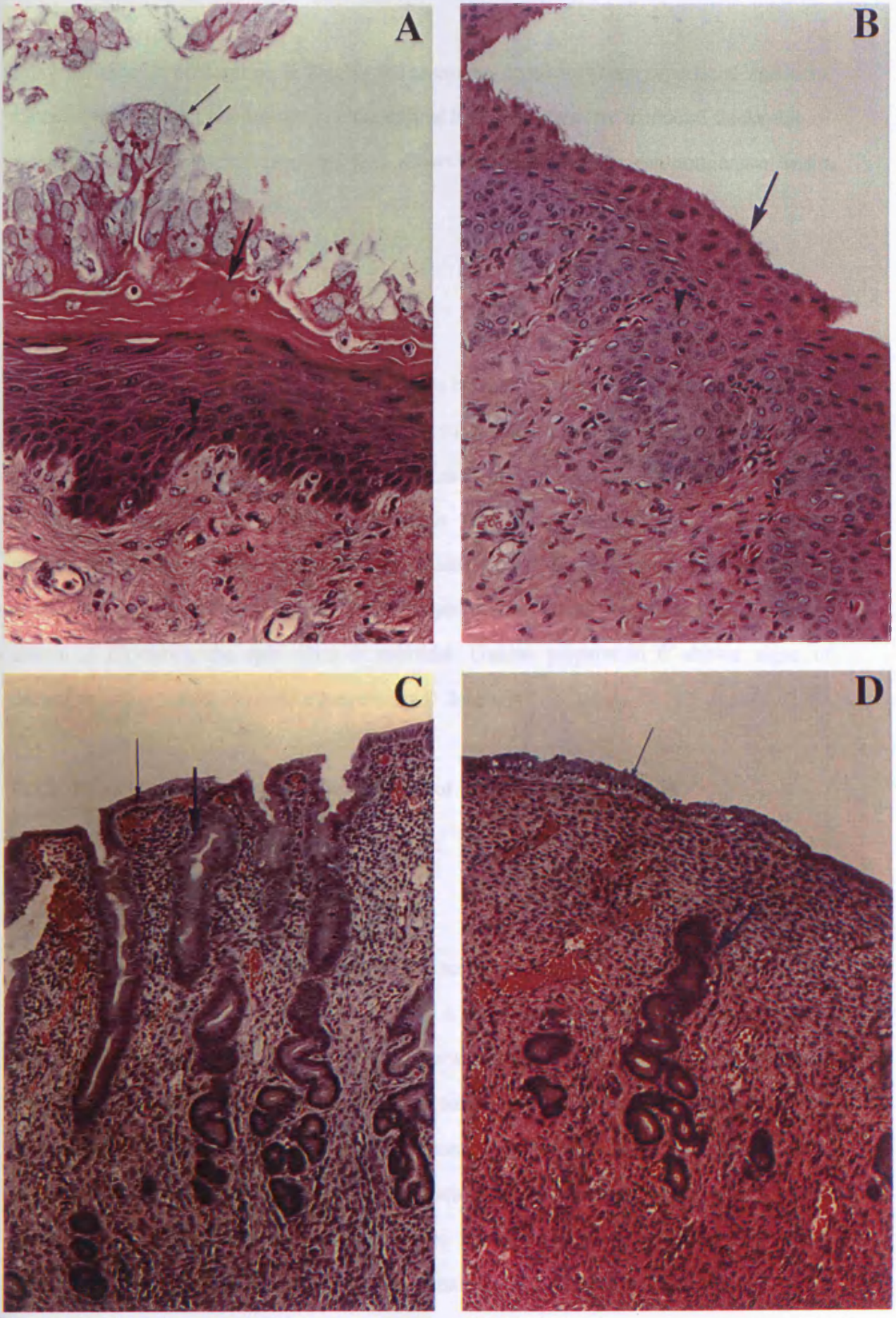
6.3.1 Diethylstilboestrol pre-treatment

As has been known for a long time, the uteri of guinea-pigs under the influence of oestrogen are congested, that is the blood vessels to the uteri are large and conspicuous, there is profound mucous secretion and the uterine horns are large and inflamed (Stockard & Papanicolaou, 1917). This could be seen in diethylstilboestrol pre-treated animals whilst those uteri from untreated guinea-pigs remained pale and unswollen and did not exhibit any secretory activity. Using histological preparations of uterus and vagina from diethylstilboestrol pre-treated animals and vehicle-injected controls, evidence of the oestrogen-dominant phase of the reproductive cycle could be examined. Although changes in the vaginal mucosa are a more easily observable indicator of endocrine influences, there were morphological differences between tissues from treated and untreated animals in both vaginal and uterine preparations which indicated that diethylstilboestrol pre-treatment does cause synchronisation to the oestrogen-dominant phase of the reproductive cycle (Figure 6.2).

Vaginal changes

The vaginal preparation from a diethylstilboestrol pre-treated animal (Preparation A) shows marked morphological differences from that from an untreated animal (Preparation B) (Figure 6.2). The most obvious difference between vaginal preparations from treated and untreated animals is seen in the keritinised epithelium (large arrow). In preparation A the stratum granulosum is highly cornified, whereas in preparation B the surface of the vagina is non-cornified and is going through a rebuilding phase of the vaginal epithelium. The arrow head indicates the basal epithelium (stratum germinatum). In preparation A it is proliferative and shows mitotic activity however the stratum germinatum of preparation B shows no such proliferation. Finally, a layer of mucal cells (small arrows) can be seen in preparation A

Figure 6.2 - Histological preparations of guinea-pig uterus and vagina from guinea-pigs untreated or pre-treated with diethylstilboestrol (0.1 mg kg^{-1}, i.p., 24h prior to sacrifice)
A - Vaginal preparation; diethylstilboestrol pre-treated
B - Vaginal preparation; no pre-treatment
C - Uterus preparation; diethylstilboestrol pre-treated
D - Uterus preparation; no pre-treatment
Tissues were sectioned and fixed using acetone. Sections were stained using a simple haemotoxylin and eosin stain. Arrows indicate morphological differences between the tissues from treated and untreated animals. See text for detailed explanation.


A
B
C
D

which is absent in preparation B. During the oestrogen-dominant phase superficial mucinous epithelial cells project and slough into the vaginal lumen followed by increased thickening of the cornified layer. Vaginal preparation A shows evidence of oestrogen-dominance whilst vaginal preparation B does not.

Uterine changes

The morphological differences in uterine sections between preparations from treated (C) and untreated (D) animals (Figure 6.2) are relatively subtler than those in vaginal sections. Large arrows indicate endometrial glands. In preparation C, these glands are much more active and proliferative than in preparation D. There is also more proliferative activity in the intraglandular stromal tissue and taller, hyperplastic change in the surface epithelium (small arrows) of preparation C compared to D. In preparation D, as would be expected in an animal in dioestrus, the epithelium is cuboidal. Uterine preparation C shows signs of oestrogen influence whilst uterine preparation D does not.

6.3.2 Pharmacological characterisation of contractile response

Antagonist affinities

Carbachol produced a concentration-dependent increase in tension of uterine smooth muscle with a pEC_{50} value of 5.79 ± 0.05 ($n = 12$), and a maximal tension of 2-4 g in these tissues (see sample trace Fig. 6.3). This response was sensitive to muscarinic antagonists which caused dextral shifts of the concentration-response curve in a parallel manner with no decrease in maximal tension for the concentrations of antagonists used, indicating that this antagonism was competitive (Figure 6.4). The Schild slopes were not significantly different from 1 ($P > 0.05$) and were thus constrained to 1 to estimate affinity values (pK_B) (Table 6.1). The antagonist affinities observed were statistically significantly different from those predicted for either M_2- or M_3-mACh receptors in homogeneous populations, based on

Table 6.1 - Antagonist affinity estimates (pK_B/pA_2) at mACh receptors mediating carbachol-induced contraction of guinea-pig isolated uterus strips

	Untreated *(pK_B)*	*'Protected'* *(pA_2)*	*M_2* *atria*	*M_3* *ileum*
Atropine	9.3 ± 0.1	n.d.	9.1[a]	9.3[a]
Methoctramine	7.1 ± 0.1	7.1 ± 0.1	7.9[a]	6.0[a]
Zamifenacin	8.3 ± 0.1	8.6 ± 0.1	6.6[a]	9.3[a]
Tripitramine	8.1 ± 0.1	8.3 ± 0.1	9.7[b]	6.5[b]
p-F-HHSiD	7.5 ± 0.1	n.d.	6.0[c]	7.9[c]
4-DAMP	8.9 ± 0.1	n.d.	7.8[c]	9.3[c]
Pirenzepine	6.8 ± 0.1	n.d.	6.8[c]	6.9[c]
MTx-3	<6.0	n.d.	n.d.	n.d.

Values shown are means ± s.e.m., n ≥ 7. n.d. = not determined. [a]Caulfield (1993); [b]Chiarini *et al.* (1995); [c]Eglen *et al.* (1994)

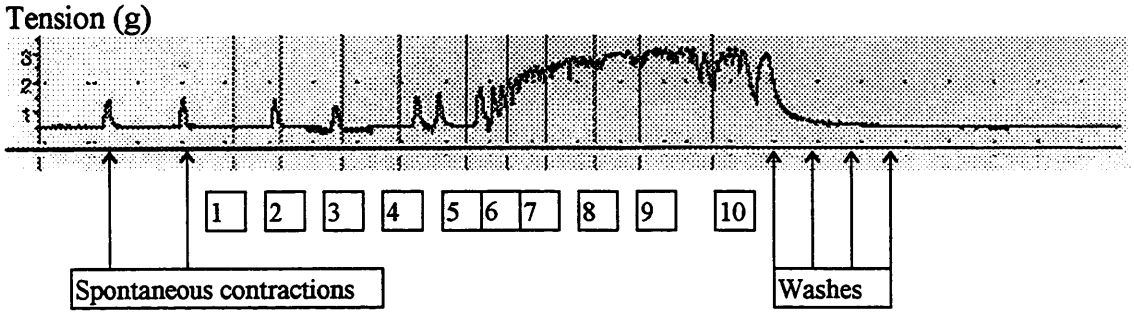
Table 6.2 - Antagonist affinity estimates (pK_i) determined by competition radioligand binding studies in EDTA-washed uterus membrane preparations

	pK_i *(log M)*	*n_H*	*M_1*	*M_2*	*M_3*	*M_4*
Atropine	8.9 ± 0.3	1.1 ± 0.2				
Methoctramine	8.1 ± 0.1	1.2 ± 0.2	7.3	7.9	6.7	7.5
Zamifenacin	7.6 ± 0.2	1.0 ± 0.2	7.4	7.6	8.0	7.6
Tripitramine	9.3 ± 0.2	0.9 ± 0.1	8.8	9.6	7.4	7.9

Values shown are means ± s.e.m., n = 3. Hill slopes are not significantly different from 1 ($P > 0.05$). Values in columns 4-7 have been determined in ligand binding studies at cloned mACh receptors expressed in CHO cells (Eglen *et al.*, 1997)

Figure 6.3 - Sample trace showing a typical concentration-response curve for carbachol in guinea-pig uterus preparation

Carbachol was added cumulatively at time points as indicated. Dots indicate 5 min time-points. Basal tension was 0.5 g. Tissue measured approximately 5 mm by 2 mm. Total bath volume was 10 ml, and the vehicle for carbachol was bathing solution (Sund's solution; see text). Baths were maintained at 32°C and aerated with 95% O_2 /5% CO_2. Tension was recorded using a force transducer connected to MacLab system.



Concentrations of carbachol added -

1 = 10^{-9} M
2 = 10^{-8} M
3 = 10^{-7} M
4 = 3×10^{-7} M
5 = 10^{-6} M
6 = 3×10^{-6} M
7 = 10^{-5} M
8 = 3×10^{-5} M
9 = 10^{-4} M
10 = 10^{-3} M

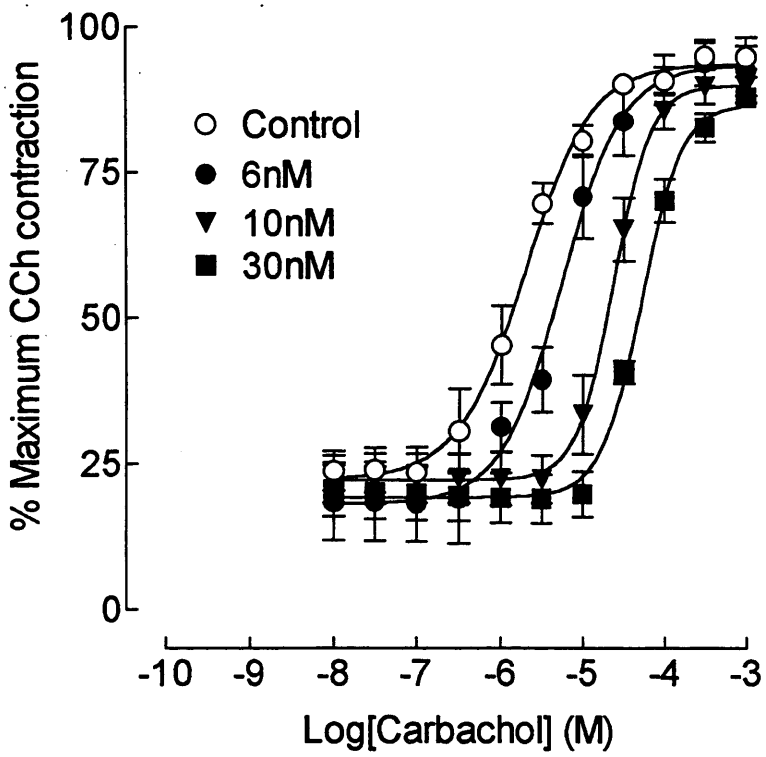


Figure 6.4 - Antagonism of carbachol-induced contraction of guinea-pig isolated uterus strips by zamifenacin

Concentration-response curves to carbachol were constructed in the absence and presence of zamifenacin (6, 10, 30 nM) which was equilibrated in the tissue for 1h prior to agonist stimulation. Data are shown as % maximum carbachol-induced contraction prior to incubation with antagonist and are expressed as means ± s.e.m. for at least 3 independent experiments. The effect of three out of five concentrations of zamifenacin used have been illustrated for clarity. Similar effects were seen with other antagonists used.

previously reported affinity estimates (Caulfield, 1993; Chiarini *et al.*, 1995), and a low affinity value for pirenzipine in this tissue (pK_B = 6.82 ± 0.08) does not support the involvement of M_1- or M_4-mACh receptors in carbachol-induced contraction of this tissue.

Contractions to carbachol were abolished by phenoxybenzamine (in the absence of methoctramine), but the response could be protected by prior equilibration with 0.1 μM methoctramine followed by extensive washing (Figure 6.5). Under these conditions a rightward shift and small decrease (10 ± 2%) in maximum response was observed. Antagonist affinities estimated at the 'protected' receptors using a single concentration of antagonist were not significantly different from estimations made in untreated tissues ($P > 0.05$) (Table 6.1), and were therefore likewise significantly different from affinity values previously reported for M_2- and M_3-mACh receptors. It would be expected that removal of a M_3-mACh receptor population, as would be predicted by alkylation procedures in a heterogeneous population, would cause the greatest change in affinity estimates for zamifenacin, despite the fact that zamifenacin may have varying affinities at M_3-mACh receptors in different tissues (Eglen *et al.*, 1997). However, as for the other antagonists, selective alkylation procedures had no effect on the estimated affinity for zamifenacin to contraction in this tissue.

It was noted that for all antagonists, the affinities in both untreated tissues and at 'protected' receptors were significantly different from those estimations previously reported at M_2- and M_3-mACh receptors ($P < 0.05$) (Table 6.1) (Caulfield, 1993; Chiarini *et al.*, 1995), and that the affinity estimates were most closely alike to estimates expected at M_4-mACh receptors. However, the M_4- selective muscarinic toxin 3 isolated from venom of the green mamba *Dendroaspis augusticeps* (MTx-3;Jolkkonen *et al.*, 1994) did not have any effect on carbachol-induced uterine contraction (n=5) suggesting that M_4-mACh receptors are not involved (Figure 6.6).

