

**Exploring agonist dependency of receptor-
G protein-coupling and constitutive activity
at muscarinic acetylcholine receptors**

**Thesis submitted for the degree of
Doctor of Philosophy
at the University of Leicester**

by

Mark R. Dowling

**Department of Cell Physiology and Pharmacology
University of Leicester**

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Exploring agonist dependency of receptor-G protein-coupling and constitutive activity at muscarinic acetylcholine receptors

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The effects of structurally diverse agonists were assessed on receptor-mediated activation of G-proteins using in Chinese hamster ovary cells stably expressing similar densities of M_1 and M_3 mACh receptors. Using a total [35 S]-GTP γ S binding protocol both receptor subtypes were shown to couple to both pertussis toxin-sensitive and -insensitive G-proteins. M_1 mACh receptors coupled with greater potency and intrinsic efficacy than M_3 receptors, indicating that in this cell-line the M_1 mACh receptor may be more efficiently coupled to its complement of G-proteins. Total [35 S]-GTP γ S binding concentration-response curves for both receptor subtypes were shallow and, in the case of the M_1 receptor, could be readily resolved into high and low potency components. The heterogeneity of $G\alpha$ subunits activated was further investigated using an immunoprecipitation technique. This strategy revealed that agonist binding to M_1 receptor caused the activation of a heterogeneous population of G-proteins, which was directly related to agonist efficacy. Full agonists were able to activate both $G\alpha_{q/11}$ and $G\alpha_{i1-3}$ subtypes, whereas partial agonists activated only the most efficiently coupled G-protein $G\alpha_{q/11}$. In contrast, the complement of G-proteins activated after M_3 receptor stimulation was not related to the efficacy of the agonist and suggested that agonists may form active conformations of the M_3 receptor that possess different G-protein coupling profiles.

To investigate the G-protein coupling of M_1 and M_3 receptors further, homologous point mutations were introduced into both subtypes, which conferred agonist-independent constitutive activity. All of the antagonists tested were able to concentration-dependently inhibit basal [3 H]-inositol phosphate accumulation and were therefore classified as ‘inverse agonists’. However, subtle differences in the results obtained for different inverse agonists via different functional and binding readouts suggested the existence of more than one conformation of the inactive receptor conformation

This study therefore provides experimental evidence to suggest that the ‘two-state-model’ of receptor activation is too simplistic to accommodate all of the empirical observations, and alternative models of receptor-G-protein activation and inverse agonist binding are proposed and discussed.

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Publications

Publications

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Differential activation of $G_{q/11}$ and G_{i1-3} proteins at M_1 and M_3 muscarinic acetylcholine receptors revealed by an immunoprecipitation-capture technique: Evidence for agonist-specific receptor conformations. In Preparation

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Differential (inverse) agonist efficacy at a constitutively-active, mutant M_3 muscarinic acetylcholine receptor. In Preparation

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Measurement of agonist-stimulated [35 S]-GTP γ S binding to assess total G protein and $G\alpha$ subtype-specific activation by G protein-coupled receptors. In "Receptor Signal Transduction Protocols" (2004) **259**: 197-259

Mistry R., Dowling M. R. and Challiss R. A. J

An investigation of whether agonist-selective receptor conformations occur with respect to M_2 and M_4 muscarinic acetylcholine receptor signalling via $G_{i/o}$ and G_s proteins. Revised version re-submitted to *Br. J. Pharmacol.* (2004)

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Activation of $G_{q/11}$ and G_{i1-3} proteins by M_1 and M_3 muscarinic acetylcholine (mACh) receptors: Evidence for agonist-specific receptor conformations. Keystone Symposium: "G-protein-coupled receptors: Evolving concepts and drug discovery", Taos, New Mexico, USA, (2004) 211P

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Preliminary characterization of a mutant, constitutively-active M_3 muscarinic acetylcholine receptor. Dahlem Symposium on cellular signal recognition and transduction (Honouring Gunter Schultz), Berlin, Germany (2003) 45P

Abbreviations

Abbreviations List

5'-AMP	Adenosine-5'-monophosphate
5-HT	5-hydroxytryptamine
7-TM	7-Transmembrane spanning domain
AC	Adenylyl cyclase
ADP	Adenosine 5'-diphosphate
AKAP	A-kinase anchoring proteins
ATP	Adenosine 5'-triphosphate
BSA	Bovine serum albumin
cAMP	Adenosine 3',5'-cyclic monophosphate
cGMP	Cyclic guanosine monophosphates
CHO-cells	Chinese hamster ovary cells
CK1 α	Casein kinase 1 α
CNS	Central nervous system
CPM	Counts per minute
CTC	Cubic ternary complex model
DAG	<i>sn</i> -1,2-diacylglycerol
DNA	Deoxyribonucleic acid
DOI	1-(4-iodo-2,5-dimethoxyphenyl)-2-aminopropane
DPM	Disintegrations per minute
EC ₅₀	The molar concentration of an agonist, which produces 50% of the maximum possible response for that agonist
EDTA	Ethylenediaminetetra-acetic acid
ER	Endoplasmic reticulum
ERK	extracellular signal-regulated kinase
ETC	Extended ternary complex model
FRET	Fluorescence resonance energy transfer
GABA	γ -aminobutyrate
GAP	GTPase-activating protein
GDP	Guanosine-5'-diphosphate
GEF	Guanine-nucleotide exchange

Abbreviations

GIRK	G protein-coupled inwardly rectifying K ⁺ channels
GPCR	G-protein coupled receptor
G-protein	Guanine nucleotide binding protein
GRK	G protein-coupled receptor kinase
GTP	Guanosine-5;-triphosphate
GTP γ S	Guanosine-5'-triphosphate-(thiophosphate)
HEK-cells	Human embryonic kidney cells
IC ₅₀	The molar concentration of an agonist, which produces 50% of the maximum possible inhibitory response for that agonist
IMPase	Inositol monophosphatase
IP ₃	Inositol 1,4,5-trisphosphate
IP ₄	Inositol 1,3,4,5 tetrakisphosphate
JNK	c-Jun NH2-terminal kinase
K _D	Equilibrium dissociation constant
K _i	Inhibition constant
LSD	Lysergic acid diethylamide
mACh	Muscarinic acetylcholine receptor
MAPK	Mitogen-Activated Protein kinase
NAD ⁺	β -nicotinamide-adenine nucleotide
nH	Hill Coefficient
NMS	N-methyl scopolamine
NSB	Non-specific binding
PDE	Phosphodiesterase
PI	Phosphatidylinositol
PI3K	Phosphoinositol 3 kinase
PIP ₂	Phosphatidylinositol 4,5-bisphosphate
PKA	Protein kinase A
PKC	Protein kinase C
PLA ₂	Phospholipase A ₂
PLC	Phospholipase C
PLD	Phospholipase D

Abbreviations

PTx	Pertussis toxin
RGS	Regulator of G-protein signalling
RNA	Ribonucleic acid
siRNA	Small interfering RNA
SPA	Scintillation proximity bead assay
TCA	Trichloroacetic acid
TCM	Ternary complex model
TFMPP	Trifluoromethylphenylpiperazine Monohydrochloride
VIP	Vasoactive intestinal peptide

Chapter One.

Introduction

1.0. Introduction

This thesis details experiments investigating the G-protein coupling profile of the M₁ and M₃ muscarinic acetylcholine (mACh) receptors employing two distinct approaches: **(1)**. Using two different [³⁵S]-GTPγS binding methodologies to assess the complement of G-protein α subunits activated in response to range of mACh receptor agonists; **(2)**. Assessing the pharmacology of agonists and antagonists at constitutively active M₁ and M₃ mACh receptors, mutated at a single amino acid residue to confer agonist-independent activity.

To aid the discussions following each experimental Chapter, this Introduction has three broad aims:

- (1)**. To provide information on the nature of some fundamental aspects of cell-signalling which are applicable to the subsequent Chapters.
- (2)**. To detail the coupling, expression profiles and therapeutic potential of the human muscarinic acetylcholine family (with particular emphasis on the M₁ and M₃ receptors). Additionally, using the M₃ mACh receptor as a model, to summarize our current understanding of how GPCRs are activated in response to agonist and subsequently couple to G-protein(s).
- (3)**. To summarise some of the key theories to explain and quantify receptor activation and to introduce the concepts of efficacy, inverse-agonism and agonist-specific receptor conformations ('agonist-trafficking').

1.1. G-protein coupled receptors, G-proteins and effectors

1.1.1. Diversity of G-protein coupled receptors

G-protein coupled receptors (GPCRs) mediate physiological responses to a diverse array of stimuli, including hormones, neurotransmitters, small peptides, proteins, lipids, ions as well as sensory stimuli such as odorants, pheromones, bitter and sweet tastants and

Chapter 1: Introduction

photons (Hall, 1999b; Hall and Lefkowitz, 2002). The number of rhodopsin-like GPCRs in the human genome has been estimated to be between approximately 620 (Venter *et al.*, 2001) to 950 (Takeda *et al.*, 2002) and as such they represent the largest class of cell-surface molecules in the mammalian genome.

All of the GPCRs share a common core domain structure consisting of seven transmembrane (7-TM) helices; and it is a change in the conformation in this region that is thought to be responsible for receptor activation (Bockaert and Pin, 1999). These regions are connected via intracellular and extracellular loops, and along with the *N*- and *C*-terminal regions, show remarkable amino acid sequence diversity. Agonist-induced changes within the 7-TM regions leads to conformational re-arrangements of key sequences of residues in the second (i2) and third (i3) intracellular loops, which is thought to initiate G-protein recognition and binding (Strader *et al.*, 1995; Wess, 1997; Wess *et al.*, 1997).

On the basis of structural and sequence similarities mammalian GPCRs are classified into (at least) three groups (Bockaert and Pin, 1999; King and Wilson, 1999; Hermans, 2003). Group A (rhodopsin-type receptor group) is the largest and is exemplified by rhodopsin and the adrenoceptor sub-families. This family also contains receptors for copious neurotransmitters (biogenic amines and nucleotides), prostaglandins and many peptides and neuropeptides. Group B (secretin/glucagon receptors) encompasses receptors for peptides and hormones such as secretin, parathyroid, calcitonin, glucagon and vasoactive intestinal polypeptide (VIP). Family C (metabotropic glutamate receptor group) incorporates GPCRs for glutamate and γ -aminobutyric acid (GABA), extracellular Ca^{2+} , as well as containing a variety of receptors associated with sensory perception (taste, smell). Class C receptors are typified by a large extracellular *N*-terminal domain, which exhibits homology with some periplasmic binding proteins of bacteria (O'Hara *et al.*, 1993; Fredriksson *et al.*, 2003).

GPCRs have proved to be invaluable therapeutic targets for the pharmaceutical industry, with between 40-50% of current marketed drugs exerting their therapeutic actions via

GPCRs (Fukuhara *et al.*, 2000a,b; Kostenis, 2001). Despite this, we are only just beginning to appreciate and comprehend the complexity and diversity of the intracellular signalling pathways and molecules regulated by these receptors. Recent investigations have highlighted intracellular signalling pathways that are regulated by these receptors independently of G-proteins (Hall *et al.*, 1999), and for this reason some researchers prefer the term ‘heptahelical’ (or 7-TM) receptors to GPCRs (see section 1.1.4.).

The GPCR signal transduction process often comprises a complex signalling network with diverging and converging steps at each coupling interface (Gudermann *et al.*, 1997). This section introduces some of the major groups of proteins involved in the GPCR transduction process (especially those revisited in later Chapters) and also considers some more contemporary views on what governs the specificity of cell-signalling. The structure of heptahelical receptors (exemplified by the muscarinic acetylcholine receptor family) and the perceived relationship to intracellular signalling is addressed separately in section 1.2.