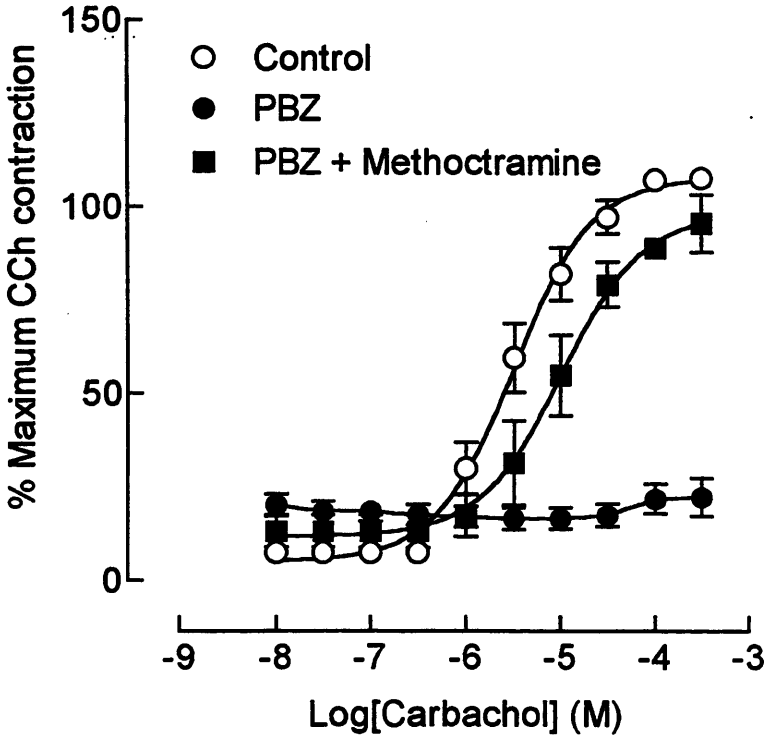


Figure 6.5 - Alkylation and protection of mACh receptors mediating carbachol-induced contraction of guinea-pig uterus strips

Phenoxybenzamine (PBZ; 3μM) was incubated for 20 min alone, or in the presence of previously equilibrated methoctramine (0.1 μM; 1h). Antagonists were then completely washed from the tissues before construction of the above concentration-response curves. Data are shown as % maximum carbachol-induced contraction prior to incubation with antagonist and are expressed as means ± s.e.m. for 6-8 independent experiments.

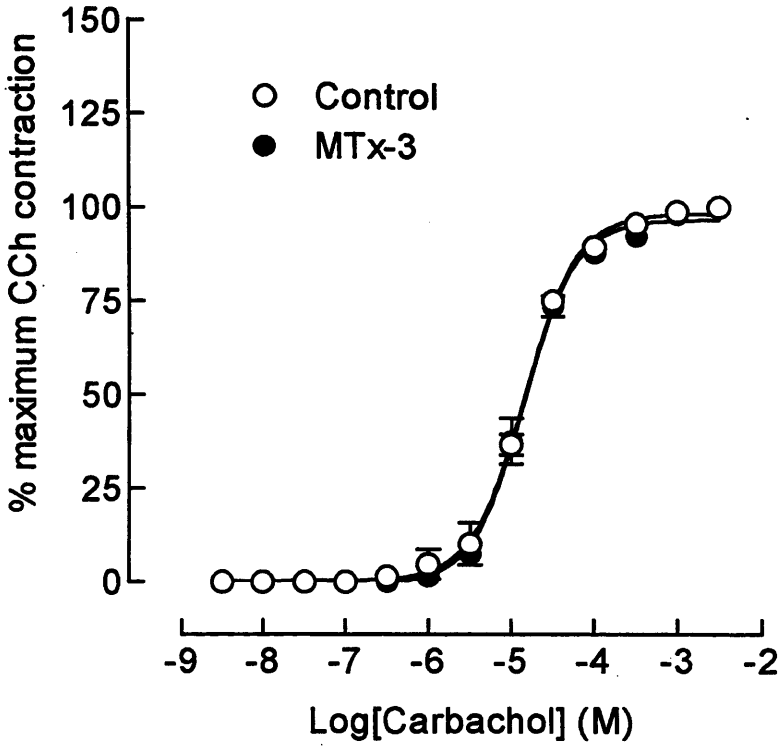


Figure 6.6 - Effect of MTx-3 (Muscarinic toxin 3) on carbachol-induced contraction of guinea-pig uterus strips.

MTx-3 (1μM) was equilibrated with the tissue for 1h prior to agonist stimulation. Data are shown as % maximum carbachol-induced contraction prior to incubation with toxin and are expressed as means ± s.e.m. of 3 independent experiments.

Agonist affinities

The affinity value (pK_A) calculated for the putative 'M_2-selective' agonist L660,863 (Harris *et al.*, 1991) of 5.44 ± 0.03 (n=6) (Figure 6.7) was significantly different from the value previously reported for its affinity at M_2-mACh receptors (7.6 ± 0.05). In contrast, the pK_A value for carbachol, which does not discriminate between muscarinic subtypes, of 4.22 ± 0.17 (n=8) agrees with that previously reported in guinea pig ileum (4.7 ± 0.5; Ford *et al.*, 1991). The fact that the estimate for carbachol, which is not selective between mACh receptors, is as expected gives us confidence in the validity of this method in estimating the affinity value for the agonist L-660,863.

Effect of propranolol on carbachol-induced uterine contraction

Propranolol had no significant effect on carbachol-induced contraction when compared to time-matched controls ($P > 0.05$) (Figure 6.8). There was also no effect on basal tension of the tissues compared to controls. This was also true when the incubation time for the antagonist was extended to 1 h and 2 h, showing that the antagonist was at equilibrium in the tissue. Since the antagonist was being replaced every 15 min it is unlikely that the antagonist was being broken down in the tissue sufficiently to account for its lack of effect on contraction. The use of cocaine and corticosterone in the bathing buffer should prevent re-uptake of any endogenously released adrenergic agonists.

6.3.3 Effect of pertussis toxin pre-treatment

Pertussis toxin pre-treatment had no significant effect on carbachol-induced contraction when compared with tissues from vehicle-injected controls ($P > 0.05$) (Figure 6.9). This contrasted with effects on adenylyl cyclase activity in collagenase-treated uterus cell preparations. Thus, carbachol caused a 35 ± 3% inhibition of forskolin-stimulated cyclic AMP accumulation after 30 min stimulation; an effect almost entirely attenuated by pertussis

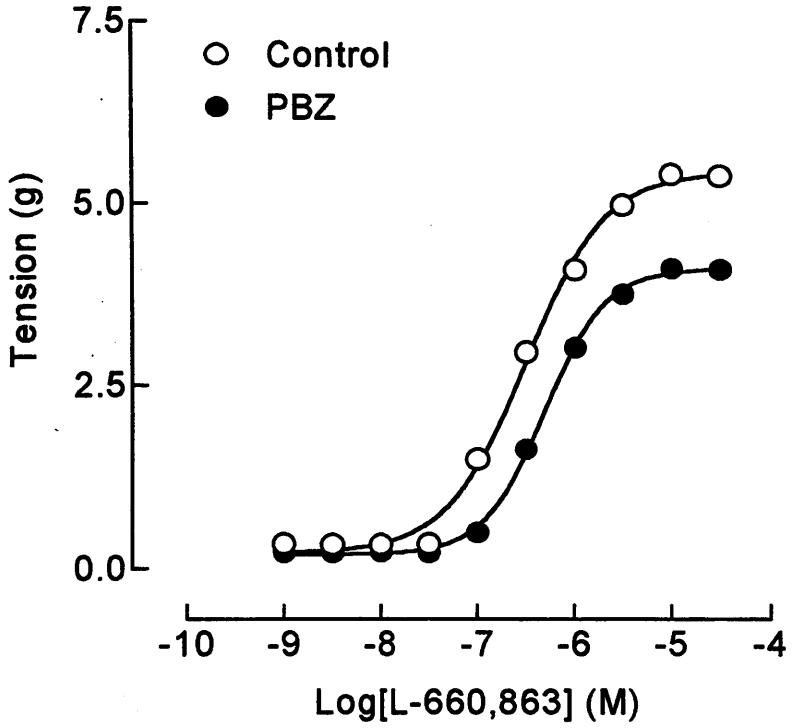


Figure 6.7 - Partial inactivation of contractile response to L-660,863, a 'M_2-selective' agonist, by phenoxybenzamine in guinea-pig uterus strips.

Phenoxybenzamine (PBZ; 0.1μM) was incubated for 20 min before construction of a second concentration-response curve to the agonist. Data are expressed as absolute tension and are from a single experiment representative of 6 independent experiments. K_A values were calculated separately for each experiment and then means and s.e.mean were derived.

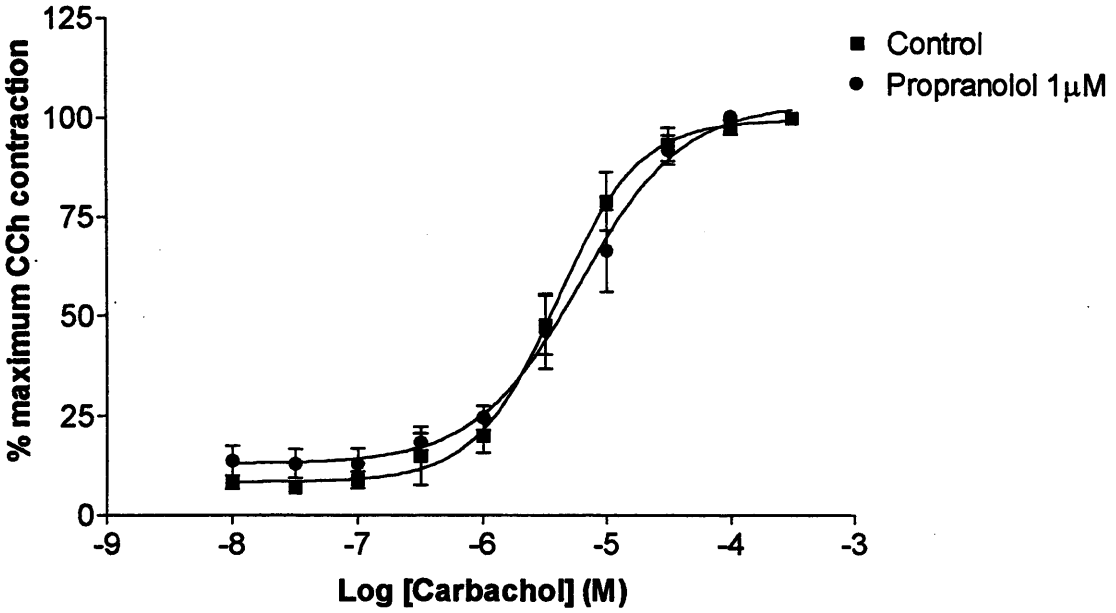


Figure 6.8 - Effect of propranolol on carbachol-induced contraction of guinea-pig isolated uterus strips

Propranolol (1 μM) was incubated for 30 min prior to agonist stimulation. Data are shown as % maximum carbachol-induced contraction prior to incubation with antagonist and are expressed as means ± s.e.m. of 3 independent experiments.

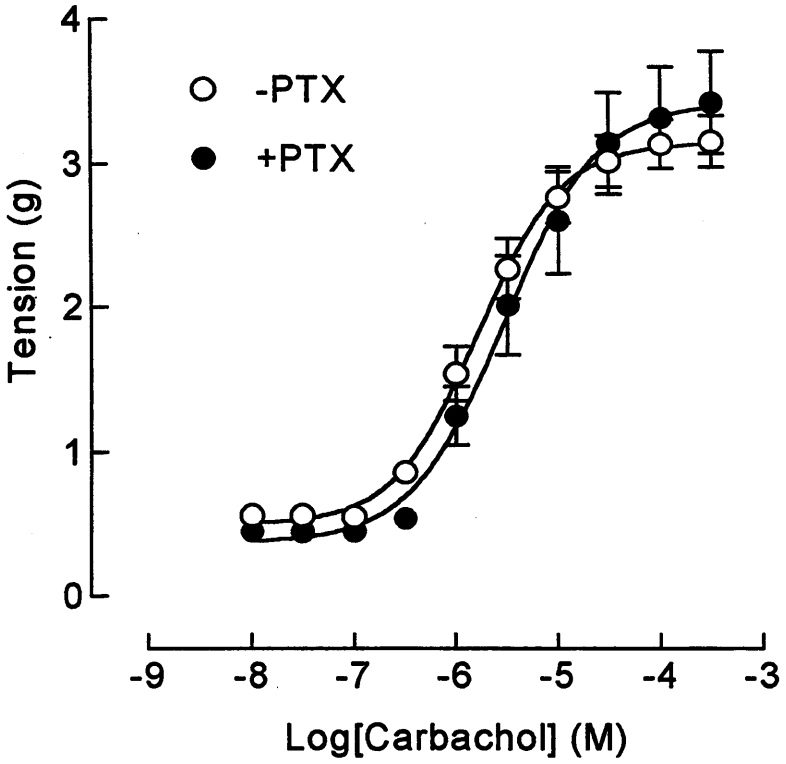


Figure 6.9 - Effect of pertussis toxin pre-treatment on carbachol-induced contraction of guinea-pig uterus strips

Animals were pre-treated with pertussis toxin (PTx; 50μg kg^{-1}) or vehicle via the external jugular vein 24 h prior to sacrifice. Single concentration-response curves were constructed after tissue equilibration. Contraction is expressed as absolute tension and presented as means ± s.e.m. for 3 separate experiments.

toxin pre-treatment (Figure 6.10). This suggests that the pertussis toxin is acting on the sensitive G-proteins in this tissue, yet is having no effect on carbachol-induced contraction.

6.3.4 Inositol phosphate accumulation

Stimulation with a maximal carbachol concentration for 30 min produced a 31 ± 7% increase in total [^{3}H]- inositol phosphate accumulation over basal levels (Figure 6.11). Although this increase was reproducible and statistically significant ($P < 0.01$), the size of the response precluded pharmacological characterisation. This was despite measurable agonist-induced increases in lipid accumulation of [^{3}H]-inositol.

6.3.5 Pharmacological characterisation of [^{3}H]-NMS binding

An equilibrium dissociation constant (K_D) of 0.12 ± 0.05 nM for [^{3}H]-NMS was obtained from saturation binding experiments (n=3) which were also used to calculate a B_{max} of 147 ± 18 fmol mg protein^{-1} for mACh receptors in this membrane preparation (Figure 6.12). The affinity values for subtype-selective antagonists were estimated using competition radioligand binding techniques in uterus membrane preparations (Table 6.2) (Figure 6.13). These affinity values were not significantly different from those expected for M_2-mACh receptors and supported previous radioligand binding studies in this tissue (Doods *et al.*, 1993; Eglen *et al.*, 1989). The Hill slopes of the displacement isotherms were not significantly different from 1, and the best-fit curves were one-site competition curves indicating that by this method only one population of mACh receptors can be detected.

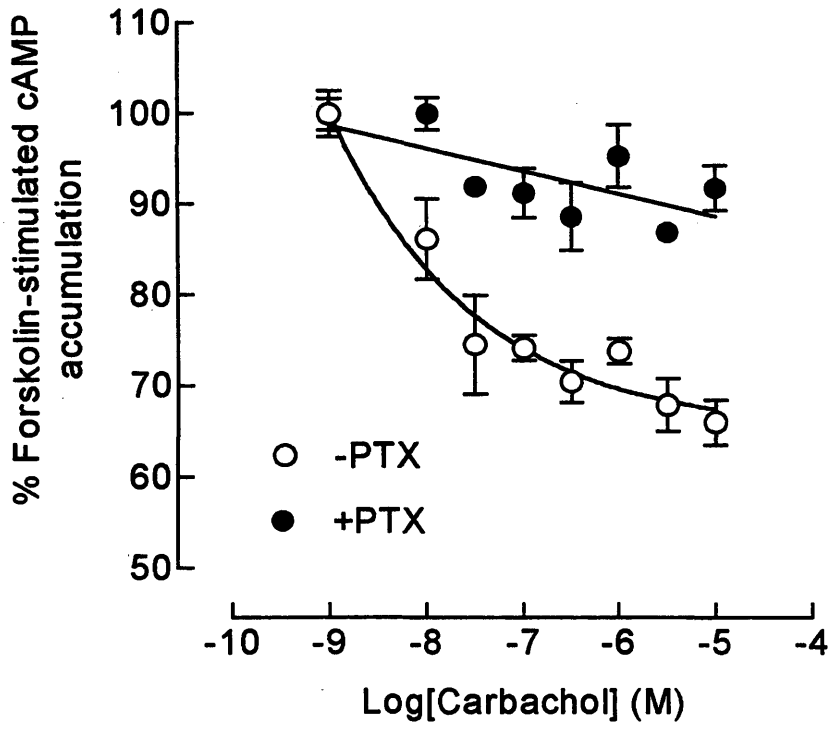


Figure 6.10 - Effect of pertussis toxin pre-treatment on carbachol-induced inhibition of forskolin-stimulated cAMP accumulation in dissociated guinea-pig uterus smooth muscle cells

Animals were treated with pertussis toxin (PTx; 50 μg kg^{-1}) or vehicle via the external jugular vein 24 h prior to sacrifice. Cells were dissociated using collagenase (1mg ml^{-1}) (see Methods). Data are expressed as % of forskolin-stimulated cyclic AMP accumulation in the absence of carbachol, and presented as means ± s.e.m. for 3 separate experiments

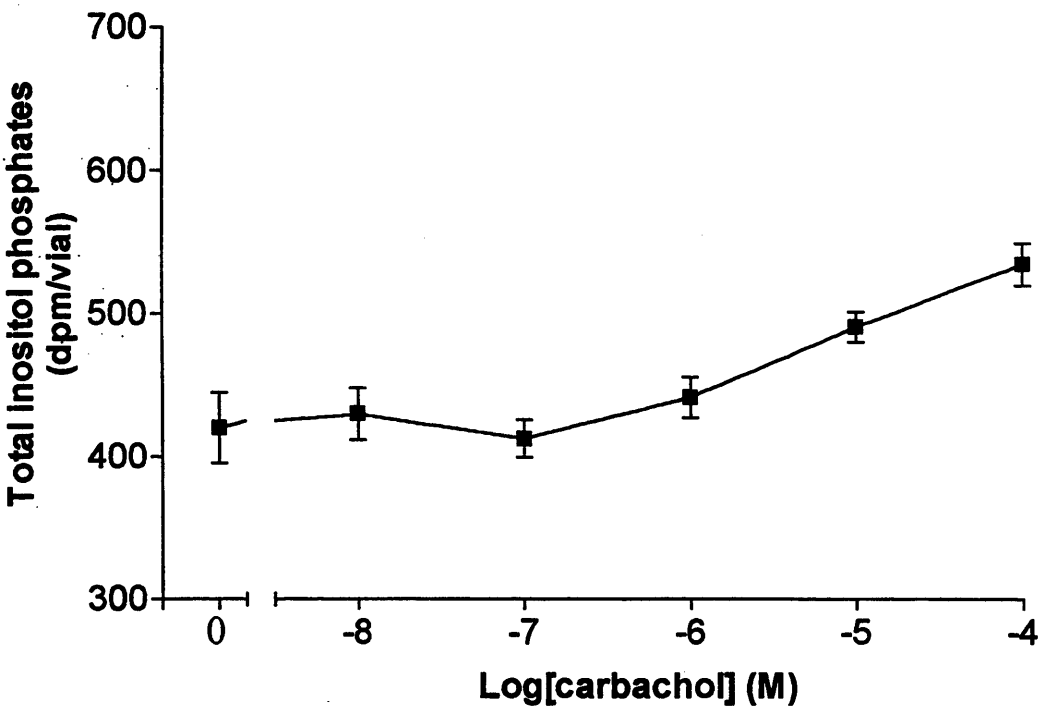


Figure 6.11 - Carbachol-induced increases in total [^{3}H]-inositol phosphate accumulation in guinea-pig uterus slices

Uterus strips were chopped using a tissue chopper into 300 × 300μm cubes. Slices were washed and packed under gravity into a pipette tip. Slices were aliqouted into insert vials and labelled using 5 μCi ml^{-1} [^{3}H]-inositol for 3h at 37°C. LiCl (10 mM) was added to the cells 10 min prior to stimulation with carbachol. The reactions were stopped with TCA (1M) and the slices pelleted by centrifugation. The supernatant was extracted with water-saturated diethylether and total [3H]-inositol phosphates determined using chromatographic methods using Dowex (Cl- form) columns. Data are expressed as d.p.m. per vial and shown as means ± s.e.m. for 3 separate experiments.

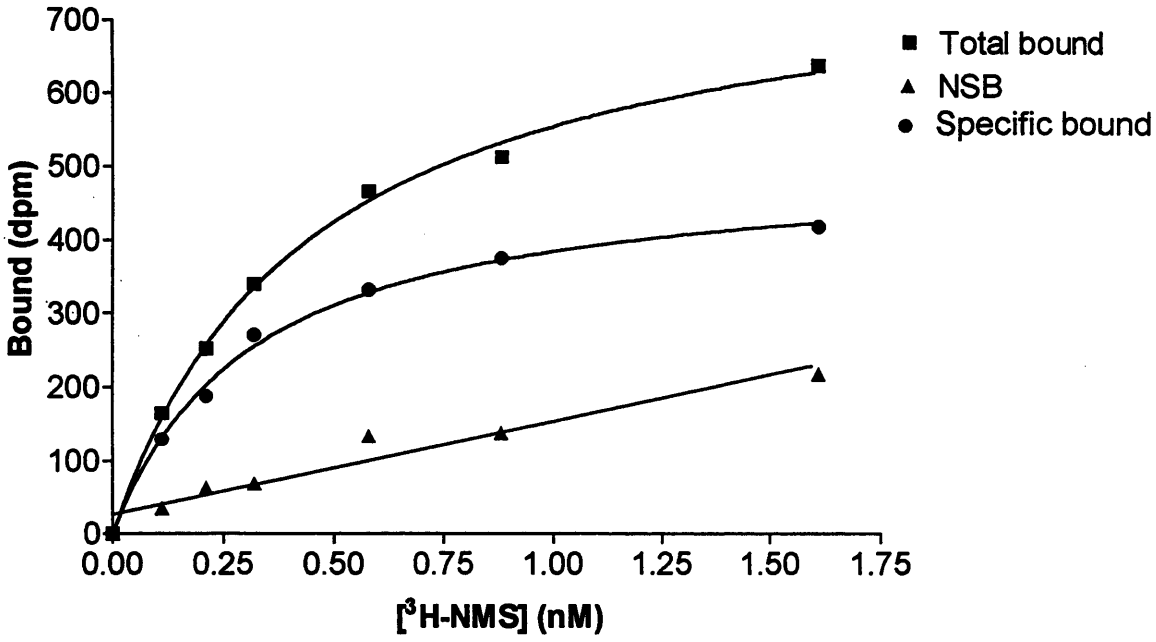


Figure 6.12 - Saturation binding of [^{3}H]-NMS in uterus membrane preparations

Uterus membranes were prepared as described in Methods. Saturation binding was carried out in HEPES buffer A using 25 μg of membranes and increasing concentrations of [^{3}H]-NMS. Incubations of 90 min were performed at 37 °C in duplicate, and the non-specific binding defined by 1 μM atropine. Data are from a single experiment, representative of 3 separate experiments.

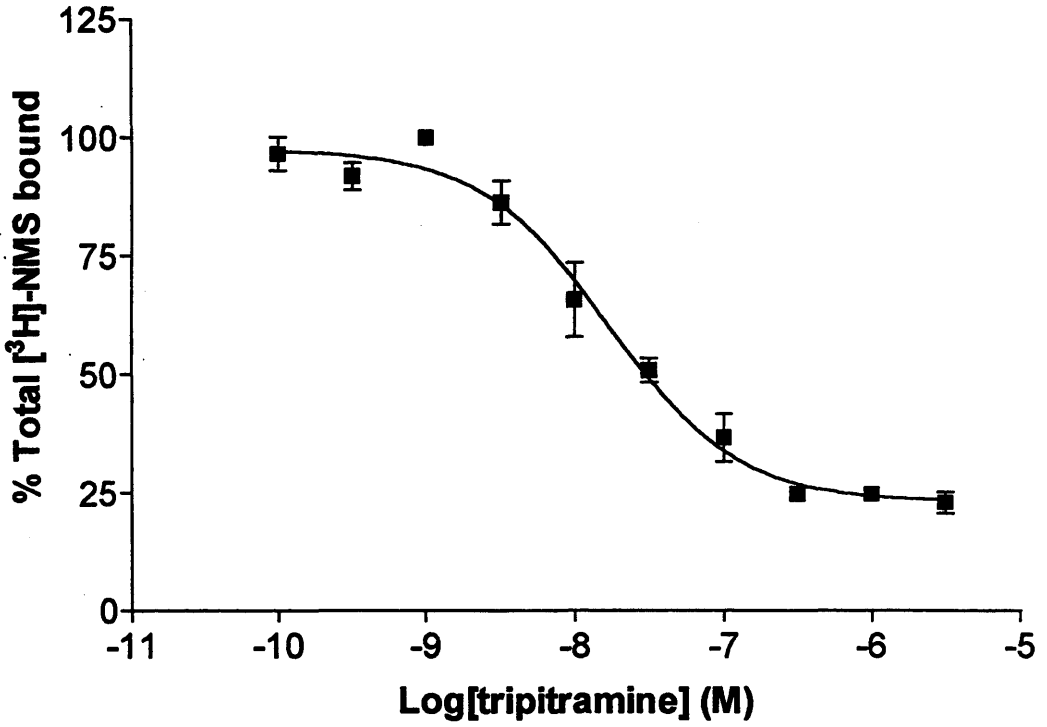


Figure 6.13 - Displacement of [^{3}H]-NMS binding by tripitramine in uterus membrane preparations

Uterus membranes were prepared as described in Methods. Displacement binding was carried out in HEPES buffer A using 25 μg of membranes, 0.5 nM [^{3}H]-NMS and increasing concentrations of tripitramine. Incubations of 90 min were performed at 37°C in duplicate. Data are expressed as means ± s.e.m. for three separate experiments

6.4 Discussion

The characterisation of mACh receptor-mediated contraction in uterine tissue has been hampered both by the inherent variability of the contractile responses observed and the lack of subtype-specific mACh receptor ligands. Moreover, several studies of this tissue fail to account for potential changes in receptor populations due to fluctuating steroid hormone levels, despite studies showing this to be a critical factor (Arkinstall & Jones, 1990; Matucci *et al.*, 1996; Riemer *et al.*, 1987; Varol *et al.*, 1989). In this study pre-treatment with diethylstilboestrol induced all guinea-pigs to enter an oestrogen-dominant phase of the reproductive cycle, thus minimising inter-animal differences in the steroid hormone levels, and implying the tissue mACh receptor levels are under a constant hormonal influence. It consequently can be assumed with confidence that the mACh receptor populations were not significantly different between pre-treated animals.

Although only a limited number of antagonists were used to characterise the muscarinic response of this tissue those chosen comprise some of the most 'M_2/M_3'-selective antagonists available. Surprisingly these have not been used before despite the large numbers of antagonists used previously (Doods *et al.*, 1993; Dorje *et al.*, 1990). Moreover, these M_2/M_3-mACh receptor-selective compounds are of interest given the coexpression of M_2- and M_3-mACh receptors in most smooth muscles, and the apparent absence of M_3-mACh receptors from guinea-pig uterus.

In the present study, the affinity estimates made for these 'M_2/M_3-selective' antagonists did not correlate with those made at M_2- or M_3-mACh receptors (Caulfield, 1993; Chiarini *et al.*, 1995). This excludes the possibility that carbachol-induced contraction of guinea-pig uterus is mediated by a homogeneous population of M_2- or M_3-mACh receptors alone, and may suggest that M_2- and M_3-mACh receptors are synergistically activated to produce atypical affinity estimates. Selective alkylation procedures were employed to clarify this point.

Alternatively the mACh receptor population responsible for carbachol-induced contraction might be atypical and remains to be classified.

Given that MTx-3, a selective M_4-mACh receptor toxin (Jolkkonen *et al.*, 1994) has no effect on contraction and that pirenzepine displays a low affinity for contraction (Caulfield, 1993) the possibility of involvement of M_4- and M_1-mACh receptors also can be excluded. These data contradict the conclusion of Dorje *et al.*, (1990) in which activation of an M_4-mACh receptor was invoked. Two caveats need to be applied when considering the use of MTx-3 as a ligand to determine involvement of M_4-mACh receptors. First, functional data on this compound is extremely limited (Olianas *et al.*, 1996). Nonetheless, unpublished data (R.M. Eglen *et al.*; personal communication) support the literature in terms of the affinity profile at recombinant mACh receptors. Second, a functional confirmation is difficult given the paucity of robust and accepted bioassays exhibiting M_4-mACh receptor pharmacology, apart from recombinant systems. The use of MTx-3 in defining the M_4-mACh receptor awaits a more extensive exploration of its pharmacological properties.

In tissues where a heterogeneous receptor population exists selective alkylation procedures have been used to isolate single subtype populations e.g. Thomas *et al.*, (1993). This technique, using a reversible, selective antagonist, enables a given receptor type to be 'protected' from inactivation by non-selective alkylating agents such as phenoxybenzamine. In the absence of a protecting ligand, when the tissues were exposed to 3 μM phenoxybenzamine and extensively washed, the responses to carbachol were abolished. In contrast, prior equilibration with methoctramine (0.1 μM) almost completely attenuated this effect of phenoxybenzamine, such that only a small dextral shift and slight depression of maximum response was seen. Methoctramine at 0.1 μM would be expected to occupy more than 85% of M_2-mACh receptors and less than 10% of M_3-mACh receptors (see Chapter 3). Therefore it would be expected that any M_3-mACh receptors in this tissue would have been inactivated by phenoxybenzamine whilst the M_2-mACh receptors would be protected from such inactivation.

This approach has been used to try to dissect a direct role for M_2-mACh receptor activation in contraction in other smooth muscles. Some studies have shown that M_2-mACh receptors may cause contraction via a physiological antagonism of β-adrenoceptor-induced relaxation in various different smooth muscle tissues, by using selective inactivation procedures to remove M_3-mACh receptor-mediated responses (Fernandes *et al.*, 1992; Thomas *et al.*, 1993; Thomas & Ehlert, 1996; Torphy *et al.*, 1983). However, use of a similar protocol in other tissues (notably tracheal preparations) has led other groups to conclude that there is no evidence for a role of M_2-mACh receptors in functional antagonism of β-adrenoceptor-mediated relaxation, and direct smooth muscle contraction (Roffel *et al.*, 1993; Roffel *et al.*, 1995; Watson *et al.*, 1995). This approach has not been used to characterise the mACh receptor-mediated contraction in uterus smooth muscle until now, and may be an important method to use to decide whether the atypical affinity estimates observed are due to synergism between M_2- and M_3-mACh receptors in a previously unseen heterogeneous receptor population. This is especially important given the evidence suggesting the relative importance of M_2-mACh receptors in this tissue (Doods *et al.*, 1993; Eglen *et al.*, 1989).

However, characterisation of antagonist affinities at these 'protected' receptors showed no differences between these and affinity estimates made in untreated tissues. This suggests that the dextral shift and slight depression of maximum was caused by removal of part of a single population of receptors as opposed to the removal of a single subtype of receptors from a heterogeneous population. Contraction is therefore not caused by concurrent activation of M_2- and M_3-mACh receptors.

Scepticism abounds as to the usefulness of agonist affinity estimates in characterisation of receptors. However, these data may be supporting evidence for other methods used in the pharmacological characterisation of receptors (Keen, 1991; Leff, 1995). Hence, the finding that the agonist affinity value for the putative 'M_2-selective' agonist L-660,863 differed from that expected at a M_2-mACh receptor is further evidence to support conclusions based on

atypical antagonist affinity profiles. Since the affinity estimate for carbachol (which is not selective between muscarinic receptor subtypes) agrees with that previously reported in the guinea-pig ileum (Ford *et al.*, 1991), collectively the contractile data of this study would suggest that contractions to carbachol in guinea-pig isolated uterus are mediated not by M_2-, M_3- or M_4-mACh receptors as previously reported, but by a single population of mACh receptors with an atypical operational profile.

As propranolol has no effect on carbachol-induced contraction or basal tension of the isolated guinea-pig uterus it can be assumed that there is no intrinsic adrenoceptor-induced relaxant tone. This rules out the possibility that atypical affinity estimates for carbachol-induced contraction can be explained by varying degrees of adrenoceptor stimulation by endogenous adrenoceptor agonists, and hence relaxant tone. In rat isolated urinary bladder it has been shown that there may be a functional role for M_2-mACh receptors in the reversal of β-adrenoceptor-mediated relaxation (Choppin *et al.*, 1997). In this study, a sustained contraction was induced and then addition of adrenergic agonists caused a concentration-dependent relaxation in opposition to this contractile tone. Muscarinic agonist application then caused 're-contraction' of the tissue. Subsequent pharmacological analysis of this mACh receptor-induced response showed it to be mediated by M_2-mACh receptors. This has also been shown in other tissues by other groups (Thomas *et al.*, 1993; Thomas & Ehlert, 1996; Watson *et al.*, 1995). The relationship between β-adrenoceptor-induced relaxation and M_2-mACh receptor activation could not be studied in this tissue, as contraction to a variety of spasmogens was not sufficiently sustained to carry out 're-contraction' studies.

Estimates of antagonist affinities in radioligand binding studies were not in agreement with those estimated in functional studies (cf. Tables 6.1 and 6.2). The affinity profile of the chosen 'M_2/M_3-selective' antagonists was consistent with the presence of a homogeneous population of M_2-mACh receptors. The Hill slopes of competition curves of antagonists to [^{3}H]-NMS would suggest that only one type of muscarinic receptor subtype is expressed in this tissue. However, it has been shown by immunological methods and Northern blotting

techniques that both M_2- and M_4-mACh receptors can coexist in uterine tissues (Dorje *et al.*, 1991; Eglen *et al.*, 1992), so it may be that these antagonists are still insufficiently selective between mACh receptor subtypes to detect coexpression, particularly if expression levels of one receptor subtype are low. These data however support previous evidence from Eglen *et al.*, (1989) and Doods *et al.*, (1993), in which radioligand binding data suggested that M_2-mACh receptors alone were responsible for contraction in this tissue. Thus, it would seem that whilst there are conflicting functional data regarding the nature of the response, all radioligand studies carried out on guinea-pig uterus concur that M_2-mACh receptors only can be detected.