1.1.2. Guanine nucleotide-binding proteins (G-proteins)

G-proteins act as molecular switches that are able to link extracellular stimuli such as hormones, neurotransmitters and light, to influence the activities of second messenger-generating enzymes and ion channels (Neer, 1994). Ubiquitously expressed in all eukaryotic cells, their function is to couple to heptahelical receptors and act as binary switches, interconverting between an active GTP-bound form and an inactive GDP-bound form (Hall, 1999b), to convey a signal from an activated receptor to the appropriate effector. They are heterotrimeric proteins consisting of an α , β and γ subunit and are characterised by the identity of the $G\alpha$ subunit (Marinissen and Gutkind, 2001). Sixteen genes in the mammalian genome have been identified encoding α -subunits and there are 5 and 12 genes respectively for the β - and γ - subunits (Downes and Gautam, 1999). Free $G\beta\gamma$ subunits function as dimeric complexes (Downes and Gautam, 1999).

The guanine-nucleotide binding α subunit and the $\beta\gamma$ subunits are able to influence the activities of a distinct, but overlapping array of effector molecules. Although most

experimental evidence indicates that the α and $\beta\gamma$ subunits function as separate signalling complexes (Janetopoulos *et al.*, 2001; Yi *et al.*, 2003), recent FRET-based assays have suggested that the $G\alpha_i$ $\alpha\beta\gamma$ complex, undergoes conformational changes upon GDP/GTP exchange, but does not dissociate into α - and $\beta\gamma$ -subunits (Bunemann *et al.*, 2003). More detailed structural analysis should show whether retention of the α -GTP- $\beta\gamma$ trimeric subunit complex during cell-signalling is more common than initially suggested. Deactivation of G-protein signalling is regulated in part by the intrinsic GTPase activity of the α -subunit. Therefore, heterotrimeric G-proteins are part of a large family of GTPases, which also includes the monomeric GTPase proteins Ras, Rho, Rab, Arf and Ran (Hall, 1999a).

1.1.2.1. The G-protein cycle

Fig. 1.1. depicts a simplified summary of our current understanding of how G-proteins are activated in response to agonist-stimulation of GPCRs. In the absence of agonist the G-protein resides in the heterotrimeric form, with GDP bound at the α subunit, and the complex is tethered to the inner leaflet of the plasma membrane via lipid modifications to both α - and γ -subunits (Downes and Gautam 1999) (**Step 1**). A conformational change occurs in GPCRs following agonist binding, which increases the receptor affinity for the G-protein and facilitates guanine nucleotide exchange (Hall, 1999b). Site-directed mutagenesis studies have indicated various cytoplasmic domains of GPCRs that constitute the specific interaction surfaces between the receptor and the G-protein (Wess, 1997; Gether and Kobilka, 1998). The precise mechanism by which activated receptors (**R***) promote the release of GDP from the α subunit (Breivogel *et al.*, 1997; Mukhopadhyay and Ross, 1999) is not comprehensively understood and several regions of the receptor are likely to be involved (Gudermann *et al.*, 1997). The guanine nucleotide-free G-protein and agonist-bound activated receptor form a transient ternary complex which exhibits high-agonist affinity (Samama *et al.*, 1993) (**Step 2**). The more abundant guanine-nucleotide, GTP, then binds to a specific site on the α subunit buried deep in a cleft between its GTPase and α -helical domains (Gudermann *et al.*, 1997) (**Step 3**). Agonist-receptor binding/activation may also facilitate GDP/GTP exchange by actually increasing the affinity of the α -subunit for GTP (Morris and Malbon, 1999).

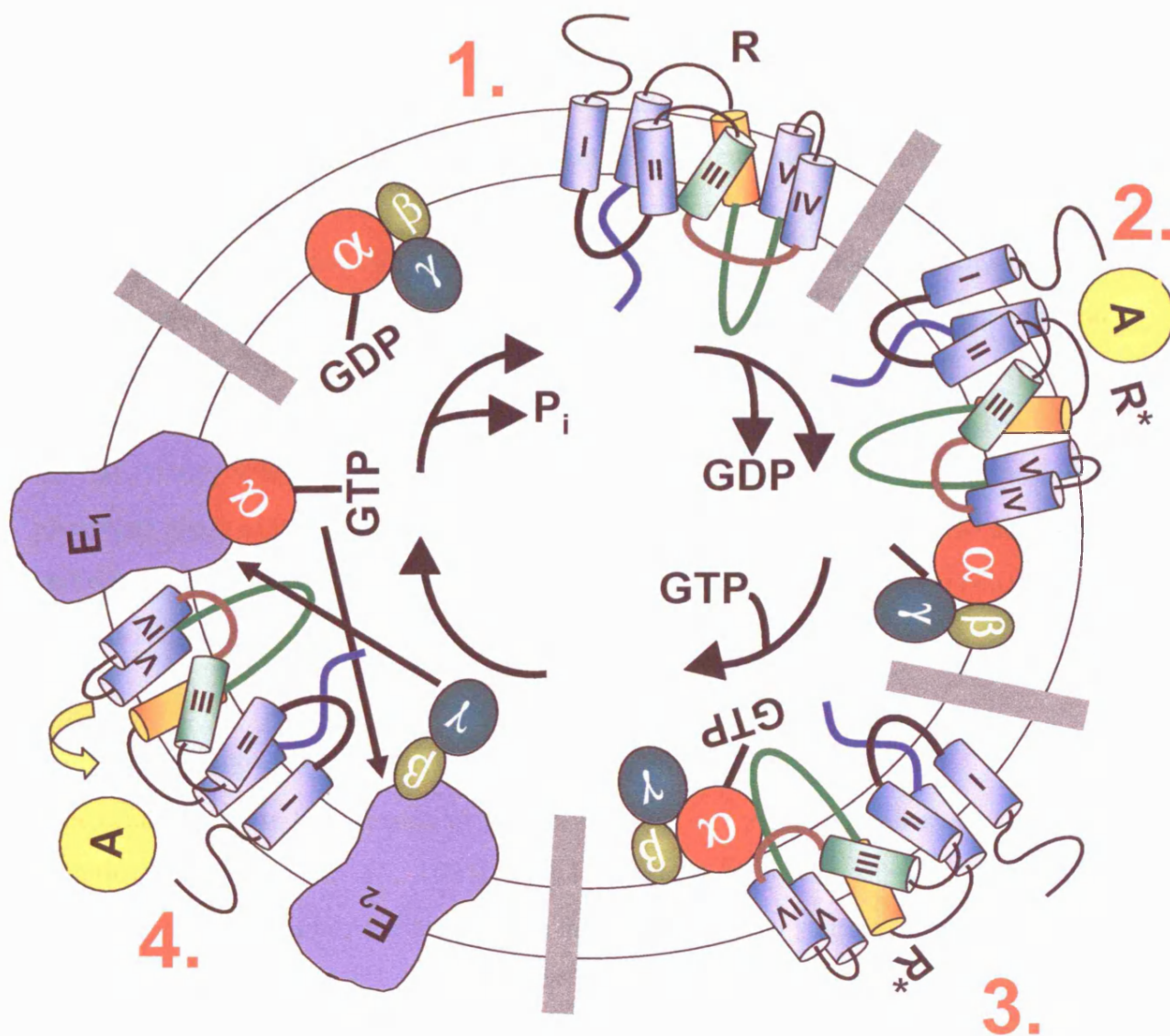


Fig. 1.1. Schematic diagram of the G-protein activation cycle

General overview of the G-protein activation cycle illustrating the four main steps (highlighted in **red**). A full explanation of the details involved can be found in the text (section 1.1.2.1.).

Upon GTP binding, the G-protein undergoes several conformational re-arrangements within the ‘switch-regions’ of the α subunits (Bunemann *et al.*, 2003). This leads to a weakening of the interactions between the proteins, which ultimately causes dissociation of the $\alpha\beta\gamma$ trimer into α -GTP and $\beta\gamma$ subunits (Morris and Malbon, 1999). Both the α -GTP and $\beta\gamma$ subunits are able to influence the activity of a number of different downstream effectors, and this may provide diversity and the potential for pathway cross-talk (Hur and Kim, 2002) (**Step 4**). The intrinsic GTPase activity of the α subunit, and the acceleration of this activity by other regulatory proteins, including effectors themselves and regulators of G-protein signalling (RGS) proteins (Arshavsky and Bownds, 1992, Scholich *et al.*, 1999, Ross and Wilkie, 2000), hydrolyse the terminal phosphate group of the GTP molecule. This process dictates the life-time of $G\alpha$ activation and initiates re-association of the α -GDP and $\beta\gamma$ subunits to form an inactive heterotrimer (Morris and Malbon, 1999) (**Step 1**).

1.1.2.2. Signalling by the $G\alpha$ subunit

G-proteins are classified according to the amino acid sequence similarity of their α subunits (Gudermann *et al.*, 1997). Twenty-three distinct $G\alpha$ subunits have been identified, including splice variants, which are encoded by 16 genes (Kehlenbach *et al.*, 1994, Nurnberg *et al.*, 1995). These are divided into four major groups, $G\alpha_s$, $G\alpha_i$, $G\alpha_q$ and $G\alpha_{12/13}$, based on sequence homology, which exhibit diverse expression patterns (Offermanns, 2003). The individual $G\alpha$ subunit family members, their expression profile and the major effectors, whose activities are influenced by the specific $G\alpha$ -subunits are summarised in **Table 1.1**.

Table 1.1. G-protein α subunits and their major effectors

Sub-family	Members	Tissue Distribution	Major Cellular effects KEY: (+) activate, (-) inhibit
Gα_s	α_s (short and long isoforms)	ubiquitous	+ AC, + cardiac L-type Ca^{2+} channels + cardiac Na^+ channels + cardiac Cl^- channels
	α_{sXL}	neuroendocrine	unclear
	α_{olf}	olfactory neuroepithelium low levels in brain	+ AC
Gα_i	α_{i1}	wide-spread	- AC, + K^+ channels (K_{ATP} & K_{ir}), - neuronal N type Ca^{2+} channels - epithelial Cl^- channels
	α_{i2}	ubiquitous	-AC, + K^+ channels (K_{ATP} & K_{ir}) - neuronal N type Ca^{2+} channels - epithelial Cl^- channels
	α_{i3}	widespread	-AC, + K^+ channels (K_{ATP} & K_{ir}) - epithelial Na^+ channels - epithelial Cl^- channels
	α_{oa}	neuronal, neuroendocrine	- AC, + phosphoinositide 3-kinase - epithelial Cl^- channels
	α_{t1} α_{t2}	retinal rod cells retinal cone cells	+ retinal cGMP PDE + retinal cGMP PDE
	α_{gust}	taste buds	- AC (?)
	α_z	predominantly brain	unclear
Gα_q	α_q	ubiquitous	+ $\text{PLC}\beta$
	α_{11}	widespread	+ $\text{PLC}\beta$
	α_{14}	stromal/epithelial	+ $\text{PLC}\beta$
	α_{15}	haematopoietic lineage/ myeloid	+ $\text{PLC}\beta$
	α_{16}	myeloid	+ $\text{PLC}\beta$
Gα_{12}	α_{12}	ubiquitous	Na^+/H^+ exchanger
	α_{13}	ubiquitous	Na^+/H^+ exchanger

Data taken from Gudermann *et al.*, 1997; Downes and Gautam, 1999, Offermanns, 2003

1.1.2.2.1. $G\alpha_s$ subunits

The $G\alpha_s$ family comprises the ubiquitously expressed $G\alpha_s$ and the highly-tissue specific $G\alpha_{olf}$, both of which primarily stimulate membrane-bound adenylyl cyclases (Krumins *et al.*, 1997a,b,c). Recent evidence has suggested that after agonist-induced activation, the $G\alpha_s$ subunit rapidly translocates from the plasma membrane to the cytoplasm (Yu and Rasenick, 2002), however other studies have indicated that the activated $G\alpha_s$ subunit remains plasma membrane-bound (Huang *et al.*, 1999a) and therefore the functional significance of this effect remains open to debate. The $G\alpha_s$ gene can give rise to several splice variants; two short forms and two long forms which appear to be structurally closely related and functionally indistinguishable (Offermanns, 2003). All of the cloned nine isoforms of adenylyl cyclase are stimulated by G_s and its splice variants (Simonds, 1999). Another splice variant $G\alpha_{sXL}$ (extra-long) has been described although it is not clear whether it can be activated by GPCRs under physiological conditions (Bastepe *et al.*, 2002). $G\alpha_{olf}$ is expressed in olfactory tubercles and some neurons of the CNS, such as the striatum and nucleus accumbens (Zhuang *et al.*, 2000). Mice lacking $G\alpha_{olf}$ expression display motoric abnormalities in addition to olfactory defects, indicating a more complex role than solely being involved in the sensory perception of odours (Offermanns, 2003).