This is interesting given the insensitivity of the contractile response to prior systemic administration of pertussis toxin, which abolishes M_2-mACh receptor-mediated responses. This supports data produced by *in vitro* pre-treatment of guinea-pig uterus with pertussis toxin carried out by Marc *et al.*, (1988). In the present study it has been shown that pertussis toxin abolishes the G_i-mediated inhibitory effect of carbachol on forskolin-stimulated cyclic AMP accumulation, giving confidence that systemic administration allows effective delivery of the toxin to the uterus and consequent ADP-ribosylation of sensitive G-proteins. The insensitivity of the contractile response to pertussis toxin indicates that it is not mediated by G_i-proteins.

It remains possible that the contractile response is in fact mediated by M_2-mACh receptors. The results of the present study are not inconsistent with the conclusion that M_2-mACh receptors mediate carbachol-induced contraction via a G-protein which is not sensitive to pertussis toxin, such as by phosphoinositide cycle activation via a $G_{q/11}$ G-protein. In recombinant systems M_2-mACh receptors can interact with multiple G-proteins (Ashkenazi *et al.*, 1987) and there is some evidence to show that receptor promiscuity can occur physiologically (Kenakin & Boselli, 1991; Pinkas-Kramaski *et al.*, 1990). The existence of constitutively active receptors has revealed the inverse agonist nature of some muscarinic 'antagonists' (Jakubik *et al.*, 1995); a phenomenon that can be demonstrated in physiological

systems as well as in recombinant cells (Noguera *et al.*, 1996), and this fact may be of the utmost importance when making functional affinity measurements of so-called 'antagonists'. Agonists act by preferentially binding to the active state of the receptor, inverse agonists act by preferentially binding to the inactive receptor state, whilst neutral antagonists bind with equal affinity to both receptor states. In a two-state model of receptor activation, when an inverse agonist is used to antagonise an agonist the affinity is system dependent, and varies according to the ratio of receptors in inactive and active states (Leff, 1995).

The three state receptor theory has been proposed to allow for receptor promiscuity and explain how this in turn may affect measurable ligand affinities (Leff *et al.*, 1997). In the simplest case, two active conformations of a receptor exist, each activating a single G-protein. Therefore, agonist affinity depends on three affinity constants, which govern the distribution of the receptors between the three states. The measured affinity of an agonist will be different in the intact state to that measured when experimental conditions are used which eliminate one pathway while studying the other. Ligands acting with higher affinity for the inactive state over both active states are inverse agonists through both pathways and are governed by the same rules. Changes in receptor affinity measured, according to this model, can be due to differences in the assay system, the response measured or the level of constitutive activity of the receptors, and therefore do not necessarily indicate an atypical receptor subtype. In other words, this model provides an explanation of how differences in agonist or inverse agonist affinity may not only indicate different receptor subtypes, but different G-protein coupling i.e. different receptor states of a single receptor population.

The main argument against this explanation is that in a system where the active state of the receptor is predominant there should be a measurable response in the absence of agonist. Although no increase in basal tone is observed upon incubation with antagonists, the experimental conditions do reduce the possibility that increased basal tone can be measured. The low levels of Ca^{2+} and increased levels of Mg^{2+}, which are used to reduce the incidence of spontaneous contraction, may mask any small changes in basal tension. In this tissue, it is

possible that an increased incidence of R* causes an increase in the occurrence of spontaneous contractions, rather than an increase in sustained tone, and that the experimental conditions prevent this from being observed.

There are other models which can predict that G-protein coupling efficiency can also influence the measured affinity of an agonist (Krumins & Barber, 1997). Krumins and Barber have used a restricted version of the ternary complex model to explain differences in the rate of breakdown of the ternary complex for different β_2-adrenoceptor agonists. They concluded that the G-protein interaction does affect the measured agonist affinity and that receptor activation of the effector system is more complicated than agonist stabilisation of the active receptor conformation. Their findings may in theory be applied to inverse agonism, but further mathematics and modelling studies would need to be performed before this could be declared conclusively.

So bearing these models in mind, if M_2-mACh receptors in the uterus cause direct contraction, potentially by coupling to $G_{q/11}$ and hence the phosphoinositide pathway, an atypical operational profile may be expected if the antagonists used were in fact inverse agonists, and the ratio of R* to R was high. In contrast, affinity estimates made using radioligand binding in membrane preparations would result in typical M_2-mACh receptor values, since the estimates of affinity are made independent of activity. The assay conditions for radioligand binding are different to those used in the intact smooth muscle preparation. The addition of high NaCl and $MgCl_2$ into the binding assay could cause changes in coupling of the receptor, but commonly are used in measurements of binding affinity of a ligand to a receptor. Unfortunately, this conclusion could not be tested by the pharmacological characterisation of the phophoinositide response due to the small agonist-induced increase in [^{3}H]-inositol phosphate accumulation. It is unknown whether the responses observed here are different from those reported by others, because in previous work where total inositol phosphate accumulation has been characterised in guinea-pig

uterus e.g.Leiber *et al.*, (1990) the response has been normalised and the size of response not stated.

Whilst activation of PLC and inhibition of adenylyl cyclase have been used extensively to observe activation of mACh receptors, it should not be forgotten that a number of other methods of signalling can be utilised by this receptor class, not least via the direct regulation of a number of ion channels. mACh receptor subtypes m2 and m4 have been shown to interact with an inwardly rectifying K^+ channel in cardiac myocytes (GIRK1) (Kubo *et al.*, 1993; Kurachi, 1995), with large conductance Ca^{2+}-dependent K^+ channels (BK_{Ca}) in many tissues including smooth muscle (Cole & Sanders, 1989; Kume & Kotlikoff, 1991), and with non-specific cation channels in smooth muscle tissues (Benham *et al.*, 1985). The importance of mACh receptor activated ion conductance in uterine smooth muscle must not be overlooked. The non-selective cation channel activated by acetylcholine in mammalian smooth muscle cells first described by (Benham *et al.*, 1985) has since been shown to be directly activated via a G-protein (Inoue & Isenberg, 1990; Komori *et al.*, 1992) and this G-protein activation modulates voltage-dependence as well as gating of these channels (Zholos & Bolton, 1994). It has been shown that the activation of these channels results in depolarisation of the smooth muscle cell membrane (mainly due to Na^+ entry) giving rise to an increase in action potential discharge, and hence Ca^{2+} entry via voltage-sensitive Ca^{2+} channels (Inoue *et al.*, 1987). The release of Ca^{2+} from intracellular stores can be shown to potentiate this response (Pacaud & Bolton, 1991). Therefore, it can be said that these G-protein linked channels may have a profound influence on the degree of contraction induced by muscarinic receptor activation in smooth muscle cells. It has been shown that activation of these channels is pertussis toxin-sensitive (Komori *et al.*, 1992), and it has been proposed that the activation of such non-selective cation channels is due to coupling of the M_2-mACh subtype to G_i and that co-stimulation of the M_3-mACh receptor subtype causes a potentiation of this response (Zholos & Bolton, 1997). Therefore, it is possible this channel activation is another mechanism by which M_2-mACh receptors may cause contraction directly. If this was the case, it might be expected that the contraction would be sensitive to pertussis toxin.

However it has been shown that muscarinic atrial K^+ channels and some Ca^{2+} channels can be activated by βγ subunits of not only $G_{i/o}$, but also other types of G-proteins (Ikeda, 1996; Logothetis *et al.*, 1987; Takao *et al.*, 1994; Wickman *et al.*, 1994). Activation by these G-protein subunits and not $G_i\alpha$-GTP has not been ruled out for the non-specific cation channel. This means that activation of the channel by M_2-mACh receptors in the uterus need not necessarily be through the G_i family of G-proteins. Therefore pertussis toxin insensitivity and an atypical antagonist profile for contraction would not be unexpected due to potential receptor promiscuity as described above. Further investigations must be carried out to determine whether this channel exists in uterine smooth muscle, and whether it may be involved in contraction of this smooth muscle via M_2-mACh receptors or an atypical mACh receptor.

Emerging evidence suggests that G-protein βγ-subunits also regulate effector systems including isoforms of phospholipase C in recombinant cell systems (Camps *et al.*, 1992; Katz *et al.*, 1992). Earlier evidence suggested that the phosphoinositide-specific phospholipase C-γ family of phospholipases is activated by receptor complex formation and tyrosine phosphorylation (for review see Rhee, 1992), whilst the phosphoinositide-specific PLC-β family is activated by interaction with Gα-GTP subunits of the pertussis toxin-insensitive G_q class of G-proteins (Smrcka *et al.*, 1991; Taylor *et al.*, 1991). A further mode of activation of PLC enzymes was sought given that in some cases certain receptors can activate phosphoinositide signalling pathways in a pertussis toxin-sensitive manner (Ashkenazi *et al.*, 1987; Dell'Acqua *et al.*, 1993; Schmidt *et al.*, 1995; Vogel *et al.*, 1995) (for reviews see Meldrum *et al.*, 1991; Richards, 1991). More recent evidence has shown that this activation may be via βγ-subunits of G-proteins and that these interactions may be specific for each subtype of PLC-β (Camps *et al.*, 1992; Carozzi *et al.*, 1993; Katz *et al.*, 1992). AlF_4^- converts $G_{q/11}$ G-proteins to their active form by interacting with the inactive GDP-bound forms of the monomeric as well as the heterotrimeric forms and hence activates PLC, and this effect of AlF_4^- can be potentiated by βγ subunits (Boyer *et al.*, 1989). Tomura *et al.*, (1997) have shown in model cells that βγ subunits originating from pertussis toxin-

sensitive G-proteins can activate phospholipase C alongside other signalling pathways activated by the $G_i\alpha$ subunit, and coactivation of other G-proteins by another receptor type can potentiate this response, presumably by increasing available $\beta\gamma$ subunits. It is possible that a bifurcation of signal transduction via the M_2-mACh receptor is adequate to cause activation of these effectors in uterus, despite the high levels of free $\beta\gamma$-subunits which are likely to be required (Smrcka & Sternweis, 1993). This theory would however be dependent on the $\beta\gamma$ subunits being derived from pertussis-toxin insensitive G-proteins as the contraction observed is also pertussis toxin-insensitive. Nonetheless it remains a possible theoretical mechanism for activation of multiple signalling pathways via a single receptor population.

Therefore, in conclusion the mACh receptors in oestrogen-dominant guinea-pig uterus display an atypical pharmacological profile when examined functionally, but display classical M_2-mACh receptor pharmacology when characterised using a radioligand binding approach. This could be due to the existence of a small population of atypical mACh receptors that mediate carbachol-induced contraction, but which cannot be distinguished using competitive radioligand binding studies. Alternatively, the atypical operational profile observed may be explained by the promiscuous coupling of M_2-mACh receptors to pertussis toxin-insensitive G-proteins activating phosphoinositide-specific phospholipases C or a receptor-operated non-specific cation channel, in order to cause contraction directly.

CHAPTER 7 - Concluding discussion

Much remains unknown about the function of M_2-mACh receptors in smooth muscle contraction. This receptor-subtype is widely expressed in smooth muscle tissues of most mammalian species but debate continues as to whether M_2-mACh receptors can activate contraction directly, or whether contraction of smooth muscle is mediated solely by activation of the M_3-mACh receptor population. Although appearing disparate at first glance, the work in uterus and that in a model cell-line have both, in their own way, rendered important information about the possible role(s) of M_2-mACh receptors in smooth muscle, and have thus enabled evaluation of its importance. The main findings of this Thesis, the questions which were answered and those subsequently raised, and further work which could continue from this Thesis are summarised below:

Can M_2/M_3-selective antagonists be used to quantify M_2- and M_3-mACh populations in a mixed mACh receptor population?

In the Aims of this Thesis, I stated that experiments to determine the specificity of so-called 'M_2/M_3-mACh receptor-selective' antagonists would indicate whether competition binding methods could be used to quantify M_2- and M_3-mACh receptors in a mixed population. This quantification was crucial to the correct planning and interpretation of the subsequent work, and the full characterisation of newly created co-expressing cell-lines. Previous investigations into the function of mACh receptors have been hindered by the lack of available subtype-selective ligands. Obviously, studies of cross-talk between receptors from different families are much simplified by the use of two agonists that can specifically target and activate one, and not the other, receptor type of interest. No M_2/M_3-mACh receptor subtype-specific agonists are available, however it was thought that some mACh receptor antagonists may offer adequate subtype selectivity for the purposes of these studies. Indeed, when the concentration of methoctramine was increased in the presence of a constant concentration of a known radiolabelled ligand, it was shown that methoctramine

discriminated sufficiently between M_2- and M_3-mACh receptors, in mixed membrane preparations (CHO-M2 and CHO-M3), to produce a biphasic displacement curve. From this binding curve, the fractions of binding sites which make up the low affinity and high affinity sites were estimated and compared to those expected according to the proportions in which the membranes from the two cell-lines were mixed. The IC_{50} measurements for each part of the curve were compared with affinity estimates made at the homogeneous mACh receptor populations. Perhaps most remarkable was the way in which actual competition binding data curves resembled theoretical curves, constructed using known relative percentages of the two receptor subtypes, and the affinity estimates made for methoctramine at homogeneous populations of these receptors in the same cell-type. The similarity of the data to the best possible predicted outcome showed that this antagonist would be invaluable for the discrimination of M_2- and M_3-mACh receptor subtypes, and by using bovine tracheal smooth muscle preparations it was shown that it could also be used to differentiate these receptors in native tissues. The use of tripitramine, which only became commercially available after the initial studies with methoctramine had been performed, further improved upon the discrimination seen using methoctramine. Consequently, not only did the combination of saturation and competition binding techniques become a relatively simple way to quantify mACh receptor subtype expression, but also it was observed that tripitramine can be used, over a specific range of concentrations, to block M_2- and not M_3-mACh receptors. In this way, the expectations for this part of the Thesis were actually surpassed.

What are the signalling properties of mACh receptors in homogeneous populations in CHO cells?

The signalling pathways of M_2- and M_3-mACh receptors were investigated in homogeneous populations of receptors in CHO cells. As expected, inhibition of forskolin-stimulated cAMP was observed on stimulation of CHO-SLM2 cells with methacholine, but no phosphoinositide hydrolysis could be observed by measurements of total inositol phosphate

accumulation, $InsP_3$ mass measurement or CMP-PA accumulation. The inhibition of adenylyl cyclase was blocked completely by pre-treatment with tripitramine (30nM), but only partially inhibited by pre-incubation with pertussis toxin. This indicated that although the inhibition is mediated by M_2-mACh receptors alone, these receptors are acting to reduce cAMP production via more than one G-protein at this receptor density, in this cell-line. This observation is in agreement with conclusions by Burford & Nahorski, (1996) that the coupling of M_2-mACh receptors to pertussis toxin-sensitive or -insensitive G-proteins is dependent on receptor expression levels in this cell-type, and that it must not be assumed that M_2-mACh receptors always signal via the G_i G-protein family alone. This fact is important, given the apparent agonist-stimulated increase in CMP-PA accumulation in cells from the first transfection, in which no M_3-mACh receptors could be detected by immunological means, as discussed below.

In CHO-M3 cells, a typical profile of $InsP_3$ production, in terms of concentration-dependence and time-course, was observed upon stimulation with methacholine. Agonist-induced increases in total inositol phosphates and CMP-PA were also measured in the presence of Li^+, and profiles of activation were as expected for this cell-line. An increase in accumulation of CMP-PA, in the presence of Li^+, could also be measured after 20 min incubation with methacholine (1 mM) in CHO-VT-9 cells, which express a considerably lower density of M_3-mACh receptors at the cell surface than CHO-M3 cells. Application of methacholine caused stimulation of adenylyl cyclase activity in CHO-M3 cells, which was unaffected by either pertussis toxin or tripitramine (30nM) pre-treatment. This is in direct contrast to experiments in the same cell-line where pertussis toxin pre-treatment resulted in a potentiation of the agonist-stimulated increase in cAMP levels (Burford *et al.*, 1995). Both treatments also had no statistically significant effect on the concentration-dependence or time-course of $InsP_3$ production in CHO-M3 cells. This suggests that both adenylyl cyclase and phosphoinositide signalling pathways are activated by M_3-mACh receptors via pertussis toxin-insensitive G-proteins. The phosphoinositide pathway is activated by $G_q\alpha$ and adenylyl cyclase stimulation is probably caused by promiscuous coupling of M_3-mACh

receptors to G_s G-proteins, as for M_1-mACh receptor-stimulated adenylyl cyclase activity in CHO-M1 cells (Burford & Nahorski, 1996).

Can a successful cell model of M_2/M_3-mACh receptor co-expression be achieved?