1.1.2.2.2. $G\alpha_i$ subunits

The $G\alpha_i$ subfamily consists of the widely expressed $G\alpha_{i1}$, $G\alpha_{i2}$ and $G\alpha_{i3}$, and the more tissue-specific $G\alpha_{oA/B}$, $G\alpha_{i1/2}$, $G\alpha_{gust}$ and $G\alpha_z$. $G\alpha_{i1/2/3}$ proteins are implicated in the inhibition of certain isoenzymes of adenylyl cyclase, via either $G\alpha_i$ -GTP or the release of $\beta\gamma$ subunits (Simonds, 1999). There is considerable functional similarity between the three $G\alpha_i$ isoforms, which may result in redundant functions exemplified by the lack of major phenotypic changes in $G\alpha_{i1}$ or $G\alpha_{i2}$ knockout mice (Offermanns, 2003). The three $G\alpha_i$ proteins are also implicated in the regulation of several ion channels and in the modulation of phospholipase C activity, probably via the release of free $\beta\gamma$ subunits (Morris and Malbon, 1999). A recent study has examined the kinetics of GIRK activation by $G\beta\gamma$ subunits released from $G\alpha_o$ and $G\alpha_i$ subtypes and reported that faster activation of the GIRKs by $G\alpha_o$ is perhaps due to differences in receptor (M_2 mACh) catalyzed GDP release which influences the rate of $G\beta\gamma$ production (Zhang *et al.*, 2004). $G\alpha_o$ is the most

predominate G-protein expressed in neuronal tissue, but despite this its physiological functions are poorly characterised to date and it has been suggested that its major actions are mediated via release of associated $\beta\gamma$ subunits (Offermans, 2003). $G\alpha_z$ is the only $G\alpha_i$ family member to be pertussis toxin-insensitive (see 1.1.2.2.2.1.) and is expressed in a limited range of tissues, including platelets, neurons and the adrenal gland (Fields and Casey, 1997). Like, $G\alpha_o$ its physiological role is still poorly understood (Offermanns, 2003), although $G\alpha_z$ has been implicated in diverse cellular signalling process such as proliferation, differentiation and apoptosis (Tu *et al.*, 1997; Ho and Wong, 1998, 2001). $G\alpha_{\text{gust}}$ and $G\alpha_{1/2}$ are expressed in specific tissues and perform specialized sensory roles, which will not be discussed further here.

1.1.2.2.2.1. Pertussis Toxin

The S1 subunit of pertussis toxin (PTx), isolated from *Bordetella pertussis*, possesses ADP-ribosyl transferase activity and catalyses the transfer of an ADP-ribose moiety of NAD^+ to a specific amino acid residue (Fields and Casey, 1997). For $G\alpha_i$ proteins the target residue for modification is a cysteine found near the C-terminal, which is not present on other $G\alpha$ subtypes (Wess *et al.*, 1995) and this covalent modification prevents G-protein interaction with the cognate, activated heptahelical receptors. The uncoupling of receptor from a functional pathway, following the pre-incubation with PTx is therefore evidence that $G\alpha_{i/o/t}$ proteins are intermediaries (Mumby, 1999).

1.1.2.2.3. $G\alpha_q$ subunit

The $G\alpha_q$ family comprises 5 members, $G\alpha_q$, $G\alpha_{11}$, $G\alpha_{14}$, $G\alpha_{15}$, and $G\alpha_{16}$, whose α subunits are expressed from individual genes and possess differential expression patterns (Offermanns, 2003). $G\alpha_q$ and $G\alpha_{11}$ are widely expressed in mammalian tissues, although the expression of $G\alpha_q$ often exceeds that of $G\alpha_{11}$ (Offermanns, 2003). The expression of $G\alpha_{14}$, the murine $G\alpha_{15}$ and its human counterpart $G\alpha_{16}$ is restricted to stromal and epithelial cells and also to those found in the kidney, testis and lung (Morris and Malbon, 1999). All five members are notable regulators of the β -isoenzymes of phosphoinositide-specific phospholipase Cs, leading to hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2), generating inositol 1,4,5-trisphosphate (IP_3) and *sn*-1,2-diacylglycerol (DAG) and

subsequently causing a rise in intracellular calcium levels and activation of protein kinase Cs, respectively (Exton, 1996,1997; Rhee, 2001). More recently $G\alpha_q$ (and its cognate $\beta\gamma$ dimer) has been shown to directly modulate the activity of a novel delayed rectifier-like K^+ current (I_{KM3}) via the muscarinic M_3 mACh receptor in canine atrial myocytes (Shi *et al.*, 2004). The extent of (novel) ion-channel modulation directly by $G\alpha$ subunits from the $G\alpha_{q/11}$ -family remains to be explored.

One salient feature of this PTx resistant class of G-protein is the relative slow intrinsic GTPase activity when compared to some other $G\alpha$ subunit types (Fields and Casey, 1997). Their k_{cat} value for GTP hydrolysis is typically between 10-15 times lower than that for $G\alpha_i$ (or $G\alpha_s$) subunits, and this may have a structural basis in the so-called *GAGES* region in the nucleotide-binding domain, which is found only in the $G\alpha_q$ family (Fields and Casey, 1997). The observation that signalling mediated via the $G\alpha_q$ family is rapidly 'switched-off', led to a search for proteins that may modulate the intrinsic GTPase activity. PLC- β_1 was shown to act as GTPase accelerating protein (GAP) for $G\alpha_q$, accelerating the rate of bound GTP by over a 1000-fold (Berstein *et al.*, 1992, Mukhopadhyay and Ross, 1999). In addition to some effector molecules, a family of proteins known as regulators of G-protein signalling (RGS proteins) have been shown to augment the GTPase activity of $G\alpha$ subunits (Iyengar, 1997; Kozasa, 2001; Rebois and Hebert, 2003). Apart from a highly conserved domain termed the RGS box, the RGS family are highly diverse in size and structure which may permit these proteins to play a role in signalling specificity (Brady and Limbird, 2002). RGS proteins have been demonstrated to stimulate the rate of GTP hydrolysis of the $G\alpha$ subunit by over 40 fold (Hunt *et al.*, 1996), but may also attenuate signalling by directly competing for $G\alpha$ effector activity (Brady and Limbird, 2002).

The physiological relevance of the diversity of the $G\alpha_q$ family is not completely understood, as GPCRs activating this pathway do not appear to discriminate between $G\alpha_q$ and $G\alpha_{11}$ (Offermanns, 2003). Given the restricted expression pathway of $G\alpha_{14/15/16}$ it is perhaps surprising that mice possessing inactivating mutations of each of these $G\alpha$ subtypes, showed no obvious phenotypic changes (Offermanns, 2003). $G\alpha_{11}$ deficient mice

also appear normal, whilst $G\alpha_q$ deficient mice exhibit motor co-ordination and functional cerebellar cortex defects (Offermanns, 2003).

1.1.2.2.4. $G\alpha_{12/13}$ subunit

The $G\alpha_{12/13}$ family consists of the ubiquitously expressed $G\alpha_{12}$ and $G\alpha_{13}$ who share 67% amino acid identity (Gudermann *et al.*, 1997). The effectors regulated by these $G\alpha$ subunits are poorly characterised, although they have been shown to activate both the c-Jun NH2-terminal kinase (JNK) (Prasad *et al.*, 1995) and phospholipase D (Rumenapp *et al.*, 2001) pathways and to regulate different isoforms of the Na^+/H^+ exchanger (Lin *et al.*, 1996; Wadsworth *et al.*, 1997). The precise functions of the $G\alpha_{12/13}$ family are still incompletely understood, although activated $G\alpha_{12/13}$ has been shown to lead to stress-fibre formation (Gohla *et al.*, 1999), blood vessel development in mouse embryogenesis (Gu *et al.*, 2002), and oncogenic transformation of fibroblast cell-lines (Dhanasekaran and Dermott, 1996; Dhanasekaran *et al.*, 1998; Dermott and Dhanasekaran, 2002).

1.1.2.3. Signaling via the $\beta\gamma$ subunit

Five distinct $G\beta$ subunits and 12 distinct $G\gamma$ subunits have been identified (Schwindinger and Robishaw, 2001). Although it is still unclear what combinations of $\beta\gamma$ subunits form physiologically, it is now widely appreciated that these dimers are signaling molecules in their own right (Gudermann *et al.*, 1997). One major question to be addressed is how effectors modulated by $\beta\gamma$ dimers maintain specificity (Bunemann *et al.*, 2003) as there appears to be little difference between various $\beta\gamma$ combinations with regard to their ability to regulate effector enzymes (Clapham and Neer, 1997). $\beta\gamma$ dimers have been implicated in the modulation of G-protein regulated inwardly rectifying K^+ channels (GIRKs), particular isoforms of adenylyl cyclase and phospholipase C, and voltage-operated calcium channels (Exton, 1994; Sunahara *et al.*, 1996; Yamada *et al.*, 1998; Zamponi and Snutch, 1998a,b; Simonds, 1999; Offermanns, 2003). Additionally, we are just beginning to appreciate the potential role for the $\beta\gamma$ dimer in stimulating proliferation via activation of MAP-kinase pathways and in cell survival by the modulation of phosphatidylinositol-3-kinase (PI3K) (Vanhaesebroeck *et al.*, 2001; Schwindinger and Robishaw, 2001).

1.1.3. Major effectors modulated by G α subunits

The diversity of intracellular enzymes, receptors and other proteins regulated by G α subunits is extensive and a full description is beyond the scope of this Introduction. This section therefore focuses on the two main targets for G α_q and G α_i activation (which are the main G-proteins discussed within this Thesis), phospholipase C and adenylyl cyclase. For further discussion on other proteins modulated by G-proteins, such as ion channels and the MAP-kinase pathways, please see Dascal, 2001; Marinissen and Gutkind, 2001 for recent reviews.

1.1.3.1. Phospholipase C- β

There are four main families of PLCs classified as PLC- β , PLC- γ , PLC- δ , and PLC- ϵ with multiple isoforms in each class (Morris and Malbon, 1999). The recently characterised PLC- ϵ contains two Ras-binding domains and a Ras-specific guanine nucleotide exchange factor (GEF) domain, and may link signalling by heterotrimeric G-proteins and Ras-related GTPases (Kelley *et al.*, 2001; Evellin *et al.*, 2002). All four families share common structural features, with conserved domains for substrate binding and catalysis, however only isoforms of PLC- β are directly regulated by heterotrimeric G-proteins (Exton, 1997; Rhee, 2001). PIP₂ is the major substrate for PLC- β , and the products of its hydrolysis, IP₃ and DAG, are both intracellular second messenger molecules (Berridge, 1984).

There are 4 isoforms of PLC- β and transient expression of these proteins along with the G α subunits indicates differences in the susceptibility of individual PLC- β s to activation by G α_q (Wu *et al.*, 1992,1993a,b). Although much debated, the order of sensitivity to activation by G $\alpha_{q/11}$ for the PLC- β isoforms is reported as PLC- $\beta_1 \geq$ PLC- $\beta_3 >$ PLC- $\beta_4 >$ PLC- β_2 (Rhee, 2001). The other members of the G α_q family show a preference for the activation of PLC- β_2 , which shows a similar limited pattern of expression (Morris and Malbon, 1999). PTx sensitivity of the PLC- β -mediated response can be demonstrated in some systems (Boyer *et al.*, 1989,1992) and this has been attributed to activation by $\beta\gamma$ subunits released from G_{i/o} heterotrimers (Boyer *et al.*, 1994; Paterson *et al.*, 1995; Waldo *et al.*, 1996). The rank order of PLC- β isoform responsiveness to $\beta\gamma$ subunit activation is

reported as PLC- $\beta 3 > \text{PLC-}\beta 2 > \text{PLC-}\beta 1$, with little or no PLC- $\beta 4$ sensitivity (Stehno-Bittel *et al.*, 1995; Morris and Malbon, 1999). The overall sensitivity of the PLC- β s to $\beta\gamma$ subunits appears to be less than $G\alpha$, as the concentration of agonist required for half-maximal activation tends to be at least two orders of magnitude higher (Exton, 1997).

1.1.3.1.1. Inositol 1,4,5-trisphosphate: signalling and metabolism

The activation of PLC catalyses the hydrolysis of PIP_2 , one of the two products of this reaction is IP_3 . IP_3 is able to diffuse through the cytosol and bind to IP_3 receptors on the surface of the endoplasmic reticulum (ER) (Kiselyov and Muallem, 1999; Thrower *et al.*, 2001). The ER is a cellular store of Ca^{2+} and binding of IP_3 leads to opening of the IP_3 -gated channel and subsequent Ca^{2+} release. The IP_3 receptor is both inhibited and stimulated by Ca^{2+} , and therefore the spatial and temporal aspects of Ca^{2+} release are subject to tight regulation through positive and negative ‘feedback’ regulation (Thrower *et al.*, 2001).

The regeneration of inositol, required for re-incorporation into lipids including PIP_2 , is achieved via a complex network of chemical reactions, with some of the intermediary molecules also implicated in cell signalling (Hilton *et al.*, 2001; Irvine and Schell, 2001).

Fig. 1.2. (adapted from Attack *et al.*, 1995; Hancock, 1997; Williams and Harwood, 2000; Irvine and Schell, 2001) illustrates a simplified overview of the phosphoinositide cycle. Whilst the recycling of DAG is relatively straightforward the inositol polyphosphate pathways are complex and can proceed along at least two major routes. After the production of IP_3 , this signalling molecule can be sequentially dephosphorylated, the ultimate step being the dephosphorylation of inositol monophosphates by the enzyme inositol monophosphatase (IMPase). Alternatively IP_3 may be phosphorylated to form inositol 1,3,4,5-tetrakisphosphate (IP_4), via IP_3 3-kinase, which is itself regulated by Ca^{2+} and modulated by PKC and/or PKA phosphorylation (Hancock, 1997). A signalling role for IP_4 has been suggested on the basis of its apparent ability to stimulate Ca^{2+} influx (Cullen, 1990; Luckhoff and Clapham, 1992). IP_4 is also sequentially dephosphorylated to inositol before reincorporation into the inositol phospholipid pool.

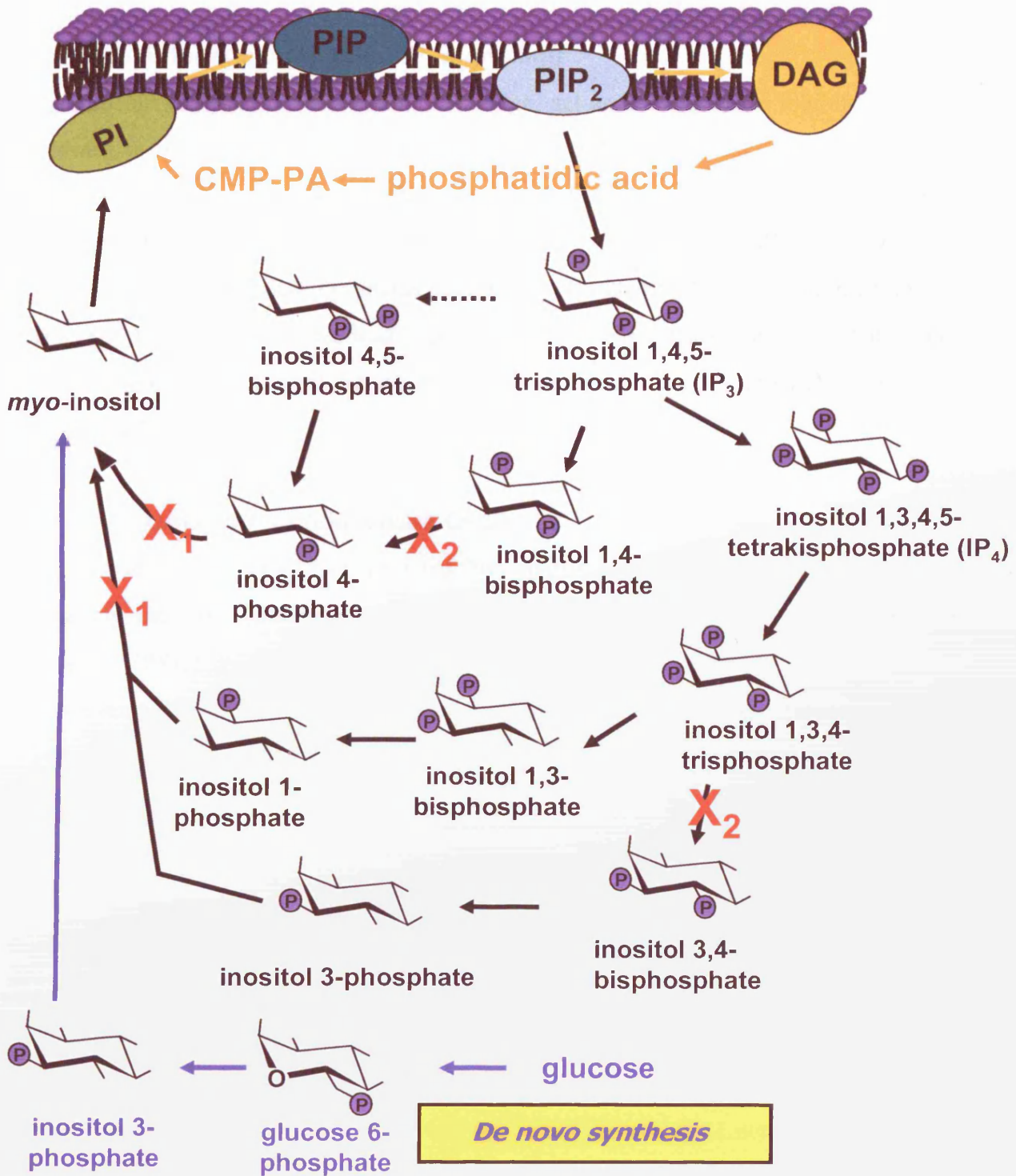


Fig. 1.2 The phosphoinositide cycle

Simplified overview of the phosphatidylinositol 4,5-bisphosphate (PIP₂)/inositol 1,4,5-trisphosphate (IP₃)/diacylglycerol (DAG) signalling pathway. DAG is metabolized via phosphatidic acid to cytidine monophosphorylphosphatide (CMP-PA) before incorporation into inositol phospholipids (orange). The re-cycling of IP₃ is more complex, and occurs via either sequential dephosphorylation to IP₂, or via phosphorylation to IP₄. Li⁺-sensitive steps are indicated by with an X_x (For details please see section 1.1.3.1.1.).

1.1.3.1.2. Effects of lithium on IP₃ signalling

Lithium ions (Li⁺), in the low millimolar range, act as uncompetitive inhibitors of two enzymes in the inositol polyphosphate recycling pathway (Nahorski *et al.*, 1991). Li⁺ inhibits both IMPase (X₁) and inositol polyphosphate 1-phosphatase (X₂), both of which are key enzymes necessary for the recycling (and in the case of IMPase the *de novo* synthesis) of *myo*-inositol (Williams and Harwood, 2000). Assays performed in the presence of Li⁺, will therefore lead to an accumulation of inositol monophosphates (and some inositol bisphosphates) that can be used as an index of PLC activity (Wojcikiewicz and Nahorski, 1993).

1.1.3.1.3. DAG and protein kinase Cs (PKCs)

The second product of PLC-β-mediated PIP₂ hydrolysis, DAG is also an intracellular signalling molecule (Berridge, 1984). DAG is implicated in the activation of protein kinase Cs (PKC), as well as a number of other proteins (e.g. protein kinase D, RasGRP (Ras guanine nucleotide-releasing protein), MUNC-13 and a subset of DAG kinases) (Yang and Kazanietz, 2003). All of these DAG-regulated proteins possess C1 domains that bind DAG resulting in their activation.

Protein kinase Cs represent a large family of at least 12 serine/threonine kinases encoded by 12 distinct genes (Mellor and Parker, 1998). They are divided into three subfamilies based on their structure and substrate requirements (Way *et al.*, 2000). Group I or conventional PKCs (cPKC) contain α, βI, βII and γ isoenzymes and are Ca²⁺-dependent and activated by both DAG and phosphatidylserine (PS). Group II, also known as the novel PKCs (nPKC), comprises the δ, ε, η and θ isoenzymes, these are also regulated by DAG and PS, but are Ca²⁺-independent. The last group are termed atypical PKCs (aPKC) are neither Ca²⁺- nor DAG-dependent, although they are regulated by PS. Members in this group include the ζ and λ isoenzymes.

In general, PKC isoenzymes are widely distributed, although some isoenzymes exhibit a restricted tissue expression. Each PKC is a single polypeptide with an *N*-terminal regulatory region and a *C*-terminal catalytic domain. The polypeptide structure comprises

four conserved (C1-C4) and five variable (V1-V5) domains. Regions C1 and C2 are required for membrane association and also for interaction with DAG, Ca^{2+} and PS. As few selective pharmacological agents exist the precise functions of each isoenzyme has been difficult to ascribe, although through the use of antisense oligonucleotides, short interfering double stranded RNA (siRNA) and over-expression of dominant-negative forms, progress is being made (Yang and Kazanietz, 2003). The phosphorylation targets of PKC isoenzymes include proteins involved in cell proliferation, differentiation, survival, transformation and apoptosis, and a key factor in isoenzymic specificity is likely to be PKC compartmentalization through association with PKC-interacting proteins (Yang and Kazanietz. 2003).

1.1.3.2. Regulation of adenylyl cyclases

Molecular cloning has allowed the identification of at least nine isoenzymes of the membrane-spanning effector, adenylyl cyclase (AC). The AC structure comprises an intracellular *N*-terminal region followed by a bundle of 6 transmembrane-spanning α -helices forming a membrane-anchoring region (M1), a large cytoplasmic domain (C1) followed by a second bundle of 6 transmembrane helices (M2) and another cytoplasmic domain (C2) leading to the intracellular *C*-terminus (Simonds, 1999). The C1 and C2 domains share a high degree of homology with adenylyl cyclases from yeast and various guanylyl cyclases. The C1 and C2 domains interact with each other in a 'head-to-tail' manner and together are presumed to form the catalytic site, as point mutations in either C1 or C2 domain can abolish enzymatic activity (Sunahara, *et al.* 1996).

All of the cloned mammalian ACs are activated by $\text{G}\alpha_s$ and all but AC9 are also robustly activated by the plant diterpene forskolin (Simonds, 1999). There appears to be little difference between the AC isoenzymes with respect to their quantitative or qualitative response to $\text{G}\alpha_s$ activation. The activation of AC leads to the hydrolysis of ATP to generate adenosine 3'-5'-cyclic monophosphate (cAMP) and pyrophosphate (PP_i). Cyclic AMP levels directly regulate the activity of protein kinase A (PKA) leading to the phosphorylation of target proteins. The duration of signal is governed, in part, to a group

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of enzymes termed cyclic nucleotide phosphodiesterases (PDEs) that hydrolyze cyclic AMP to 5'-AMP.