Initial efforts to use measurements of CMP-PA accumulation and cAMP inhibition to screen transfectants were not successful. Increases in CMP-PA, upon stimulation with methacholine, were measured in most of the new clones. This was despite no signal for M_3-mACh receptors being detectable in crude membrane preparations of these cells by Western blot analysis using a highly specific and high affinity antibody for M_3-mACh receptors. Nor could a biphasic competition binding curve be observed using tripitramine as the competing agent, for these cell-lines. This could be because M_3-mACh receptors were expressed at levels so low that they were not detectable by the antibody, and could not be discerned in the competition binding assay due to the small size of this population, which is of lower affinity than the major mACh receptor population with respect to the competing ligand, in this case tripitramine. Alternatively, there were no M_3-mACh receptors expressed in the cell-lines and the CMP-PA response was due to promiscuous coupling of M_2-mACh receptors to phospholipase C. This latter option seemed most likely, given that full concentration-response curves for these clones, measuring CMP-PA accumulation ($+Li^+$), lie considerably to the right of that for CHO-M3 cells, and that it was also shown by the use of pertussis toxin pretreament that M_2-mACh receptors may couple to G-proteins other than those from the G_i family when expressed at this density (see above).

Western blotting was therefore used to screen the clones resulting from the second round of transfection. In this case, clones positive for M_3-mACh receptor antibody binding also gave clear biphasic competition binding curves with tripitramine as the competing agent. There are important points to emphasise from the failure of the first transfection to produce clones expressing M_3-mACh receptors at levels equal to those seen in smooth muscle tissues.

Firstly, it is important to titre the selection antibiotic carefully to optimise the selecting conditions to the particular population of cells to be used, and secondly, it is not ideal to use measurements of intracellular signalling as the primary method of screening. The selecting agent, in this case hygromycin, allowed growth of transfected clones only within a narrow concentration range. At concentrations higher than this total cell death occurred, even in those cells successfully expressing the selection marker, however at lower concentrations even those cells not expressing this marker may have survived the selection. It cannot therefore be stressed strongly enough that construction of detailed 'kill curve' data will dramatically improve the selection of clones which are 'true positives' from a population of transfected cells. This process should be performed before every transfection, regardless of whether the transfection vector has been used with this cell-type previously, as different populations of cells (e.g. number of passages, or different batch of stored cells) may have different resistance to the selecting agent. This procedure was not performed prior to the first transfection in this case, and the concentration of hygromycin used was not sufficient to provide truly selecting conditions. This problem was compounded by the fact that the first round of the screening process did not adequately indicate 'false positives'. It would seem at first glance that an obvious distinguishing characteristic between M_2- and M_3-mACh receptors is the different pathway through which each signals, however, this has only been shown in homogeneous populations. Nothing was known about the way in which co-transfection or sub-cloning of the initial parent cell-line would affect the interaction of the receptors with signalling pathways. In retrospect, since the very nature of the aims of the Thesis were to investigate what effect co-transfection of M_2- and M_3-mACh receptors would have on mACh receptor signalling, it was not a good idea to use measurement of increases in second messenger molecules as an indication of successful transfection. If immunological methods had been used to indicate M_3-mACh expression from the start, cells that had survived the selection by hygromycin but did not express M_3-mACh receptors at the cell membrane would have been identified at an earlier stage before complex and time-consuming binding studies were carried out. The entire screening process would therefore have been more efficient, despite the fact that Western blotting is a more demanding 'primary screen'

than one-point CMP-PA assays, and full characterisation could have gone ahead at an earlier stage in the project.

The round of second transfection did produce cells that co-expressed M_3-mACh receptors and M_2-mACh receptors. Using a combination of saturation and competition binding analyses using [^{3}H]-NMS and tripitramine, several clones expressing physiologically relevant levels of M_3-mACh receptors, whilst maintaining expression of M_2-mACh receptors at a level equal to that in the parent cell-line, were selected for further investigation. It was shown that the peak $InsP_3$ response to methacholine was not necessarily proportional to M_3-mACh receptor number in the cell-lines, and that not only did the new cell-lines represent a range of expression levels of M_3-mACh receptors, but perhaps also varying levels of receptor coupling efficiency.

How does co-expression of M_2- and M_3-mACh receptors affect mACh receptor signalling pathways in these cells?

Very interesting results were observed in one newly created cell-line (B3), which expressed a ratio of approximately 50:50% M_2:M_3-mACh receptors. It was shown that the profile of $InsP_3$ production with time upon methacholine stimulation was statistically significantly different from the response in CHO-M3 cells. Although maximum peak levels of $InsP_3$ were of similar concentration dependence and magnitude to those seen upon stimulation of CHO-M3 cells, the second phase of $InsP_3$ production was not a plateau in these co-expressing cells. Rather, the production of $InsP_3$ began to increase once more, and was still increasing after 20 min of stimulation. This was an exciting finding, and it was hypothesised that this increase could represent a cross-talk event between M_2- and M_3-mACh receptors, and therefore a possible role for M_2-mACh receptors of potential importance. This enhancement of the $InsP_3$ response was shown to be sensitive not only to pertussis toxin pre-treatment, but also to the presence of tripitramine (30 nM). This finding suggests that M_2-mACh receptors act via G_i-type G-proteins to cause an increase in the sustained phase of agonist-

stimulated phosphoinositide hydrolysis in this cell-line during concurrent M_3-mACh receptor stimulation.

The most important question to result from these studies is, does M_2-mACh receptor co-expression alter Ca^{2+}-signalling events in this cell-line? In order to determine a possible physiological outcome of the augmentation of phosphoinositide hydrolysis by M_2-mACh receptors, it is important to determine the down-stream consequences of this enhancement. Can a similar potentiation of M_3-mACh receptor-induced Ca^{2+}-release by M_2-mACh receptors be observed? Augmentation by opioid receptors of Ca^{2+} signalling induced by mACh receptor-activation has been described by Connor and Henderson (1994). It has been proposed that a universal cross-talk mechanism exists between G_i and G_q G-proteins which accounts for this observation, so perhaps similar down-stream events would be expected to result from the cross-talk between M_2- and M_3-mACh receptors as from the cross-talk between opioid and mACh receptors. It would appear from preliminary results using single cell Ca^{2+}-imaging, presented in this Thesis, that an obvious enhancement of Ca^{2+}-signalling compared to M_3-mACh receptors in these cells is not apparent, and the Ca^{2+} response to MCh-stimulation appears to be completely PTx-insensitive. However, there does appear to be some effect of co-expression on the time-course of Ca^{2+}-release, which warrants further examination. Care must be taken in the design of more complex Ca^{2+} imaging experiments using these cells, as CHO cells cannot be used for long periods of time in Ca^{2+}-imaging or fluorimetric studies since Fura-2 and other fluorencent dyes leak relatively rapidly from the cells.

Further control experiments need to be performed to establish whether any increases in phosphoinositide hydrolysis are observed if the M_3-mACh receptor population is selectively removed, leaving the M_2-mACh receptor population intact. This would show whether M_2-mACh receptors are able to influence this signalling pathway in this cell-line, without the need for concurrent stimulation of the pathway by M_3-mACh receptors. Connor & Henderson, (1996) observed, in SH-SY5Y cells, an additional increase in agonist-stimulated

increases in $[Ca^{2+}]_i$, if mACh receptors and opioid receptors were stimulated concurrently, compared to stimulation caused by muscarinic agonists alone. They measured no increase in $[Ca^{2+}]_i$ when opioid receptors alone were stimulated. It is likely that this will be the case in the present study, in that M_2-mACh receptors are unlikely to be able to activate phosphoinositide hydrolysis alone, as they cannot do so when expressed as a homogeneous population at the same density in the same cell type. The experiments to test this are reasonably complex due to the complete lack of selective muscarinic agonists. In order to isolate M_2-mACh receptors in this cell-line, M_3-mACh receptors could be inactivated by as selective an alkylating agent as possible, e.g. 4-DAMP mustard, whilst M_2-mACh receptors are 'protected' from this alkylation by concurrent incubation with tripitramine at a M_2-selective concentration. Extensive washing should then leave an isolated and intact M_2-mACh receptor population. This technique of 'selective alkylation' has been successfully employed in uterine isolated smooth muscle preparations in this Thesis and many other studies using isolated smooth muscle preparations (Eglen *et al.*, 1994). It should be used to demonstrate whether M_2-mACh receptors affect phosphoinositide signalling solely by their influence on the signalling of M_3-mACh receptors via this pathway, in the same cell.

Connor & Henderson (1996) were able to show that potentiation by opioid receptor activation of mACh receptor-induced $[Ca^{2+}]_i$ increases were independent of the concentration of methacholine used to stimulate mACh receptors, in SH-SY5Y cells. Unfortunately, it is not possible to demonstrate in this way that the measured effect on $InsP_3$ plateau levels produced by M_2-mACh receptor stimulation is independent of the level of stimulation of M_3-mACh receptors, since both receptor subtypes are activated by the same agonist. Another more complex way to approach this, may be by the graded removal of M_3-mACh receptors by alkylation/protection procedures. The M_3-mACh receptor population could be alkylated in steps, whilst the M_2-mACh receptors are protected by co-incubation with methoctramine, as described above. By varying the M_3-mACh receptor number but stimulating with the same concentration of methacholine, it may be possible to see whether the M_2-mACh receptor-induced potentiation is affected by altering M_3-mACh receptor number, or is independent of

the level of M_3-mACh receptor stimulation. Obviously, this is not as clear cut as using selective agonists, but may offer more information about the interaction between M_2- and M_3-mACh receptors.

What are the mechanisms underlying M_2/M_3-mACh receptor cross-talk?

The findings discussed so far indicate that M_2-mACh receptors can act via G_i-proteins to modulate phosphoinositide hydrolysis, a second messenger system not usually thought to be associated with this receptor subtype or this G-protein interaction. Since $G_i\alpha$ G-protein subunits cannot stimulate PLC, it is more likely that M_2-mACh receptors are having this effect on phosphoinositide signalling via βγ-subunits derived from G_i heterotrimers. Although βγ-subunits alone produce only a small increase in PLC or adenylyl cyclase activity in general, this kind of potentiation of effector activation by βγ-subunits could be a way in which receptors signalling through G_i G-proteins act as universal modulators of G_q- and G_s-linked signalling. This kind of effector activation by βγ-subunits has been suggested previously by many groups, for example by Tang and Gilman (1991) to explain how receptors acting via G_i G-proteins may act synergistically with receptors coupled to G_s G-proteins to increase adenylyl cyclase activation; by Gerwins and Fredholm (1992) to illustrate how adenosine A1 receptors increase stimulation of $InsP_3$ production and Ca^{2+} release mediated by bradykinin receptors; by Connor and Henderson (1996) to explain how opioid receptor activation may potentiate intracellular Ca^{2+} release by muscarinic receptors; and by Dickenson and Hill (1996) to explain the synergy between adenosine A1 and cholecystokinin receptor activation of increases in total inositol phosphate accumulation. Tomura *et al.* (1997) have suggested that adenosine A1 receptors increase thyrotropin-mediated activation of PLC via βγ-subunits, but propose that this may not be by the direct stimulation of PLC, but rather by increasing the formation of G_q heterotrimers which can interact with TSH receptors. Boyer *et al.* (1989) have shown directly that in turkey erythrocyte membranes, the stimulation of PLC by P_{2Y} purinoceptors could be stimulated by reconstitution with βγ-subunits, but the stimulation of PLC activity by AlF_4^- could not be

potentiated in this manner. This perhaps supports the proposal that it is an interaction at the receptor-G-protein level and not the stimulation of effector by G-protein α and βγ-subunits, which allows the synergistic activation of PLC by receptors coupling to G_q and G_i G-proteins. Further experiments need to be carried out to see whether the M_2-mACh receptor enhancement of M_3-mACh receptor activation of phosphoinositide hydrolysis is via βγ-subunits from G_i G-proteins. Perhaps reconstitution experiments using membrane preparations of this cell-line and βγ-subunits may be appropriate, but these types of experiments need to be interpreted with care, since the usual cellular environment of the receptors is compromised by the use of membranes, and factors important to the mechanism of cross-talk may be lost. Further studies of agonist stimulation of intracellular Ca^{2+} increases are necessary to establish whether the potentiation of $InsP_3$ production has any effect on the Ca^{2+} signalling of the cells, or whether the ultimate effect of this potentiation is on protein kinase cascades stimulated by concurrent increases in DAG. The Ca^{2+} imaging studies undertaken in this study are very preliminary, and need to be expanded to address this issue.

The other most immediately obvious way in which M_2-mACh receptors could influence phosphoinositide signalling is via non-selective cation channels. It is a method of cross-talk which could explain very simply the data presented here. M_2-mACh receptors have long been known to activate non-selective cation channels via mechanisms involving pertussis toxin-sensitive G-proteins. Also, it is a well established fact that the plateau phase of $InsP_3$ production is dependent on Ca^{2+} entry across the plasma membrane. The activation of non-selective cation channels may increase the likelihood of Ca^{2+}-channel opening, and therefore increase Ca^{2+} entry. Therefore, it is tempting to connect the two events and conclude that M_2-mACh receptors may increase the second phase of $InsP_3$ production by their ability to activate non-selective cation channels and thereby provide a route for Ca^{2+}-influx across the membrane. This mechanism could also explain why the plateau phase of $InsP_3$ is potentiated by M_2-mACh receptor co-expression, whilst the peak phase remains unaffected. Since this sustained phase of $InsP_3$ production is thought to be more important in the activation of

protein kinase cascades, via concurrent production of DAG, than the initiation of Ca^{2+} release from intracellular stores, it can be speculated that the role of M_2-mACh receptors in smooth muscle might lie in maintenance of contraction, rather than in its rapid initiation. However, whilst production of DAG from this source may be important at relatively early time points, it is more usual for the most important source of DAG at later time points to be from phosphatidylcholine hydrolysis by phosphatidyl choline-preferring PLCs. It would be interesting to observe what contribution this pathway has to DAG formation at the later time-points at which enhancement of DAG production by phosphoinositide hydrolysis occurs.

Experiments altering external Ca^{2+} concentration would appear to be the most simple way to ascertain whether the enhancement of phosphoinositide hydrolysis by M_2-mACh receptors is dependent on Ca^{2+} entry. However, if measurements of $InsP_3$ production are carried out in nominally Ca^{2+}-free solutions little useful information will will gained, as the plateau phase of M_3-mACh receptor induced $InsP_3$ production is dependent on Ca^{2+} entry. It is expected that M_2-mACh receptor-stimulation of PLC is dependent on concurrent M_3-mACh receptor activation in this cell-line and therefore removing the ability of M_3-mACh receptors to cause a sustained increase in $InsP_3$ could also affect M_2-mACh receptor effects indirectly i.e. decreases in the augmentation of the second phase of $InsP_3$ production may not be directly caused by decreases in $[Ca^{2+}]_e$, but may be due to the decrease in the ability of M_3-mACh to induce $InsP_3$ production at these time-points. This is also true if a cocktail of Ca^{2+} entry blockers were used. Perhaps the first and only obvious first step to assess whether M_2-mACh receptor-mediated augmentation of PLC is dependent on Ca^{2+}-entry would be to see whether a mACh receptor-activated non-selective cation current can be measured, using electrophysiological methods, which is pertussis toxin-sensitive and blocked by pre-incubation with tripitramine at 30 nM, in this cell-line. It may be that there is no significant M_2-mACh receptor-induced ionic current at all, which would rule out this explanation. If such a current can be measured, then it may be difficult to link definitively activation of the channel by M_2-mACh receptors to potentiation of $InsP_3$ production by M_3-mACh receptors, but may offer an explanation if other methods of cross-talk can be ruled out.

Another important issue which needs to be addressed is, does this potentiation of $InsP_3$ signal occur at lower concentrations of methacholine? In the current experiments, a maximal concentration of MCh has been used and $InsP_3$ production measured with time. Experiments using lower concentrations of MCh need to be performed. Perhaps $InsP_3$ could be measured at 10 min for a variety of MCh concentrations to allow potency estimations for MCh for the enhancement of the second phase of $InsP_3$ production. This estimate of potency could then be compared to those estimates for other effects, such as MCh-induced increases in $[Ca^{2+}]_i$. It may be possible to rule out, for example Ca^{2+}-influx induced by M_2-mACh receptor-activation of non-selective cation channels as a mechanism, if there are discrepancies between the ability of MCh to cause channel opening and its ability to potentiate $InsP_3$ production caused by M_3-mACh receptor activation.

Some of the work which is necessary for better interpretation of the data reported in this Thesis is currently being carried out within this laboratory. Western blotting techniques using newly available specific antibodies are being used to discern the isotypes of PLC and confirm the families of G-proteins involved in mACh receptor signalling in CHO cells. This could also be done in isolated smooth muscle preparations so that a comparison between the effectors and G-proteins involved in mACh receptor signalling in CHO cells and at least one M_2/M_3-mACh receptor co-expressing smooth muscle could be made. This may help to add credance to this model as a good one for investigating mACh receptor signalling in smooth muscle.

Is the model cell-line a good model for smooth muscle mACh receptor signalling?