$G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$, $G\alpha_o$ and $G\alpha_z$ have been shown to interact with AC (at a poorly characterized binding site, distinct from the $G\alpha_s$ binding site) to cause inhibition of AC activity. However, unlike $G\alpha_s$ it appears that the different isoenzymes of AC have different sensitivities to $G\alpha_i$ mediated inhibition, allowing a degree of signal discrimination/integration at the level of the effector (Simonds, 1999). In short, recombinant expression studies have shown that AC isoenzymes 3, 5 and 6 are sensitive to $G\alpha_i$ mediated inhibition, whereas the forskolin or Ca^{2+} /calmodulin, rather than the $G\alpha_s$, mediated activation of AC1 can also be inhibited by $G\alpha_i$ -type α subunits. $\beta\gamma$ -subunits can also modulate the activity of particular isoenzymes of AC, however this modulation is conditional on co-incident activation by $G\alpha_s$ and may also serve as a mechanism to integrate signals that would normally be insufficient to affect AC activity (Simonds, 1999). In the presence of $G\alpha_s$, $\beta\gamma$ -dimers have been shown to inhibit AC1, while they stimulate AC2 and AC4, and to a lesser extent AC7.

As well as different tissue expression patterns, specificity of AC isoenzymic activation is also achieved via differential sensitivity to regulatory proteins. ACs are important targets for regulation by PKA (providing a modulatory feedback control), PKC, Ca^{2+} and Ca^{2+} /calmodulin. For example, experimental evidence indicates that only isoenzymes AC5 and AC6 are sensitive to PKA regulation, whereas phosphorylation by PKC seems to enhance activities of AC1, AC2, AC3 and AC7, but to inhibit AC4 (Sunahara *et al.*, 1996). The differential regulatory properties of the AC isoenzymes means that both specificity and diversity of cell signalling can be achieved not only from $G\alpha_s$ / $G\alpha_i$ -coupled receptors, but also by integrating signals from GPCRs, ion channels and receptor tyrosine kinases linked to PLC-activation and Ca^{2+} mobilization.

1.1.4. Factors regulating the specificity of G-protein signalling

1.1.4.1. Scaffolding proteins

It was initially proposed that receptor activation of G-proteins occurred in a 'random-transient collision' manner (Chidiac, 1998). However, a growing body of experimental evidence has shown that two distinct receptors expressed in the same cell, can elicit agonist-mediated coupling to quite different cellular responses even though they are coupled to the same G-protein sub-population(s) (Graeser and Neubig, 1993). This had lead investigators to propose that there exists either a compartmentalization of receptors and effector molecules within subcellular micro-domains, or a translocation of receptors between cellular compartments (Brady and Limbird, 2002; Marinissen and Gutkind, 2001; Albert and Robillard, 2002; Ostrom, 2002; Rebois and Hebert, 2003). The list of proteins implicated in the higher structural organisation of receptors, G-proteins and effector molecules grows weekly, and this added complexity is further compounded by the observation that many receptor-interacting proteins or 'scaffolding-proteins' may have signalling properties themselves.

Scaffolding proteins provide a structural link between receptor, G-protein and effector and maintain, at least in part, the integrity of intracellular signalling pathways (Hur and Kim, 2002; Itoh and DeCamilli, 2000). A recent review suggested that scaffolding proteins may interact with receptors and effectors to perform four specialized functions (Hall and Lefkowitz, 2002): *(i)* interact directly to mediate receptor signalling; *(ii)* to control receptor localization and/or trafficking; *(iii)* to act as allosteric modulators of receptor conformation to alter receptor function (and/or pharmacology), and finally *(iv)* to act as scaffolds physically linking the receptor to various effector components.

Scaffolding proteins have been implicated in the maintenance of cell signalling integrity of the well-characterized pathways activated by the β_2 -adrenoceptor. Gravin (AKAP-250) is a scaffolding protein that displays increased association with the β_2 -adrenoceptor upon agonist binding. Suppression of Gravin expression using antisense oligonucleotides disrupts agonist-dependent association of the receptor with GRK2, β -arrestin and clathrin,

as well as altering the kinetics of receptor recovery from desensitization (Lin *et al.*, 2000). Lavine *et al.*, (2002) demonstrated stable association of the β_2 -adrenoceptor with adenylyl cyclase and Kir3 ion channels. The formation of such signalling complexes is likely to involve many scaffolding proteins and may provide a way of ensuring intracellular signal specificity (Bray, 1998).

A dramatic effect of a receptor-scaffold protein interaction would be an alteration in the specificity of G-protein coupling. The D₁ dopamine receptor couples to G α_s to mediate activation of adenylyl cyclase. A glycosylated 24-kDa transmembrane protein called Calcyon interacts with a 16 amino acid domain located in the C-terminal region of the D₁ receptor. In the presence of Calcyon the D₁ receptor is able to mediate robust Ca²⁺ activation, which has been proposed to be due to an increase in receptor interaction with G $\alpha_{q/11}$ (Lezcano *et al.*, 2000). The significance of differential accessory protein expression in the altered pharmacology of receptors in different cell background remains to be established.

It is likely that hundreds of proteins will be identified with the potential to act as scaffolding proteins, whilst some are likely to be ubiquitously expressed, many may be cell or receptor-effector specific. Therefore the same receptor may interact with different scaffolding proteins in different cell-types (Milligan and White, 2001) which may further complicate the interpretation of data obtained in different cellular backgrounds (Yu and Rasenick, 2002).

1.1.4.2. Microdomains and translocation

The plasma membrane is not a homogeneous lipid bilayer, but contains areas that are enriched in certain lipids (e.g. cholesterol) and proteins. Caveolae are key components of the plasma membrane and along with so-called 'lipid-rafts' form specialized microdomains that can concentrate signalling-protein complexes. It has been suggested that some receptors may translocate between these signalling domains and this may be responsible for some of their G-protein coupling properties (Ostrom *et al.*, 2002). The β_1 - and β_2 -adrenoceptors are reportedly concentrated in caveolin-rich areas within the plasma

membrane within cardiac myocytes (Ostrom *et al.*, 2000, Ostrom *et al.*, 2001). The effector enzyme AC6 is almost exclusively expressed within these microdomains. The β_1 - and β_2 -adrenoceptors robustly activate AC6 via $G_s\alpha$, whilst prostanoid receptors, which couple to the same $G\alpha$ subunit, but are excluded from these microdomain, cannot (Ostrom *et al.*, 2001). Interestingly, the β_2 -adrenoceptor is less efficacious than the β_1 - and this has been attributed to its movement (translocation) out of the microdomain subsequent to agonist-exposure, whilst the β_1 -adrenoceptor remains within the microdomain (Ostrom *et al.*, 2001).

1.1.4.3. Phosphorylation, endocytosis and G-protein coupling

Activated GPCRs are phosphorylated by a family of proteins, termed the G-protein coupled receptor kinases (GRKs). Subsequent to phosphorylation the receptors bind another family of proteins called β -arrestins. These generally bind to the third intracellular loop and the region of the C-terminal domain closest to the membrane (Ferguson *et al.*, 1998a; Zhang *et al.*, 1997; Hall *et al.*, 1999). This results in the desensitization of the GPCR and also targets the receptor for internalization through β -arrestin-mediated interactions with clathrin (Bockaert and Pin, 1999). It is beyond the scope of this general introduction to detail all the proteins and mechanisms involved in the desensitization and endocytic process and many excellent reviews exist (see Koenig and Edwardson, 1997; Ferguson *et al.*, 1998b; Ferguson, 2001; Maxfield and McGraw, 2004). However, an emerging view is that binding by β -arrestins, and other proteins, not only terminates existing G-protein coupling events, but also can initiate a new set of signalling outputs (Hall *et al.*, 1999). This has led to the appreciation of an unsuspected diversity of cellular signalling by GPCRs, and furthermore to the demonstration that some pathways can be activated independently of G-proteins (Marinissen and Gutkind, 2001).

By acting as scaffolding proteins, arrestins have also been shown to facilitate the formation of multi-protein complexes leading to the activation of several MAP-kinase pathways (Van Biesen *et al.*, 1996; McDonald *et al.*, 2000). Binding of β -arrestin to the β_2 -adrenoceptor has been shown to recruit the tyrosine kinase, Src (Luttrell *et al.*, 1999), and this in turn leads to the activation of specific MAP-kinase pathways (Hall and

Lefkowitz, 2002). Arrestins also interact with proteins involved in the machinery of endocytosis and it has been suggested that this may be a mechanism by which receptors can regulate the endocytic process (Hall and Lefkowitz, 2002).

The majority of GPCRs are phosphorylated at serine and threonine residues that are often arranged in clusters within the third intracellular loop and the C-terminal domains (Tobin, 2002). Agonist-occupied receptors initiate complex and incompletely understood, hierarchical and sequential phosphorylation of these residues. This can be mediated by either second-messenger-dependent kinases or G protein-coupled receptor kinases (GRKs), with phosphorylation serving as signal that leads to the desensitization and internalization of the receptor. However, it has recently been proposed that the phosphorylation of the receptor may also initiate a new set of signalling events that are distinct from those regulated by the initial receptor-G protein coupling event (Pierce *et al.*, 2001; Pierce and Lefkowitz, 2001).

Perhaps the best example of phosphorylation-dependent coupling has been obtained from studies on the β_2 -adrenoceptor. When expressed in a HEK-293 cell line the β_2 -adrenoceptor couples robustly to activate adenylyl cyclase activity via $G\alpha_s$. Daaka *et al.*, (1997) demonstrated that this leads to PKA-mediated phosphorylation of specific serine residues in the third intracellular loop of the β_2 -adrenoceptor. This phosphorylation event appears to switch the coupling specificity of the receptor away from $G\alpha_s$ to activate $G\alpha_i$, and the released $\beta\gamma$ subunits can stimulate ERK1/2 activation. Subsequent experiments showed that the alanine replacement of specific serine residues produced a mutant β_2 -adrenoceptor that mediated robust cyclic AMP accumulation, but was unable to activate ERK (Zamah *et al.*, 2002). It has also been shown that a specific phosphodiesterase, PDE4, is recruited to the β_2 -adrenoceptor signalling complex via intermediary β -arrestin binding and this is also essential for the G-protein 'switching mechanism' (Baille *et al.*, 2003).

These data suggest that phosphorylation-dependent G-protein switching brings about receptor desensitization with respect to $G\alpha_s$ coupling, but also initiates a second wave of

signalling via other $G\alpha_i$ -dependent pathways (Daaka *et al.*, 1997). However, the prevalence of G-protein switching is presently uncertain, as other groups have been unable to find experimental support for phosphorylation-dependent G-protein coupling changes. Other groups have demonstrated that ERK activation (subsequent to β_2 -adrenoceptor activation) occurs with $G\alpha_s$ as an intermediary leading to the PKA mediated activation of the small G-protein Rap-1 and the serine – threonine kinase B-raf (Schmitt and Stork, 2000; Friedman *et al.*, 2002). It has been suggested that clonal variation and cell-line differences (of HEK-293 cells) may be responsible for the different effector activation in these experiments (Lefkowitz *et al.*, 2002). However it is appreciated that the over expression of receptors can lead to GPCR-coupling promiscuity (Wenzel-Seifert and Seifert, 2000) and so it remains to be firmly established whether the dual-coupling of the β_2 -adrenoceptor is phosphorylation dependent or as a result of altered receptor:G-protein stoichiometry (Hill and Baker, 2003).

Finally, there is also evidence for protein kinases, other than the second messenger-regulated kinase and GRK families, phosphorylating receptors which may modify their signalling activity. Casein kinase 1 α (CK1 α) is a single polypeptide protein distinguished by relatively short *N*- and *C*-terminal extensions around the catalytic domain. It has been identified as a kinase capable of phosphorylating the M_3 mACh receptor in an agonist-dependent manner (Tobin, 2002). Furthermore, it has been demonstrated that over-expression of a kinase-dead, dominant-negative CK1 α mutant results in a dramatic decrease in the ability of the M_3 mACh receptor to activate ERK1/2 while its ability activate $G\alpha_{q/11}$ is largely unaffected (Budd *et al.*, 2001). These authors suggested that there are two mechanisms to activate ERK via the M_3 receptor, one dependent on the coupling to $G\alpha_{q/11}$ and one independent of G-protein coupling, but dependent on receptor-phosphorylation by CK1 α . The possibility that signalling can be regulated not only via G-protein coupling, but also by other distinct proteins (including novel receptor kinases as well as scaffolding proteins) is an exciting development in cell signalling.

1.2. The muscarinic acetylcholine receptor family

Acetylcholine (ACh) was the first chemical to be firmly established as a neurotransmitter and its physiological effects are mediated by both ionotropic and metabotropic receptors (Gainetdinov and Caron, 1999). This biogenic amine is the principal neurotransmitter of the parasympathetic nervous system where it is released at ganglionic synapses and parasympathetic post-ganglionic neuroeffector junctions (Eglen *et al.*, 1996a). Nicotinic ACh receptors are ligand-gated ion channels that exert their effects via changes in membrane potential (Felder *et al.*, 2000), and are not discussed further in this work. The metabotropic mACh receptors are heptahelical membrane-spanning glycoproteins that are encoded by five distinct but related, intronless genes (Hulme, 1990; Hulme *et al.*, 1990; Eglen and Nahorski, 2000). The products of the genes have been designated by the nomenclature M₁-M₅ (Caulfield and Birdsall, 1998) and the pharmacology, tissue-expression and therapeutic targeting of these receptors is briefly summarized in this section.

1.2.1. Muscarinic acetylcholine receptors: pharmacology and coupling

The mACh receptors possess a sequence (and putative structure) similar to that of the well characterized rhodopsin molecule, which is comprised of seven hydrophobic α -helical domains spanning the plasma membrane connected by hydrophilic intracellular and extracellular loops (Felder *et al.*, 2000). The high degree of amino acid sequence homology between the subtypes probably contributes the frustrating pharmacology of this receptor family, where few selective agonists and antagonists have been reported to date (Eglen *et al.*, 1994a,b; Gainetdinov and Caron, 1999; Eglen and Nahorski, 2000). The heterogeneity of mACh receptor expression in native tissues, coupled with the lack of receptor subtype-specific ligands has meant that assigning particular physiological functions to a precise subtype(s) has been difficult (Hosey, 1992).

The cloning and subsequent expression of the five receptors in recombinant systems has greatly facilitated our understanding of the major pathways modulated by these receptors (Bonner *et al.*, 1987; Bonner *et al.*, 1988). Although displaying a high degree of homology between subtypes, the amino acid sequence is highly divergent in the third

intracellular loop and as such is probably accounts for the different G-protein coupling properties of the subtypes (Eglen, 1996a). A simplified view of their coupling properties is that the even numbered M_2/M_4 couple primarily to the $G\alpha_i$ family to inhibit adenylyl cyclase activity, whilst the odd numbered $M_1/M_3/M_5$ receptors couple preferentially to the activation of phosphoinositide hydrolysis, via $G\alpha_q$ -like G-proteins (Eglen *et al.*, 1999). The coupling of subtypes to these pathways is by no means exclusive, especially in highly expressing recombinant systems (Eglen *et al.*, 1996b). Other G-proteins activated by mACh receptors and the plethora of pathways modulated is discussed further in the Results sections.

Although the mACh receptor ligands pilocarpine, muscarine and atropine have been known and employed for over a century there has been relatively few advances in the development of subtype-specific agonists or antagonists (Felder *et al.*, 2000). Although no ligands with absolute selectivity have been described there are both agonists and antagonists that possess relative (functional) selectivity. Agonists and antagonists with reported selectivity of greater than ten-fold are shown in **Table 1.2**. Most of data have been obtained from recombinant receptors expressed in surrogate cells. Data from such model systems must be treated with caution as factors such as membrane lipid composition; glycosylation and palmitoylation can cause discrepancies in affinity values for ligands at receptors expressed in different environments.

Table 1.2. Muscarinic receptor distribution, agonists and antagonists

	Distribution	Agonists	Antagonists*
M₁	- widely distributed in the CNS, especially in forebrain areas including the cerebral cortex and hippocampus and striatum. - autonomic ganglia and certain secretory glands, e.g. submaxillary gland. - high density in vas deferens	McN343 reported to show some selectivity	pirenzepine telenzepine MT-7 ^A

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M₂	- relatively low levels in CNS – however high restricted expression in pons/medulla and cerebellum. - high density in the heart and smooth muscle, e.g. bladder and ileum - sympathetic ganglia	n/a	AQ-RA 741 ¹ methoctramine himbacine ² AF-DX116 ³ AF-DX 384 ⁴ tripitramine (S)-(+)-dimethindene malate ⁵
M₃	- distributed widely in neuronal cells where expression pattern overlaps with M₁ (although at significantly lower levels). - smooth muscle, e.g. urinary bladder, trachea, ileum. - lung. - peripheral ganglia, exocrine glands, submaxillary gland.	n/a	pFHHSiD darifencin
M₄	- limited CNS expression, mainly in hippocampus and striatum. - high expression in lung of some species	n/a	MT-3 ^A himbacine ² PD 102807 AQ-RA 741 ¹ AF-DX 384 ⁴ tropicamide
M₅	- limited data, but low expression in the CNS, including substantia nigra and cerebellum.	n/a	

*Selectivity on the basis of mean affinity values (based on pK_i and pK_b estimates) taken from Caulfield and Birdsall, (1998); Eglen *et al.*, (1999); Eglen and Nahorski, (2000); Wess, 2003; GPCR facts-book (author, year); www.komabiotech.co (2004). ^ASnake toxins originally extracted from the mamba; ¹also moderately selectivity for M₄; ²M₂/M₄ selective; ^{3/4}less than ten-fold selective; ⁵less than ten-fold selective over M₁.

1.2.2. Expression profile and physiological function

The mACh receptor subtypes are widely expressed in neuronal and non-neuronal cells (Saffen *et al.*, 1999) where they have been implicated in such diverse physiological functions as heart rate, cognition, sensory processing, motor control, muscular tone and smooth muscle contraction (Caulfield, 1993; Eglen *et al.*, 1994b; Caulfield and Birdsall, 1998; Nathanson, 2000). The expression of the individual subtypes has been shown to be under the control of elements (Mieda *et al.*, 1996; Mieda *et al.*, 1997), such as neuron-restrictive silencer element/restriction element-1 (NRSE/RE-1) (Saffen *et al.*, 1999). Therefore, cells possessing different promoter binding proteins can exhibit divergent and developmentally regulated mACh receptor subtype expression profiles (Nadler *et al.*, 1999). The lack of subtype-specific agonists and the paucity of absolutely selective antagonists have made ascribing physiological functions to specific subtypes difficult. The development of transgenic mice lacking individual mACh receptors has greatly aided this task (Eglen *et al.*, 2001a). The main conclusions drawn so far from studies of knockout mice are summarized in **Table 1.3.**, however caution should be taken in the interpretation of transgenic phenotypes, due to receptor redundancy, compensatory mechanisms, and specific species differences (Wess, 2003; Wess *et al.*, 2003). However, these results give some insights into the major physiological processes regulated by mACh receptors. Each of the major tissues expressing mACh receptor subtypes and the potential for therapeutic intervention is briefly summarized below:

Table 1.3. mACh receptor functions delineated from genetically modified mice

Genetic model	Functional responses to agonists and phenotypic effects
M₁ k/o	- impaired pilocarpine-induced seizures - lack of M current K⁺-channel modulation in sympathetic neurones - loss of agonist-induced oscillatory network activities at γ frequencies - decreased activation of PLC and MAPK in forebrain - reduction in agonist-mediated phosphoinositide hydrolysis in primary cortical neurones - reduced hippocampal-based memory and learning (however, this phenotype maybe related to the hyperactivity observed –see below) - reduced long-term potentiation at Schaffer-collateral-CA1 synapses - loss of ‘slow’ regulation of Ca²⁺ channels in the superior cervical ganglion - loss of agonist-mediated circadian rhythm phase advance - pronounced increase in locomotor activity related to decreased inhibition of dopamine release
M₂ k/o	- impaired agonist-induced tremor/analgesia/hypothermia/akinesia - attenuated mACh receptor regulation of heart-rate (bradycardia) - (slight) reduction in potency of mACh receptor agonists to induce smooth muscle contraction and increased relaxant effects of forskolin and isoprenaline on agonist-mediated smooth muscle contractions - loss of autoreceptor-mediated regulation of ACh release and altered time-course of evoked ACh release at the neuromuscular junction - loss of ‘fast’ regulation of Ca²⁺ in the superior cervical ganglion (via N- and P/Q-type Ca²⁺ channels) - loss of agonist-mediated increases in serum corticosterone levels
M₃ k/o	- attenuated contractility of smooth muscle, e.g. ileum, stomach fundus, trachea and urinary bladder in response to agonist (however absolute reductions varied between 40-95% in different tissues)

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	- reduction in urinary bladder voiding and bladder distension (more prevalent in males) - retardation of neonatal growth - impaired salivation response (but with normal basal salivary flow) - impaired pupillary constriction - decreased body weight (associated with decreased food intake) and fat mass (associated with decrease in peripheral fat deposits and reductions and serum leptin, insulin and serum triacylglycerol levels) - reduced expression levels of melanin-concentrating hormone
M₄ k/o	- increased basal locomotor activity - increased D₁ dopamine receptor-mediated locomotor activity - preserved hypothermia, salivation, tremor, analgesia (although implicated as a minor pathway for mACh receptor-mediated analgesia) induced by agonist - absence of agonist-mediated inhibition of electrically stimulated noradrenalin release from vas deferens - reduced autoinhibition of ACh release in heart atria and urinary bladder
M₅ k/o	- impaired maintenance phase of secretory response to agonist (minor pathway for mACh receptor-mediated salivation) - increased drinking after water deprivation - large decrease in mACh receptor-mediated dopamine release (mainly in <i>Substantia nigra</i>) - impaired agonist-mediated dilation of cerebral arteries and arterioles - reduced rewarding effects of morphine administration

Data taken from Gainetdinov and Caron, (1999); Eglen *et al.*, (2001a); Birdsall *et al.*, (2001); Wess, (2003); Wess *et al.*, (2003).

1.2.3. Physiological role of mACh receptors and their therapeutic potential

The lack of subtype-specific mACh receptor agonists and antagonists has frustrated the development of therapeutically useful compounds. A number of drug candidates have been unsuccessful due to lack of selectivity leading to the high incidence of unwanted side-effects, which is compounded by a lack of understanding of the role of cholinergic transmission in disease. The common side-effects with antagonists include dry mouth, blurred vision, dizziness and tachycardia (Widzowski *et al.*, 1997), whilst those with agonists are reported as CNS disturbances, hypotension, bradycardia, gastrointestinal and genitourinary effects (Eglen *et al.*, 1999). The following section reviews tissues and systems in which normal cholinergic transmission is implicated, and the diseases where mACh receptor-directed therapy has potential or has already proved useful.