Other concerns about the interpretation of the model cell data are whether the mACh receptor expression levels are 'physiological' and whether this cross-talk can occur at different levels of M_2- and M_3-mACh receptor expression. Previously in this Chapter, I have described how

progressive alkylation of M_3-mACh receptors might be employed to investigate the relationship betwen M_2- and M_3-mACh receptor signalling in the clone B3. Another important approach to this is to reassess other clones from the second transfection process, to see whether cross-talk between M_2- and M_3-mACh receptors can also be demonstrated in other clones with different mACh receptor subtype expression levels to Clone B3. The second round of transfection did result in a large number of clones in which M_3-mACh receptors could be detected using Western blotting techniques. Although this approach is not quantitative, it would appear from the intensity of the bands representing M_3-mACh receptor that there are varying M_3-mACh receptor expression levels in the cell-lines produced. Further characterisation by binding studies, as before, and of signalling, should be carried out in more clones. Signalling data may confirm whether the cross-talk described here is an anomaly observed for this cell-line alone, or hopefully that it occurs at different levels of mACh receptor expression.

If cross-talk does occur in smooth muscles where M_2- and M_3-mACh receptors are co-expressed, cholinergic activation will always result in this cross-talk and the M_2-mACh receptor will always modulate M_3-mACh receptor-induced signalling. The endogenous ligand, acetylcholine, cannot distinguish between the receptor subtypes, therefore on its release it stimulates both receptor subtypes. M_2-mACh receptors cannot therefore initiate occasional modulation under specific circumstances, but must be intrinsic to the very nature of mACh receptor-induced contraction of M_2/M_3-mACh receptor co-expressing smooth muscle tissues.

What contribution do M_2-mACh receptors make to carbachol-induced contraction of guinea-pig isolated uterus?

Given previous evidence, it was expected that the guinea-pig uterus may be one of the few smooth muscle tissues in which M_3-mACh receptors did not initiate muscarinic agonist-induced contraction. It has been shown by Northern blotting techniques that only m2- and

perhaps m4-mACh receptor mRNA can be detected in this tissue, and competition binding experiments can only disclose the presence of a homogeneous M_2-mACh receptor population. Studies of antagonist affinity have indicated that M_2-mACh receptors may be responsible for contraction, but proposals that it is mediated by M_3-mACh receptors, M_4-mACh receptors or a synergistic relationship between M_2- and M_3-mACh receptors have been put forward based on studies of contraction and of biochemical indicators of mACh receptor signalling in this tissue. In this present study, non-pregnant guinea-pigs were pretreated with oestrogen in order to normalise the effects of reproductive cycle, since oestrogens have been shown to affect mACh receptor expression. It was expected that M_2-mACh receptors may have a more dominant role in this tissue type than in other smooth muscles, and therefore could offer interesting clues as to the involvement of M_2-mACh receptors in mACh receptor-mediated contraction. Radioligand binding studies in this tissue using M_2/M_3-mACh receptor-selective antagonists indicated that the mACh receptor population in guinea-pig uterus was apparently homogeneous and consisted solely of M_2-mACh receptors. However, affinity estimations of the same antagonists, using Schild analysis of contractions induced by carbachol, indicated an atypical antagonist affinity profile. This atypical profile was not altered by a protocol which would selectively remove M_3-mACh receptors in a mixed population of M_2- and M_3-mACh receptors. This indicated that contraction was not brought about by a synergistic activation of contraction by M_2- and M_3-mACh receptors. Therefore, although only an M_2-mACh receptor population could be detected using radioligand binding studies, the affinity estimates for antagonists made against contraction indicated that it was mediated by an atypical mACh receptor. The contraction was insensitive to pertussis toxin pretreatment, indicating that the response was not mediated by G_i-Gproteins. It was discussed in Chapter 6 that the atypical affinity estimates could be due to M_2-mACh receptors coupling promiscuously to G_q G-proteins to cause contraction, which would alter the measured affinites of antagonists. Alternatively, there may be an atypical mACh receptor mediating this response which cannot be detected using radioligand binding techniques.

Is uterus an ideal tissue for the study of M_2-mACh receptor function as it relates to smooth muscle contraction?

Although the results from the guinea-pig uterus studies were very interesting in their own right, and again emphasised the usefulness of relatively new antagonists with improved M_2/M_3-mACh receptor selectivity, they did not contribute as much as was expected to the elucidation of the function of M_2-mACh receptors in smooth muscle. This study did however confirm that mACh receptor-induced contraction is not always mediated by M_3-mACh receptors. This is useful information, as it goes some way to disproving some claims that M_3-mACh receptors are the only mACh receptors involved in contraction and that M_2-mACh receptors have no role in increases in smooth muscle tone induced by muscarinic agonists.

Concluding remarks

In summary, it appears that M_2-mACh receptors may have a more important role in mACh receptor-induced contraction that has sometimes previously been suggested. I have created a cell-line in which M_2- and M_3-mACh receptors are co-expressed. Using these model cells, I have shown that M_2-mACh receptors can potentiate M_3-mACh receptor signalling via phosphoinositide hydrolysis. This may be due to cross-talk via $\beta\gamma$-subunits of G_i G-proteins or via activation by M_2-mACh receptors of non-selective cation channels which may increase Ca^{2+} influx across the plasma membrane, to increase PLC activation. I have also confirmed that M_3-mACh receptors may not be the universal mediator of contraction in smooth muscle tissues, since this subtype is not responsible for contraction in guinea-pig uterus, but rather an atypical mACh receptor, or M_2-mACh receptors may activate contraction via G_q G-proteins in this tissue. This work shows that M_2-mACh receptors may have functions fundamental to smooth muscle contraction, and perhaps indicates that the search for drugs for pathologies of smooth muscle should not solely centre around the quest for M_3-mACh receptor-selective ligands.

APPENDIX 1– Krebs-Henseleit buffered solution

HEPES buffer	10mM
NaCl	118 mM
$NaHCO_3$	25 mM
KCl	4.7 mM
$MgSO_4$	1.2 mM
KH_2PO_4	1.2 mM
$CaCl_2$	0.13 mM
Glucose	10 mM

pH 7.4

APPENDIX 2 - Western blotting gel composition and solutions

Solutions for gel preparation

H_2O

30% Acrylamide mix (stored at 4°C)

1.5 M TRIS (pH 8.8) (stored at R.T.)

1.0 M TRIS (pH 6.8) (stored at R.T.)

10% Sodium dodecyl sulphate (SDS) (stored at R.T. for no more than 1 month)

10% ammonium persulphate (APS) (stored at 4°C for no more than 1 week)

TEMED (N,N,N',N'- tetramethyl-ethlenediamine) (stored at 4°C)

Resolving gel for Tris-glycine SDS-polyacrylamide gel electrophoresis (10% w/v)

(All volumes in mls)

Gel cast volume	5 ml	10 ml	15 ml	20 ml	25 ml	30 ml	40 ml	50 ml
H_2O	1.9	4.0	5.9	7.9	9.9	11.9	15.9	19.8
30% acrylamide	1.7	3.3	5.0	6.7	8.3	10.0	13.3	16.7
1.5 M TRIS (pH 8.8)	1.3	2.5	3.8	5.0	6.3	7.5	10.0	12.5
10% SDS	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
10% APS	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
TEMED	0.002	0.004	0.006	0.008	0.01	0.012	0.016	0.02

Stacking gel for Tris-glycine SDS-polyacrylamide gel electrophoresis (5%)

(All volumes in mls)

Gel cast volume	1 ml	2 ml	3 ml	4 ml	5 ml	6 ml	8 ml	10 ml
H_2O	0.68	1.4	2.1	2.7	3.4	4.1	5.5	6.8
30% acrylamide	0.17	0.33	0.5	0.67	0.83	1.0	1.3	1.7
1.5 M TRIS (pH 8.8)	0.13	0.25	0.38	0.5	0.63	0.75	1.0	1.25
10% SDS	0.01	0.02	0.03	0.04	0.05	0.06	0.08	0.1
10% APS	0.01	0.02	0.03	0.04	0.05	0.06	0.08	0.1
TEMED	0.001	0.002	0.003	0.004	0.005	0.006	0.008	0.01

Buffers and solutions

Sample buffer (x2)

100mM Tris-HCl (pH6.8)

2% SDS

0.1% bromophenol blue

10% glycerol

Running buffer

25 mM Tris-base

250 mM Glycine

0.1% SDS

Blotting buffer

39 mM Glycine

48 mM Tris-base

0.037% SDS

20 % Methanol

pH 8.3

Stored at R.T.

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Characterization of an atypical muscarinic cholinoceptor mediating contraction of the guinea-pig isolated uterus

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1 In many smooth muscle tissues a minor M_3-muscarinic acetylcholine (mACh) receptor population mediates contraction, despite the presence of a larger M_2-mACh receptor population. However, this is not the case for guinea-pig uterus where radioligand binding and functional studies exclude a dominant role for M_3-mACh receptors.

2 Using tissue from animals pre-treated with diethylstilboestrol, estimates of antagonist affinity were made before and after selective alkylation procedures, together with estimates of agonist affinity to characterise the mACh receptor population mediating carbachol-induced contraction of guinea-pig isolated uterus.

3 Antagonist affinity estimates made at 'protected' receptors were not significantly different from those made in untreated tissues. However all estimations were significantly different from those reported in guinea-pig ileum and atria. The rank order of affinities were atropine > zamifenacin = tripitramine > methoctramine. Carbachol-induced contractions were insensitive to the M_4-selective muscarinic toxin MTx-3, or PD102807 (0.1 μM) ruling out a role for M_4-mACh receptors.

4 The agonist affinity value for L-660,863, a putative 'M_2-selective' agonist of 5.44 ± 0.30 ($n=6$) was significantly different from that reported in guinea-pig atria. In contrast, the pK_A value for carbachol (4.22 ± 0.17; $n=8$) agrees with that reported for guinea-pig ileum.

5 Carbachol-induced contractions were insensitive to pertussis toxin although carbachol-induced inhibition of forskolin-stimulated cyclic AMP production was attenuated, ruling out the involvement of G_i-proteins in contraction.

6 Radioligand binding studies revealed a K_D for N-[^{3}H]-methylscopolamine of 0.12 ± 0.05 nM and a B_{max} of 147 ± 18 fmol mg protein^{-1}. Antagonist affinity estimates made using competition binding studies supported previous data suggesting the presence of a homogenous population of M_2-mACh receptors.

7 These data suggest a small population of mACh receptors with an atypical operational profile which can not be distinguished using radioligand binding studies may mediate carbachol-induced contraction of guinea-pig isolated uterus.

Keywords: Muscarinic acetylcholine receptors; tripitramine; zamifenacin; L-660,863; selective alkylation; smooth muscle contraction; guinea-pig (uterus)

Introduction

Uterine muscle contracts rhythmically both *in vivo* and *in vitro*, and these contractions are intrinsic to the tissue, requiring no hormonal or neural inputs to maintain them (Wray, 1993). Nevertheless, smooth muscle contractility can also be modulated by a variety of neurotransmitters and hormones. The uterine body of many species including human, is extensively innervated by cholinergic neurones of the autonomic nervous system (Traurig & Papka, 1993; Tetsuro *et al.*, 1994) and this probably allows co-ordinate control of contraction and blood flow (Sato *et al.*, 1996). Evidence has been presented to show that the populations of muscarinic acetylcholine (mACh) receptors and adrenoceptors responding to post-ganglionic neurotransmitter release may change in response to hormonal changes, particularly in oestrogen levels, during the oestrus cycle (Riemer *et al.*, 1987; Varol *et al*, 1989; Arkinstall & Jones, 1990; Matucci *et al.*, 1996). Although it can be shown that mACh receptors which couple to phosphoinositide responses and Ca^{2+} mobilization are present in this tissue, a role for this family of receptors in uterine physiology has not been firmly established (Eglen *et al.*, 1994; Challiss & Blank, 1997).

In most smooth muscle tissues, M_2- and M_3-mACh receptor subtypes form the postjunctional mACh receptor population, and in the majority of these tissues it can be shown that M_3-mACh receptors initiate contraction, presumably *via* their coupling to the phosphoinositide cycle (Roffel *et al.*, 1990; Yang *et al.*, 1991; Mahesh *et al.*, 1992; Nahorski *et al.*, 1994). However, this may not be the case for uterine smooth muscle. In contrast to most smooth muscles, myometrial contraction may be mediated by a subtype other than the M_3-mACh receptor. Initial studies in isolated guinea-pig uterus suggesting that M_2-mACh receptors are responsible for contraction (Eglen *et al.*, 1989) were supported by subsequent functional studies using an extensive series of mACh receptor antagonists (Bognar *et al.*, 1992; Doods *et al.*, 1993). Alternative suggestions that M_2- and M_3-mACh receptors may be jointly responsible (Leiber *et al.*, 1990), or M_4-mACh receptors may mediate this response (Dörje *et al.*, 1990) have also been proposed. Although immunological methods have demonstrated the presence of M_2- and M_4-mACh receptors in rabbit

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uterus (Dörje *et al.*, 1991) and Northern blot analysis of guinea-pig uterine tissue revealed evidence for m2 and m4 mRNA transcripts (Eglen *et al.*, 1992) it is unclear whether such receptors can mediate a contractile response in myometrial tissue. Inhibition of adenylyl cyclase and stimulation of phosphoinositide-specific phospholipase C by mACh receptor agonists both can occur in guinea-pig isolated uterus, with the former being attributed to M_2- and the latter to M_3-mACh receptor activation (Marc *et al.*, 1988; Leiber *et al.*, 1990).

Due to the low receptor subtype selectivity of many of the agents used to characterize the uterine mACh receptor(s) in previous studies, and a lack of any clear conclusions, we have carried out an extensive characterization of the mACh receptor subtype(s) responsible for contraction in guinea-pig isolated uterine smooth muscle. We have used guinea-pigs pre-treated with a synthetic oestrogen in order to synchronize all animals to the oestrogen-dominant phase of the reproductive cycle. The possibility was addressed that the contraction of uterus is mediated by M_2- and M_3-mACh receptors, either alone or in a synergistic fashion, by using M_2/M_3-selective antagonists in conjunction with selective alkylation procedures, *in vitro*. A preliminary account of these data has been presented previously (Boxall *et al.*, 1997).

Methods

Animals and tissue preparation

Adult, female Dunkin Hartley guinea pigs were treated with diethylstilboestrol 0.1 mg kg^{-1} i.p. and killed by CO_2 asphyxiation 24 h later, or 48 h later if also treated with pertussis toxin (see below). Uterine horns were each cut into four longitudinal strips and mounted for contractile studies, or membranes were prepared for radioligand binding studies as described below.

Contractile studies

Uterine strips were suspended in 10 ml organ baths at an initial tension of 1 g in a modified Sund's solution (pH 7.4) containing 154 mM NaCl, 5.63 mM KCl 0.98 mM $MgCl_2$, 5.95 mM $NaHCO_3$, 0.48 mM $CaCl_2$ 2.78 mM glucose, 3 μM indomethacin, 1 μM tetrodotoxin, 30 μM cocaine and 30 μM corticosterone, at 32°C. This solution was constantly aerated with 95% O_2/5% CO_2, and contractile activity measured using an isometric force transducer. The low temperature and extracellular Ca^{2+} concentration served to reduce spontaneous contractility. The addition of tetrodotoxin prevented neuronal release of acetylcholine from intact nerve terminals thus excluding the involvement of neuronal nicotinic and muscarinic acetylcholine receptors. The use of indomethacin reduces cyclooxygenase activity and therefore synthesis of contractile eicosanoids.

Agonist additions were cumulative (0.5 log molar concns) and antagonist affinity estimates were obtained by Schild regression analysis (Arunlakshana & Schild, 1959) of carbachol-induced contractions. At least five concentrations of antagonist were used, which were equilibrated with the tissue for 1 h. This period was sufficient in each case to allow equilibration of the antagonist, as no further antagonism was seen when the incubation was increased to 2 h for each antagonist. Each uterine strip was exposed only to a single concentration of the antagonist.

Selective alkylation procedures were carried out in a similar manner to Thomas *et al.* (1993). Tissues were treated with 3 μM phenoxybenzamine for 20 min with or without prior 60 min equilibration with 0.1 μM methoctramine. The tissues were washed every 20 min in Sund's solution and antagonists replaced where necessary. Those tissues not treated with methoctramine acted as controls to observe alkylation of 'unprotected' receptors. Tissues were then washed every 5 min for 90 min to ensure that all antagonist was washed from the tissues. Responses to carbachol were not significantly different after incubation with methoctramine alone followed by this washout protocol from time-matched controls (results not shown). After washout of phenoxybenzamine and methoctramine a concentration curve to carbachol was constructed which was further characterized using a single concentration of antagonist after a period of 60 min, and pA_2 values calculated at these 'protected' receptors.

Partial receptor inactivation using alkylation procedures was used to estimate agonist affinities in this tissue. Agonist concentration-response curves were constructed, followed by incubation with 0.1 μM phenoxybenzamine for 20 min. Concentration-response curves were repeated and agonist affinities estimated.

To assess the effect of pertussis toxin pre-treatment, 24 h after diethylstilboestrol was injected, pertussis toxin (50 μg kg^{-1}) or vehicle was injected via the external jugular vein of the guinea pig. The venous cut down, injection and suture closure were carried out under ether anaesthesia and the guinea pigs allowed to recover under supervision. There were no gross adverse effects of pertussis toxin pre-treatment on the guinea pigs during the time between administration, recovery and sacrifice. The guinea pigs were killed in the same manner as before, 24 h after pertussis toxin or vehicle administration. Some uterine tissue was used for biochemical studies to measure the effects of pertussis toxin on inhibition of adenylyl cyclase (see below) while the rest was used for contractile studies as discussed above.

Measurement of adenylyl cyclase activity

Uteri from animals pretreated with diethylstilboestrol were dissected into inositol-free Ham's F12 medium at 37°C and strips were chopped using a McIlwain chopper into cubes. The cubes were washed three times with fresh medium and spun at 500 *g* for 5 min. The uterus tissue was then incubated with collagenase (1 mg ml^{-1}) at 37°C for 1 h with occasional shaking. The treated preparation was then passed through a cell sieve, washed thoroughly with medium and centrifuged as before. The medium was removed and the cells resuspended carefully in a known volume of fresh medium. The ATP pools of the cells were then labelled with 5 μCi ml^{-1} [^{3}H]-adenine for 1 h at 37°C. Cells were then washed and centrifuged as before. Antagonist or buffer was preincubated with 200 μl of [^{3}H]-adenine-labelled cell suspension (approx. 25 μg protein) for 20 min at 37°C and the following were then added simultaneously: carbachol (at various concentrations) or buffer, forskolin (10 μM) or buffer and 3-isobutyl-1-methylxanthine (IBMX; 0.5 mM). After 30 min incubation at 37°C the reaction was stopped by the addition of 2.2 M HCl, followed by mixing and placing in an ice-water bath. [^{3}H]-cyclic AMP was separated by column chromatographic methods as described by Daniels & Alvarez, (1992).

Measurement of inositol phosphate accumulation

Uteri from animals pretreated with diethylstilboestrol were dissected into Sund's solution at 37°C and roughly chopped using scissors. The tissue was cross-chopped (300 × 300 μM)

using a McIlwain chopper and then shaken in Sund's solution in a waterbath at 37°C. The tissue was washed with eight changes of buffer by allowing the slices to sediment and removing buffer and cell debris by suction. Slices were packed under gravity into a repeating pipette tip and 50 μl of slices were dispensed into insert vials. Throughout the experiment the vials were purged with O_2/CO_2 every 15 min and capped. Phospholipid pools were labelled using 5 μCi ml^{-1} [^{3}H]-inositol for 3 h at 37°C. Accumulation of label into the phospholipid pool was linear with time over 3 h and could be stimulated by agonist (results not shown). Lithium chloride (10 mM) was added to the slices 15 min prior to stimulation for 30 min with varying concentrations of carbachol. The reactions were stopped with trichloroacetic acid (1 M) and the vials were left on ice for 20 min before centrifugation at 1000 *g* for 10 min. The supernatant was extracted with 3 × 5 ml water-saturated diethylether. Total [^{3}H]-inositol phosphates were separated using column chromatographic methods using Dowex (Cl^--forms) columns as described previously (Challiss *et al.*, 1992).

Preparation of uterus membranes

For membrane preparations, guinea pigs were pre-treated with diethylstilboestrol and killed as described above, the uterine horns removed and roughly chopped with scissors into 20 ml ice-cold 10 mM HEPES, 10 mM EDTA, pH 7.4 and homogenized using a Polytron homogenizer for 10 bursts of 3 s at maximal speed on ice. The membranes were then centrifuged at 200 *g* for 5 min to remove large tissue fragments. The supernatant was spun at 40 000 *g* for 15 min at 4°C. The pellet was resuspended in a HEPES buffer (10 mM HEPES, 0.1 mM EDTA, pH 7.4) and homogenized and spun as before. The final membrane preparation was resuspended in HEPES buffer with 0.1 mM EDTA, protein concentration measured using the method of Lowry *et al.* (1951) and diluted to 1 mg ml^{-1}. Aliquots of membranes were snap frozen in liquid N_2 and stored at −80°C for later use.

Radioligand binding studies

For binding assays the buffer used contained 10 mM HEPES, 10 mM $MgCl_2$, 100 mM NaCl, pH 7.4 (buffer A). For saturation binding, 25 μg of uterus membranes were incubated with increasing concentrations of N-[^{3}H]-methylscopolamine ([^{3}H]-NMS) for 90 min at 37°C in 200 μl final volume of buffer. Incubations were performed in duplicate and the non-specific binding described using 1 μM atropine. The reactions were stopped by diluting with ice-cold buffer and vacuum filtering over GF/B Whatman glass filters using a Brandell cell harvester. Filters were washed with 3 × 5 ml buffer A and then air-dried and counted using liquid scintillation spectrophotometry. For displacement binding experiments, approximately 0.5 nM [^{3}H]-NMS was incubated at 37°C for 90 min with 25 μg uterine membranes and increasing concentrations of antagonists, to a final volume in buffer as before of 200 μl. At least 12 concentrations of each antagonist were used over six orders of magnitude. Non-specific binding was described as for saturation binding, and incubations were performed in duplicate. The incubation was terminated by rapid vacuum filtration and filters washed as previously described.

Data analysis

All data are presented as means ± s.e.mean for the number of experiments indicated. Where Schild slopes were not significantly different from one, slopes were constrained to one to calculate pK_B values. Agonist affinity values were calculated using simultaneous operational curve-fitting methods using SigmaPlot (Jandel Scientific, Corte Madera, CA, U.S.A.) as described by Wiener & Thalody (1993). In radioligand binding experiments, the equilibrium constant (K_D) for [^{3}H]-NMS and the B_{max} were estimated from curves generated by non-linear regression of saturation isotherms using GraphPad Prism (San Diego, CA, U.S.A.). Competition curves from three independent experiments for each antagonist were analysed separately using GraphPad Prism and curves for one and two binding sites were fitted to the data. The curve of best fit was used to estimate the IC_{50} for the antagonist (from which the association constant (K_i) could be derived according to Cheng & Prusoff, (1973) and the Hill coefficient (Dahlquist, 1978). Statistical significance was assessed by Student's *t*-test for unpaired observations.

Materials

[^{3}H]-adenine and N-[^{3}H]-methylscopolamine were purchased from Amersham Life Sciences (Little Chalfont, U.K.). Carbachol, diethylstilboestrol, collagenase, tetrodotoxin and pertussis toxin were purchased from Sigma Chemical Co. (Poole, Dorset, U.K.). Cocaine, corticosterone, indomethacin, phenoxybenzamine, methoctramine, atropine and zamifenacin were prepared in-house at Roche Bioscience (Palo Alto, CA, U.S.A.). Tripitramine was a kind gift from Dr C. Melchiorre (University of Bologna, Italy) or was purchased from Research Biochemicals International (Natick, MA., U.S.A.). Muscarinic toxin 3 (Mtx-3) isolated from venom of the green mamba (*Dendroaspis augusticeps*) was a kind gift from Dr E. Karlsson (Biomedical Centre, Uppsala, Sweden). PD102807 was generously provided by Dr R. Schwarz (Parke Davis).

Results

Diethylstilboestrol pre-treatment

Using histological preparations of uterus and vagina from diethylstilboestrol pre-treated animals, evidence of the oestrogen-dominant phase of the reproductive cycle could be observed. Endometrial proliferation and glandular activity of the uterus preparations and vaginal keratinization was evident, indicating that diethylstilboestrol pre-treatment leads to obvious morphological changes associated with oestrogen dominance (results not shown). This demonstrated that the reproductive cycles of the guinea-pigs were successfully synchronized.

Pharmacological characterization of contractile response

Carbachol produced a concentration-dependent increase in tension of uterine smooth muscle with a pEC_{50} value of 5.79 ± 0.05 (n = 12), and a maximal tension of 2–5 g in these tissues. This response was sensitive to muscarinic antagonists causing dextral shifts of the concentration-response curve in a parallel manner with no decrease in maximal tension for the concentrations of antagonist used, indicating that this antagonist was competitive (Figure 1). The Schild slopes were not significantly different from one ($P > 0.05$) and were thus constrained to one to estimate affinity values (pK_B) (Table 1). Contractions to carbachol were abolished by phenoxybenzamine (in the absence of methoctramine), but the response could be protected by prior equilibration with 0.1 μM methoctramine

followed by extensive washing (Figure 2). Under these conditions a rightward shift and small decrease (10±2%) in maximum response was observed. Antagonist affinities estimated at the 'protected' receptors using a single concentration of antagonist were not significantly different from estimations made in untreated tissues ($P>0.05$) (Table 1).

It was noted that for all antagonists, the affinities in both untreated tissues and at 'protected' receptors were significantly different from those estimations previously reported at M_2- and M_3-mACh receptors ($P<0.05$) (Table 1) (Caulfield, 1993; Chiarini *et al.*, 1995). The M_4-selective muscarinic toxin 3 (Mtx-3; Jolkkonen *et al.*, 1994) failed to have any effect on carbachol-induced contraction ($n=5$) suggesting that M_4-mACh receptors were not involved (Figure 3). Similar findings were obtained with PD102807 (Figure 4) and the small dextral shift observed (dose ratio = 5.46±0.87) was not significantly different to that seen with vehicle alone in control tissues (dose ratio = 3.56±0.27). Attempts to evoke a greater shift using PD102807 (1 μM) were unsuccessful due to the poor solubility of the compound and the consequent vehicle (DMSO) effects on the uterus preparations. Also, the

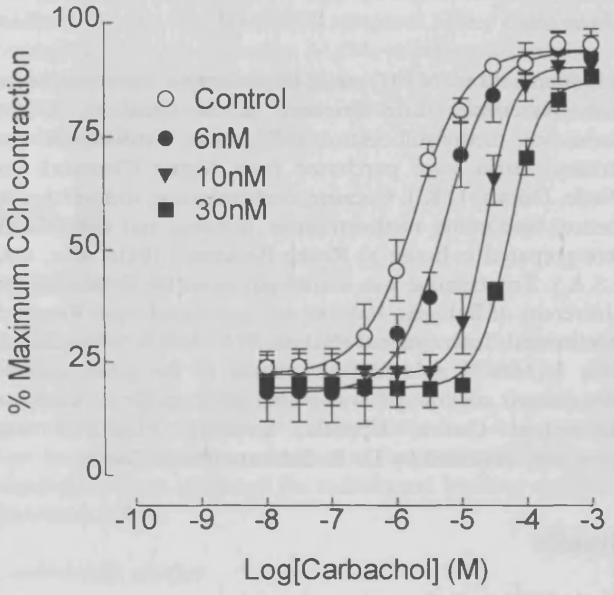

Figure 1 Antagonism of carbachol-induced contraction of guinea-pig isolated uterus strips by zamifenacin. Concentration-response curves to carbachol were constructed in the absence or presence of zamifenacin (6, 10, 30 nM), which was equilibrated in the tissue for 1 h prior to agonist stimulation. Data are shown as % maximum carbachol-induced contraction prior to incubation with antagonist and are expressed as means±s.e.mean of at least three independent experiments. The effect of three out of five concentrations of zamifenacin used have been illustrated for clarity. Similar effects were seen with the other antagonists used.

Table 1 Antagonist affinity estimates (pK_B/pA_2) at muscarinic acetylcholine receptors mediating carbachol-induced contraction of guinea-pig isolated uterus smooth muscle

	Untreated (pK_B)	*'Protected'* (pA_2)	*M_2 atria*	*M_3 ileum*
Atropine	9.3±0.1	ND	9.1[a]	9.3[a]
Methoctramine	7.1±0.1	7.1±0.1	7.9[a]	6.0[a]
Zamifenacin	8.3±0.1	8.6±0.1	6.6[a]	9.3[a]
Tripitramine	8.1±0.1	8.3±0.1	9.7[b]	6.5[b]
p-F-HHSiD	7.5±0.1	ND	6.0[c]	7.9[c]
4-DAMP	8.9±0.1	ND	7.8[c]	9.3[c]
Pirenzepine	6.8±0.1	ND	6.8[c]	6.9[c]
PD102807	<7.0	ND	ND	ND
MTx-3	<6.0	ND	ND	ND

Values shown are means±s.e.mean, n≥7. ND = not determined. [a]Caulfield (1993); [b]Chiarini *et al.* (1995); [c]Eglen *et al.* (1994).

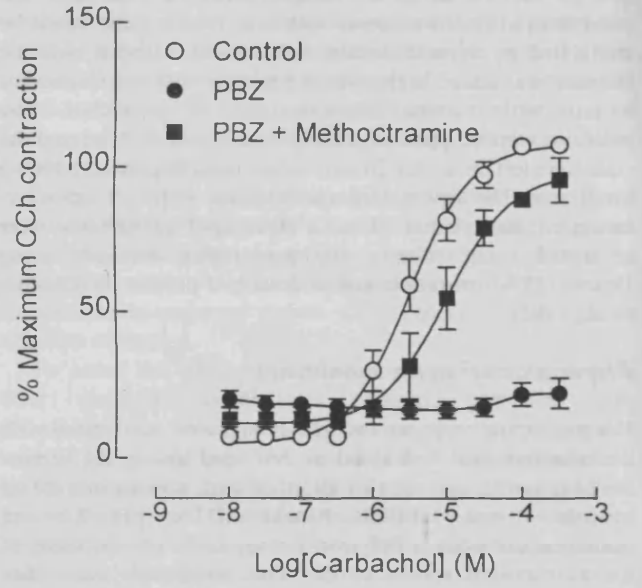

Figure 2 Alkylation and protection of mACh receptors mediating carbachol-induced (CCh) contraction of guinea-pig uterus strips. Phenoxybenzamine (PBZ; 3 μM) was incubated for 20 min alone, or in the presence of previously equilibrated methoctramine (0.1 μM; 1 h). Antagonists were then completely washed from the tissues before construction of the above concentration-response curves. Contraction is shown as a % of the maximum carbachol response prior to incubation with the antagonists, and values are expressed as means±s.e.mean for six to eight separate experiments.

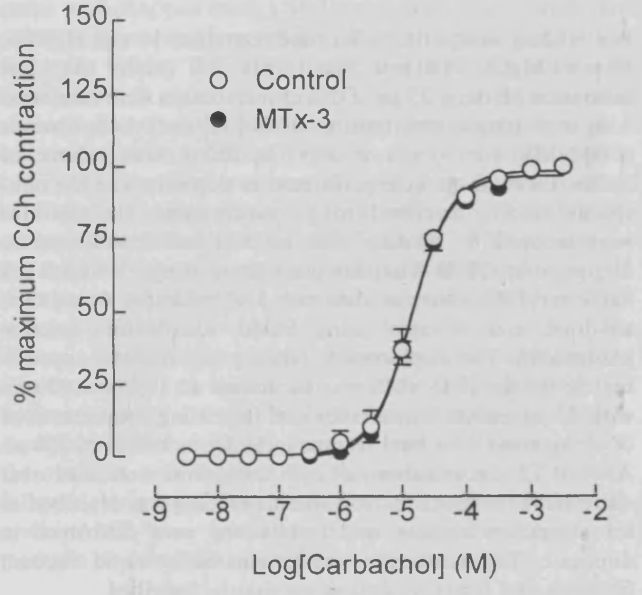

Figure 3 Effect of MTx-3 (muscarinic toxin 3) on carbachol-induced (CCh) contraction of guinea-pig uterus strips. Mtx-3 was equilibrated with the tissue for 1 h prior to agonist stimulation. Data are shown as % maximum carbachol-induced contraction prior to incubation with toxin and are expressed as means±s.e.mean of at least three independent experiments.

low affinity value for pirenzepine in untreated preparations indicated that M_4-mACh receptors were not responsible for contraction of this tissue (pK_B at M_4-mACh receptors = 7.7; Eglen *et al.*, 1997).

The affinity value (pK_A) calculated for the putative 'M_2-selective' agonist L-660,863 (Harris *et al.*, 1991) of 5.44 ± 0.03 ($n = 6$) (Figure 5) was significantly different from the value previously reported for its affinity at M_2-mACh receptor (7.6 ± 0.05). In contrast, the pK_A value estimated for carbachol, which does not discriminate between muscarinic receptor subtypes, of 4.22 ± 0.17 ($n = 8$) agrees with that previously reported in guinea pig ileum (4.7 ± 0.5; Ford *et al.*, 1991).

Effect of pertussis toxin pre-treatment

Pertussis toxin pre-treatment had no significant effect on carbachol-induced contraction when compared with tissues from vehicle-injected controls ($P > 0.05$) (Figure 6). This contrasted with effect on adenylyl cyclase activity in collagenase-treated uterus cell preparations. Thus, carbachol caused a $35 \pm 3\%$ inhibition of forskolin-stimulated cyclic AMP accumulation; an effect almost entirely attenuated by pertussis toxin pre-treatment (Figure 7).

Inositol phosphate accumulation

Stimulation with a maximal carbachol concentration for 30 min produced a $31 \pm 7\%$ increase in total [^{3}H]-inositol phosphate accumulation over basal levels. Although this increase was reproducible and statistically significant ($P < 0.01$), the size of the response precluded pharmacological characterization.