1.2.3.1. Smooth muscle

Acetylcholine released from parasympathetic neurons causes contraction of smooth muscle of the gastrointestinal and genitourinary tracts (Nathanson, 2000). This contraction modulates the direction and magnitude of peristaltic activity in the small intestine, colon and stomach and contraction in the bladder (Eglen, 2001b). Although the M₂ (and M₄) subtype seems to comprise the major population of smooth muscle mACh receptors (approx. 80%) the majority of the contractile response is almost invariably attributable to the minor M₃ mACh receptor population (Wallis and Napier, 1999), via the activation of phospholipase-C β and subsequent calcium mobilization (Ehlert, 2003). The role of the M₂ population is debatable, but a consensus is that activation of this subtype modulates the cyclic AMP-driven relaxation induced by the activation of other receptors e.g. β_2 adrenoceptors (Eglen and Watson 1996). This hypothesis is supported by the observation that forskolin and isoprenaline (a β -adrenoceptor agonist) exhibit increased relaxant effects on oxotremorine-M-mediated smooth muscle contractions in M₂ knockout mice (Matsui *et al.*, 2003). However, M₂ mACh receptors expressed in smooth muscle can also lead to opening of cation-selective channels (Zholos and Bolton, 1997; Kotlikoff *et al.*, 1999), sensitize myocyte contraction via Rho activation (Togashi *et al.*, 1998; Sohn, *et al.* 2000) and couple to p38 MAPKs (Yamboliev *et al.*, 2000; Zhou *et al.*, 2003). A role for M₂ receptor in a potentiation of the M₃ contractile response has been

suggested, via the Ca^{2+} dependent-activation of a non selective cation channel (I_{cat}) and inhibition of Ca^{2+} -activated K^+ channels (Ehlert, 2003). Additionally the contraction of human urinary bladder has been shown to mediated by the Ca^{2+} -entry activation of a Rho-kinase (Schneider *et al.*, 2004). This has led to the suggestion that the M_2 and M_3 mACh receptors mediate smooth muscle contraction via a sophisticated coupling network (Eglen, 2001), A role for the M_1 mACh receptor has also been demonstrated in the modulation of gastric acid secretion (Widzowski *et al.*, 1997).

Respiratory smooth muscle receives efferent cholinergic parasympathetic innervation from the vagus nerve, which initiates bronchoconstriction (Eglen, 1996). Similar to other smooth muscle this effect appears to be elicited from activation of the minor M_3 mACh receptor population via Ca^{2+} mobilization. The M_3 mACh receptor has also been implicated in the regulation of mucus secretion by submucosal glands and in chemotactic mediator release in alveolar macrophages (Billington and Penn, 2002). Therefore, many cellular pathways mediated by the M_3 mACh receptors are involved in the regulation of the resistance to airflow (Billington and Penn, 2002).

The central role of mACh receptors in smooth muscle contraction has lead to the development of compounds for gastrointestinal, genitourinary and respiratory disorders. Consequently, therapeutics have been designed for a number of smooth muscle disorders, such as overactive bladder, irritable bowel syndrome, chronic obstructive pulmonary disease (COPD) and muscle hyperactivity (Eglen *et al.*, 2001a).

1.2.3.2. Regulation in the heart

Activation of mACh receptors in guinea-pig heart preparations elicits a reduction in contractile force and a decrease in the rate of beating (bradycardia) (Caulfield and Birdsall, 1998). Knockout mice and antagonist binding studies have implicated a role for the M_2 mACh receptor mediating these effects probably via the inhibition of Ca^{2+} influx pathways and activation of inwardly-rectifying K^+ channels (Caulfield *et al.*, 1993; Bymaster *et al.*, 2001). Many studies have shown high and almost exclusive expression of

M₂ mACh receptors in mammalian heart (Levey, 1993), although other subtypes, especially M₃ mACh receptors (Wang *et al.*, 2004) could be involved.

1.2.3.3. Regulation in the central nervous system

The therapeutic targeting of muscarinic receptors in the central system has long been implicated in the treatment of Alzheimer's disease due to two particular aspects in pathophysiology of the condition (Growdon, 1997):

(1). The selective decrease of cholinergic neurones in ventral forebrain which form vital components of the ascending reticular activating system which are involved in memory function and attention are characteristic of Alzheimer's disease (Widzowski *et al.*, 1997; Broadley and Kelly, 2001). The major muscarinic receptor expressed in postsynaptic neurons which mediate cholinergic transmission, are proposed to be M₁. Although the neurons are damaged in the etiology of the disease, the M₁ population is unchanged are therefore a M₁ selective agonist have been suggested as a therapeutic relevant agent in the treatment of Alzheimer's (Eglen, 1999). M₂ receptors are also found on inhibitory autoreceptors for ACh release, and therefore the selective blockade of this prejunctional receptor may also be of therapeutic use (Eglen, 1999).

(2). The presence of amyloid plaques in some dying neurons of the brain are also associated with Alzheimer's disease. Amyloid deposits consist of aggregates of a peptide, a major component of which is β -amyloid peptide (A β). This peptide is derived from the cleavage of a family of glycoproteins termed amyloid precursor protein (APP) and is neurotoxic (Yankner *et al.*, 1990). M₁ agonists have been shown to reduce the secretion of A β *in vivo* (Hung *et al.*, 1993), probably via an increase in the PKC-dependent activation of a protease, α -secretase. This protease cleaves APP in the A β domain and therefore may prevent the deposition of the neurotoxic amyloid in the brain (Broadley and Kelly, 2001).

Although current therapy targeted against slowing the progression of Alzheimer's disease is limited to the employment of anticholinesterases, several reported M_1 -selective agonists are in clinical trials (Eglen, 1999).

1.2.3.4. Regulation in other peripheral tissues

The parasympathetic nervous system innervates the sphincter pupillae muscle of the iris and ciliary-body which alters the curvature of the lens (Broadley and Kelly, 2001). The contraction of the iris in response to bright light constricts the pupil, whilst contraction of the ciliary muscle facilitates near vision. Both responses are proposed to be mediated via the M_3 mACh receptor although there is a minor (5%) M_5 population present (Eglen and Nahorski, 2000). The contraction of the iris assists the drainage of the canal of Schlemm and therefore topical pilocarpine has been used to reduce the intraocular pressure that occurs in glaucoma (Broadley and Kelly, 2001).

1.3 Receptor structure and function

All G-protein coupled receptors are proposed to operate through a common mechanism, with agonist-promoted changes causing as yet poorly defined conformational changes in the integral receptor structure ultimately leading to the activation of specific G-proteins (Conklin and Bourne, 1993; Neer, 1994). In order to understand the molecular mechanisms behind receptor activation and the multiplicity of G-protein coupling, GPCRs have been subjected to intense biochemical and mutagenesis studies in order to delineate which regions of the receptor are responsible for G-protein specification and activation. However much of our current knowledge has been derived from mutagenesis experiments on adrenergic and muscarinic receptors (Strosberg *et al.*, 1995; Cotecchia *et al.*, 1998; Kostenis *et al.*, 1998; Wess, 1998). Therefore this section will concentrate on experimental data obtained from the $G\alpha_{q/11}$ coupled muscarinic receptors but also M_2/M_3 mACh chimeras and the adrenoceptors, in order to understand the regions of GPCRs involved in G-protein activation and specificity. Reference is also made to rhodopsin which is the only GPCR whose 3-dimensional structure is resolved (Rader *et al.*, 2004)

and has therefore, been invaluable for directly studying the conformational changes involved in receptor activation.

A confounding issue in GPCR structure-function studies is that activating point mutations can be identified in almost any receptor domain for an increasing number of receptors (Gether, 2000). Despite this, most mutational or hybrid studies have implicated the second intracellular loop, regions of the third intracellular loop proximal to TM5 and TM6, and to a lesser extent the C-terminal tail as important structural regions to dictating proper G-protein recognition and efficient G-protein coupling (Wess, 1998; Hermans, 2003). This section below will therefore concentrate on the experimental data obtained from mutagenesis on these regions, particularly with respect to the well-characterised M₃ mACh receptor. However, it must be appreciated that no consensus sequences for G-protein selectivity have been identified and moreover, no definitive assignments of a particular domain of the receptor in coupling to a particular G α protein subtype has been made, and therefore other regions of the receptor may also contribute to receptor-G-protein coupling (Hermans, 2003).

1.3.1. The N and C terminal domains of the third intracellular loop

Early experiments on chimeric mACh receptors and adrenoceptors have clearly identified the third intracellular loop (i3), which spans the cytoplasmic ends of transmembrane domains five and six (TM5, TM6), as a key determinant of G-protein coupling specificity (Gether *et al.*, 2002). A loss of G-protein activation results from the deletion of an N-terminal or C-terminal sequence of the putative i3 loop in the β_2 -adrenoceptor (Strader, *et al.*, 1987). Furthermore, chimaeras between β_2 - and α_2 -adrenoceptors have shown that the specificity for coupling to a particular G α subclass lies within the i3 loop (Kobilka *et al.*, 1988).

Kubo *et al.*, (1988) created a series of chimeric mACh receptors, where a region proposed to encode the i3 loop was swapped between the M₁ and M₂ mACh receptors and patterns of acetylcholine (ACh)-activated whole cell currents were observed after expression in *Xenopus* oocytes. M₁ mACh receptors mediate the activation of a Ca²⁺-dependent Cl⁻

current, which is sensitive to the intracellular injection of the calcium-chelating agent EGTA. M₂ mACh receptors primarily induce Na⁺ and K⁺ currents in a Ca²⁺-independent fashion, which are therefore unaffected by intracellular injection of EGTA, thereby allowing easy dissection of the currents elicited by the two receptor subtypes. The chimeric M₁ receptor incorporating amino acid residues 203-394 of the M₂ receptor mediated an ACh response that was almost identical to that mediated by the wild-type M₂ receptor. Similarly the 'mirror-image' chimera of the M₂ receptor (incorporating the homologous region on the M₁ receptor) produced an "M₁-like" EGTA-sensitive current, thereby implicating the i3 and the proximal transmembrane domains as determinants of selective coupling with different receptor systems.

Chimeric receptors have also been created for M₂/M₃ mACh receptors in order to delineate which particular regions of these receptors are implicated in the inhibition of adenylyl cyclase and the stimulation of phosphoinositide hydrolysis, respectively (Wess *et al.*, 1990a,b). The observation that the N-terminal of the M₁, M₃ and M₅ receptors are highly conserved but different from the sequence conserved in the M₂ and M₄ receptors lead investigators to believe that the N-terminal end of the i3 loop would be the sole determining region of G-protein specificity. Mutant M₂ mACh receptors incorporating either the whole i3 loop, or a short 17 amino acid sequence at the N-terminus of the M₃-i3 produced hybrid receptors that were able to mediate agonist-stimulated inositol phosphate accumulations comparable in magnitude to that produced by wild-type M₃ mACh receptors (Wess *et al.*, 1990a). Importantly, although both agonist and antagonist binding affinities were essentially unaltered in the chimeras, the EC₅₀ of carbachol with which the M₂-chimeras elicited inositol phosphate accumulations were 20 fold higher than that observed for the wild-type M₃ mACh receptor. It was concluded that the N-terminal end of the i3 loop was therefore an important, but not exclusive region for determining G-protein specificity. Additional experiments also revealed that whilst the chimeric M₂ receptor containing the 17-amino acid N-terminal-i3 region of the M₃ receptor sequence mediated PTx-insensitive PI hydrolysis (albeit with reduced efficacy) this hybrid retained the ability to inhibit PGE₂-stimulated cyclic AMP accumulation (Wess *et al.*, 1990b),

therefore implicating regions other than the *N*-terminal-i3 domain, as important determinants of G-protein specificity (Liu *et al.*, 1995a,b).