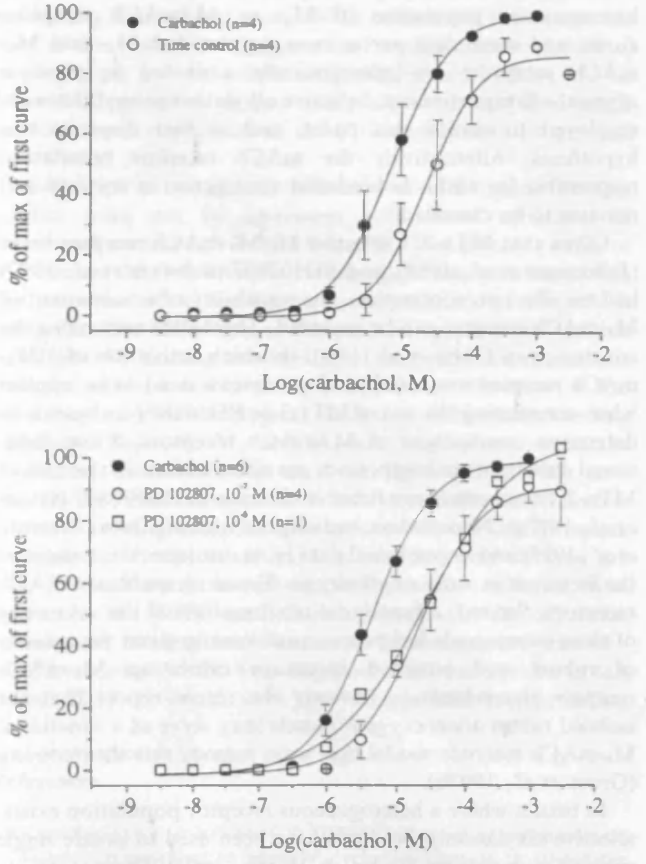

Figure 4 Effect of PD 102807 on carbachol-induced contraction of guinea-pig uterus strips. The upper panel shows time-control data. Data are shown as % maximum carbachol-induced contraction prior to incubation with antagonist and are expressed as means ± s.e.mean for number of experiments indicated in the Figure keys.

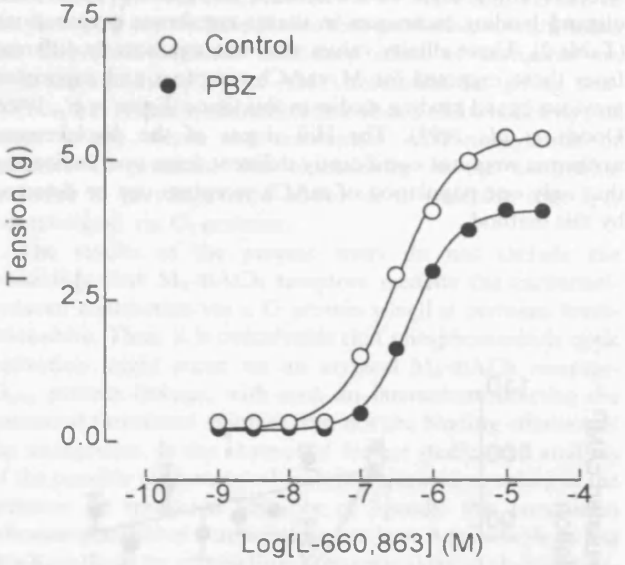

Figure 5 Partial inactivation of contractile response to L-660,863, a 'M_2-selective' agonist, by phenoxybenzamine in guinea-pig uterus strips. Phenoxybenzamine (PBZ; 0.1 μM) was incubated for 20 min before construction of a second concentration-response curve to the agonist. Data are expressed as absolute tension and are from a single experiment representative of six independent experiments. K_A values were calculated separately for each experiment and then means and s.e.mean were derived.

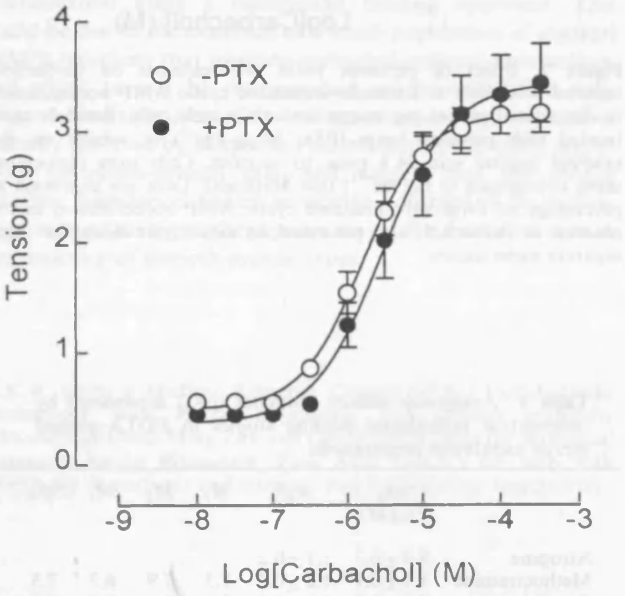

Figure 6 Effect of pertussis toxin pre-treatment on carbachol-induced contraction of guinea-pig uterus strips. Animals were pre-treated with pertussis toxin (PTx; 50 μg kg^{-1}) or vehicle via the external jugular vein 24 h prior to sacrifice. Single concentration-response curves to carbachol were constructed after tissue equilibration. Contraction is expressed as absolute tension and presented as means ± s.e.mean for three separate experiments.

Pharmacological characterization of [^{3}H]-NMS binding

An equilibrium dissociation constant (K_D) of 0.12±0.05 nM for [^{3}H]-NMS was obtained from saturation binding experiments ($n=3$) which were also used to calculate a B_{max} of 147±18 fmol mg protein^{-1} for mACh receptors in this membrane preparation. The affinity values for subtype-selective antagonists were estimated using competition radioligand binding techniques in uterus membrane preparations (Table 2). These affinity values were not significantly different from those expected for M_2-mACh receptors and supported previous ligand binding studies in this tissue (Eglen *et al.*, 1989; Doods *et al.*, 1993). The Hill slopes of the displacement isotherms were not significantly different from one, indicating that only one population of mACh receptors can be detected by this method.

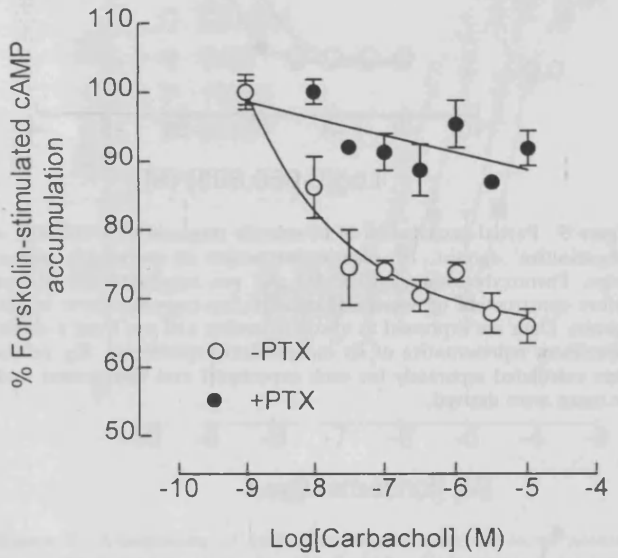

Figure 7 Effect of pertussis toxin pre-treatment on carbachol-induced inhibition of forskolin-stimulated cyclic AMP accumulation in dissociated guinea-pig uterus smooth muscle cells. Animals were treated with pertussis toxin (PTx; 50 μg kg^{-1}) or vehicle *via* the external jugular vein 24 h prior to sacrifice. Cells were dissociated using collagenase (1 mg ml^{-1}) (see Methods). Data are expressed as percentage of forskolin-stimulated cyclic AMP accumulation in the absence of carbachol, and presented as means±s.e.mean for three separate experiments.

Table 2 Antagonist affinity estimates (pK_i) determined by competition radioligand binding studies in EDTA-washed uterus membrane preparations

	pK_i (logM)	n_H	M_1	M_2	M_3	M_4
Atropine	8.9±0.3	1.1±0.2				
Methoctramine	8.1±0.1	1.2±0.2	7.3	7.9	6.7	7.5
Zamifenacin	7.6±0.2	1.0±0.2	7.4	7.6	8.0	7.6
Tripitramine	9.3±0.2	0.9±0.1	8.8	9.6	7.4	7.9

Values shown are means±s.e.mean, $n=3$. Hill slopes are not significantly different from 1 ($P<0.05$). Values in columns 4–7 have been determined in ligand binding studies at cloned muscarinic receptors expressed in CHO cells (Eglen *et al.*, 1997).

Discussion

The characterization of mACh receptor-mediated contraction in uterine tissue has been hampered both by the inherent variability of the contractile responses observed and the lack of subtype-specific mACh receptor ligands. Moreover, several studies of this tissue fail to account for potential changes in receptor populations due to fluctuating steroid hormone levels, despite studies showing this to be a critical factor (Riemer *et al.*, 1987; Varol *et al.*, 1989; Arkinstall & Jones, 1990; Matucci *et al.*, 1996). In the present study pre-treatment with diethylstilboestrol induced all guinea-pigs to enter an oestrogen-dominant phase of the reproductive cycle, thus normalizing the steroid hormone levels as much as practically possible, and implying the mACh receptor populations are under a constant hormonal influence.

Although only a limited number of antagonists were used to characterize the muscarinic response of this tissue those chosen comprise some of the most 'M_2/M_3 selective' antagonists available. Surprisingly these have not been used before despite the large number of antagonists used previously (Dörje *et al.*, 1990; Doods *et al.*, 1993). Moreover, these M_2/M_3-mACh receptor-selective compounds are of interest given the coexpression of M_2- and M_3-mACh receptors in most smooth muscles, and the apparent absence of M_3-mACh receptors in guinea-pig uterus.

In the present study, the affinity estimates made for these 'M_2/M_3 selective' antagonists did not correlate with those made at M_2- or M_3-mACh receptors (Caulfield, 1993; Chiarini, *et al.*, 1995). This excludes the possibility that carbachol-induced contraction of guinea-pig uterus is mediated by a homogeneous population of M_2- or M_3-mACh receptors alone, and these data *per se* may suggest that M_2- and M_3-mACh receptors are synergistically activated to produce atypical affinity estimates. Selective alkylation procedures were employed to clarify this point, and in fact disprove this hypothesis. Alternatively the mACh receptor population responsible for carbachol-induced contraction is atypical and remains to be classified.

Given that MTx-3, a selective M_1/M_4-mACh receptor toxin (Jolkkonen *et al.*, 1994), and PD102807 (Schwarz *et al.*, 1997) had no effect on contraction, the possibility of involvement of M_4-mACh receptor can be excluded. These data contradict the conclusion of Dörje *et al.* (1990) in which activation of a M_4-mACh receptor was invoked. Two caveats need to be applied when considering the use of MTx-3 or PD102807 as ligands to determine involvement of M_4-mACh receptors. First, functional data on these compounds are either absent in the case of MTx-3, or extremely restricted in the case of PD102807 (Gross *et al.*, 1997a). Nonetheless, radioligand binding data (Schwarz *et al.*, 1997) and unpublished data from our laboratory support the literature in terms of affinity profiles at recombinant mACh receptors. Second, a functional confirmation of the selectivity of these compounds has proven problematic given the paucity of robust and accepted bioassays exhibiting M_4-mACh receptor pharmacology, however the recent report that the isolated rabbit anococcygeus muscle may serve as a functional M_4-mACh receptor model may soon remedy this shortcoming (Gross *et al.*, 1997b).

In tissues where a heterogeneous receptor population exists, selective alkylation procedures have been used to isolate single subtype populations (Eglen & Harris, 1993; Thomas *et al.*, 1993). This technique, using a reversible selective antagonist, enables a given receptor type to be 'protected' from inactivation by non-selective alkylating agents such as phenozybenzamine. In the absence of a protecting ligand,

when the tissues were exposed to 3 μM phenoxybenzamine and extensively washed, the responses to carbachol were abolished. In contrast, prior equilibration with methoctramine (0.1 μM) almost completely attenuated this effect of phenoxybenzamine, such that only a small dextral shift and slight depression of the maximum response was seen. Methoctramine at 0.1 μM would be expected to occupy more than 85% of M_2-mACh receptors and less than 10% of M_3-mACh receptors. Therefore it would be expected that any M_3-mACh receptors in this tissue would have been largely inactivated by phenoxybenzamine, whilst the M_2-mACh receptors would have been protected from such inactivation. However, characterization of antagonist affinities at these 'protected' receptors showed no differences between these and affinity estimates made in untreated tissues. This suggests that the dextral shift and slight depression of maximum was caused by removal of part of a single population of receptors as opposed to the removal of a single subtype of receptors from a heterogeneous population. Therefore it is extremely unlikely that contraction is caused by concurrent activation of M_2- and M_3-mACh receptors.

Scepticism abounds as to the usefulness of agonist affinity estimates in characterization of receptors. However, these data may provide strong supporting evidence for other methods of pharmacological characterization of receptors (Keen, 1991; Leff, 1995). Hence, our finding that the agonist affinity value for the putative 'M_2-selective' agonist L-660,863 differed from that expected at a M_2-mACh receptor is further evidence to support conclusions made based upon atypical antagonist affinity profiles. Since the affinity estimates for carbachol (which is not selective between muscarinic receptor subtypes) agrees with that previously reported at M_3-mACh receptors in guinea pig ileum (Ford *et al.*, 1991), collectively the contractile data of this study would suggest that contractions to carbachol in guinea-pig isolated uterus are mediated not by M_2-, M_3- or M_4-mACh receptors as previously reported, but by a single homogeneous population of mACh receptor with an atypical operational profile.

Estimates of antagonist affinities in radioligand binding studies were not in agreement with those estimated in functional studies (*cf.* Tables 1 and 2). The affinity profile of the chosen 'M_2/M_3 selective' antagonists was consistent with the presence of a homogeneous population of M_2-mACh receptors. The Hill slopes of competition curves of antagonists to [^{3}H]-NMS binding would suggest that only one muscarinic receptor subtype is expressed in this tissue. However, it has been shown by immunological methods and Northern blotting techniques that both M_2- and M_4-mACh receptors can coexist in uterine tissues (Dörje *et al.*, 1991; Eglen *et al.*, 1992), so it may be that these antagonists are still not sufficiently selective between mACh receptor subtypes to detect coexpression, particularly if expression levels of one receptor subtype are low. These data however support previous evidence from Eglen *et al.* (1989) and Doods *et al.* (1993), in which radioligand binding and functional data suggested that M_2-mACh receptors alone were responsible for contraction in this tissue. Thus, it would seem that whilst there are conflicting functional data regarding the nature of the response, all radioligand studies carried out on guinea-pig uterus concur that M_2-mACh receptors only can be detected.

This is interesting given the insensitivity of the contractile response to prior systemic administration of pertussis toxin which abolishes M_2-mACh receptor-mediated responses. This supports data produced by *in vitro* pre-treatment of guinea-pig uterus with pertussis toxin carried out by Marc *et al.* (1988). In the present study we have shown that pertussis toxin abolishes the G_i-protein-mediated inhibitory effect of carbachol on forskolin-stimulated cyclic AMP accumulation, giving confidence that systemic administration allows effective delivery of toxin to the uterus and consequent ADP-ribosylation of sensitive G proteins. The insensitivity of the contractile response to pertussis toxin allows us to conclude that it is not mediated via G_i-proteins.

The results of the present study do not exclude the possibility that M_2-mACh receptors mediate the carbachol-induced contraction via a G protein which is pertussis toxin-insensitive. Thus, it is conceivable that phosphoinositide cycle activation might occur via an atypical M_2-mACh receptor-$G_{q/11}$ protein linkage, with such an interaction affecting the measured functional affinities, but not the binding affinities of the antagonists. In the absence of further studies and analysis of the possible outcome(s) of such promiscuous coupling of the receptor on measured affinities of ligands, this conclusion remains speculative. Furthermore, we have been unable to test this hypothesis by conducting a pharmacological characterization of the phosphoinositide response due to the small agonist-induced increase in [^{3}H]-inositol phosphate accumulation. Although others have assessed phosphoinositide turnover in this tissue (Leiber *et al.*, 1990), total inositol phosphate accumulations were normalized and the magnitude of response not stated.

In conclusion, we have found that mACh receptors in oestrogen-dominant guinea-pig uterus display an atypical pharmacological profile when examined functionally, but display classical M_2-mACh receptor pharmacology when characterized using a radioligand binding approach. This could be due to the existence of a small population of atypical mACh receptors that mediate carbachol-induced contraction, but which cannot be distinguished using competitive radioligand binding studies. Our findings are consistent with those recently reported by Munns & Pennefather (1998) using uterus from oestrogen-primed rats, and suggest that the mACh receptor population linked to uterine contraction in guinea-pigs, rat and perhaps other species may differ from that seen in the majority of smooth muscle types.

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