Nuclear magnetic resonance (Yeagle *et al.*, 1997a,b) and site-directed spin labelling (Farrens *et al.*, 1996) studies on rhodopsin, have shown that the *N*-terminal-i3 and *C*-terminal-i3 regions are predicted to project out into interior of the helical bundle and essentially form an α -helical extension of TM5 and TM6. A similar structural arrangement has been proposed for the M₃ mACh receptor (Blin *et al.*, 1995). These regions may define a second hydrophobic surface that is also sensitive to the movements of TM3 and TM4, and which is proposed to occur upon agonist binding (Baldwin *et al.*, 1997). It is conceivable that residues in the *N*-terminal-i3 and *C*-terminal-i3 regions contact each other in the active state and upon agonist binding and conformational rearrangement these interactions are broken and previously hidden G-protein binding domains are exposed to the intracellular surface (Wess, 1998). Mutagenesis studies on the rat M₃ mACh receptor (Bluml *et al.*, 1994a,b) and the β_2 adrenoceptor (Cheung *et al.*, 1991) have also provided evidence that the *N*-terminal-i3 forms an α -helical extension of TM5. One side of this putative helix is predicted to be composed almost exclusively of non-charged (hydrophobic) residues which form one of the proposed GPCRs important G-protein recognition sites (Bluml *et al.*, 1994b, c).

A homologous mutation to the rat ²⁵⁴Tyr-M₃ mACh receptor was created in the human M₁ mACh receptor (at ²¹²Tyr) and transiently expressed in HEK cells (Moro *et al.*, 1993; Hogger *et al.*, 1995). In agreement with previous studies (Bluml *et al.*, 1994a,b,c), replacement of the tyrosine residue resulted in a receptor that was less effective than the wild-type receptor at mediating agonist-stimulating [³H]-inositol phosphate accumulation. However, the reduced coupling was modest compared to a ²¹¹Ile point-mutation in the receptor which produced only 12-20% of the response seen for wild-type, demonstrating important species differences in the function of homologous amino acid residues.

The difficulty in attributing specific amino acid residues to receptor function across species and closely related receptor subtypes was taken as an indication that the *N*-

terminal-i3 (and C-terminal-i3) probably serve as general structural hinge regions. An agonist-induced conformation change in these hinge regions is proposed to facilitate G-protein binding (Hogger *et al.*, 1995). In addition mutagenesis experiments are frequently difficult to interpret as sequence modifications may result in profound alterations in the conformation of the larger domains of the receptor (Hermans, 2003), therefore caution should always be exercised when attributing function to a single amino acid residue. Differences in cell-type expression systems are also likely to confound analysis due to changes in absolute G-protein expression, G-protein-receptor stoichiometry, effector expression and the effect of localization through scaffolding proteins, all of which may cause conflicting results. Shapiro *et al.*, (1993) reported that a deletion a four amino acid residues from the C-terminal-i3 region of the mouse M₁ mACh receptor effectively uncoupled the receptor from PLC activation in mouse Y1 adrenal carcinoma cells, but was essentially indistinguishable from wild-type M₁ receptors when expressed in either CHO cells or Rat-2 fibroblasts. Therefore, the relative contribution of the N-terminal-i3 and C-terminal-i3 segments is likely to vary among different classes of GPCRs, among closely related members of the same receptor sub-family (Wess, 1998), and between different expression systems.

1.3.2. The second intracellular loop

Although many of the early hybrid studies showed that exchange of the i3 loop between functionally distinct receptors leads to a modification of G-protein coupling profiles, many of the studied receptors were found to be less active with respect to their potency and efficacy (Wess, 1997). Some of the hybrid receptors were still able to couple to their native G-proteins (Wess, 1990a,b) suggesting that although the i3 loop is of fundamental importance for proper G-protein recognition, it is not usually sufficient to account for the absolute coupling profile of a given receptor (Wess, 1998). In contrast to the i3 loop it has been suggested that the second intracellular loop (i2) is less important for specificity, but is involved in determining the efficiency of G-protein activation (Gether, 2000), and that the two (and possibly more) regions act in a cooperative manner to ensure effective G-protein recognition and activation (Wess, 1998).

Considerable attention has focused on the Asp/Glu-Arg-Tyr (DRY/ERY) triplet that is found at the *N*-terminal end of the i2 loop in nearly all family A GPCRs. Mutations of this triplet, especially the highly conserved arginine residue virtually abolishes G-protein coupling in the M₁ muscarinic (Zhu *et al.*, 1994), α_{1B} -adrenergic (Scheer *et al.*, 1996) and an as then uncharacterised β -adrenergic (Fraser *et al.*, 1988) receptor, implicating this triplet in efficient G-protein coupling. Receptor activation studies have suggested that the binding of agonist leads to the protonation of the Asp/Glu residue leading to release of constraining intermolecular interactions and resulting in the conversion to an active state (Gether *et al.*, 2002). Studies in the β_2 -adrenergic (Ballesteros *et al.*, 2001); the 5-HT_{2A} (Shapiro *et al.*, 2002) and AT₁ angiotensin (Gaborik *et al.*, 2003) receptors have suggested a strong ionic bond between the Asp/Arg residue, in the DRY motif, with residues at the intracellular end on TM6 which help to keep the receptor in a constrained 'inactive' state. Agonist-binding or specific point mutations may cause conformational changes which weaken this interaction leading to a relative movement of TM3 and TM6, ultimately enabling G-protein activation. Mutation of the Asp residue (within the DRY motif) in the M₁ mACh receptor significantly attenuated agonist-stimulated phosphoinositide turnover (Lu *et al.*, 1997). Mutation of the Tyr residue (¹²⁴Tyr) in the DRY motif, also lead to decrease in radioligand binding sites (Lu *et al.*, 1997), which was attributed to misfolded forms of the receptor. Interestingly deletion of the 129 amino acids from the i3 loop partially rescued expression of the mutant by ≤ 20 -fold. Charge neutralizing mutations at the ¹³⁰Asp residue in the β_2 -adrenoceptor not only lead to receptor activation, but also caused a counter-clockwise tilting of TM6, which biophysical studies have implicated as an important step in receptor activation (Rasmussen *et al.*, 1999)

Mutation of another amino acid (¹⁷³Leu) in the i2 loop to an alanine or a polar residue severely impairs G-protein coupling for the M₁ and M₃ mACh receptors (Moro *et al.*, 1997). This residue is conserved among most class A GPCRs and it has been suggested that it might serve as a general hydrophobic site for G-protein attachment (Wess, 1998). Although assignments of structure-function relationships at the level of individual amino acids are contentious, it appears that the i2 loop is less important for specificity, but is involved in the efficiency of G-protein activation (Gether, 2000).

In addition to the i2 and i3 loops, the activation of G-proteins has also been shown to involve amino acid residues located in the i1 loop of the cholecystokinin receptor (Wu, *et al.*, 1997), in the C-terminal domain of the receptor for parathyroid hormone (Huang *et al.*, 1995), as well as specific residues in various transmembrane domains. Despite this, i2 and the N- and C-terminal portions of i3 remain the major regions implicated in the proper recognition and activation of G-proteins. This is highlighted by a comprehensive structure-function analysis of the $G_{q/11}\alpha$ coupling properties of the M₃ mACh receptor (Blin *et al.*, 1995). Systematic substitution of regions of the M₃ receptor into the homologous regions of the M₂ mACh receptor were made. Using this approach it was found that a sequence of four amino acids from the i2 loop, and four amino acids from the N- and C-terminal ends of i3 were sufficient to quantitatively account for the coupling properties of the wild-type M₃ mACh receptor. It is possible that mutations in other regions of this receptor, which have been implicated in either attenuating activation of G-proteins, are involved in the stabilization of the inactive/active states of the receptor. It remains to be seen whether the coupling properties of other class A GPCRs can be accounted for by such a limited number of key amino acid residues.

1.4 GPCR-drug interactions: theory and experiment evidence

As experimental techniques have relentlessly progressed in sophistication and sensitivity so our understanding of how ligands interact with and modify their respective receptor targets has rapidly adapted to accommodate new findings. This is nowhere more evident than in the evolution of modern receptor theory to explain the pharmacological behaviours of ligands at GPCRs. From the relatively simple laws of mass action used to describe ligand ‘induction’ (Clark, 1937) to the theoretically unlimited receptor species available for ligand selection in the cubic ternary complex model (Weiss *et al.*, 1996a,b, c), the development of a GPCR-ligand binding model is seen as critical for unravelling the molecular basis for affinity and efficacy. This section summarizes the key features of contemporary receptor theory models with two key questions in mind: (1). How can the models be used to explain experimental pharmacological data?, and (2). How can these models help explain the molecular basis of efficacy and affinity?

1.4.1 Emergence of the concepts of affinity and efficacy - a brief history

A measure of the strength of interaction between a ligand and a particular receptor is called its affinity, and the subsequent modification of the behaviour of the receptor (either positive or negative) is known as efficacy. With the development of radiolabelled ligands and receptor-binding techniques, affinity became a quantifiable property, however there continues to be some debate of the physiological relevance of values obtained with compounds employed to measure the 'ground-state' affinity of the receptor; e.g. in the presence of guanine nucleotides (Strange, 1999). The exact mechanism and subsequent measurement of the efficacy of a ligand has remained somewhat elusive and at present pharmacologists are, at best, only able to estimate the ability of a ligand to initiate a biochemical response.

Fig. 1.3A-C and Fig. 1.4A-C illustrate the evolution of intrinsic efficacy into a seemingly quantifiable parameter (adapted from Clarke and Bond, 1998; Kenakin, 2004b). A.J. Clark (1937) applied the Langmuir adsorption isotherm (**Fig. 1.3A**), traditionally used to describe the adsorption of gas molecules on to a surface, proposing that the fractional response produced by an agonist is equal to the fractional occupancy of the receptor by the ligand (**Fig. 1.3B**). Although useful for quantifying the way drugs bind to receptors, its fundamental assumption that 100% occupancy of receptors was necessary to evoke a maximal response meant that this model was unable to account for two commonly seen pharmacological phenomena: the ability of some agonists to produce maximal responses at occupancies of less than 100%, and conversely that some agonists can occupy 100% of their respective receptors without evoking a maximal response (**Fig. 1.3C**).

As a consequence this model was refined by inclusion of a proportionality factor, α , termed *intrinsic activity* (Ariens, 1954), which could assume values between 0 and 1, thus giving a quantitative scale to the ability of a ligand to elicit a response (Kenakin, 1997c) (**Fig. 1.4A**). Ariens observed that some compounds were unable to elicit a maximal system response despite occupying all available binding sites. Therefore, he proposed that the response to an agonist was dependent on both its binding (K_D or affinity component)

A.

Langmuir adsorption isotherm:

Rate of diffusion to surface = α

Rate of dissociation = V_1

Area bound by molecule = θ_1

Concentration of molecule = μ

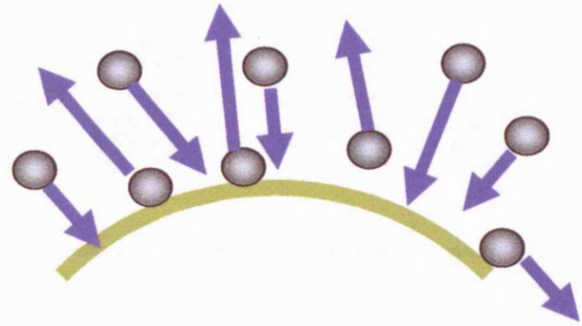
Rate of diffusion towards surface = $\alpha\mu(1-\theta)$

Rate of dissociation = $V_1\theta_1$

At equilibrium: $\alpha\mu(1-\theta)=V_1\theta_1$

So area bound by molecule...

$$\theta_1 = \frac{\alpha\mu}{\alpha\mu + V_1}$$



B.

In pharmacological nomenclature (Clark, 1937)

$\mu = [A]$ molar concentration of drug

$V_1 = [K_A]$ equilibrium dissociation constant

$\theta = \rho$ fraction of maximal binding

$$\rho = \frac{[AR]}{[R_T]} = \frac{[E_A]}{[E_M]} = \frac{[A]}{[A] + K_A}$$

If response (E_A) of a maximum (E_M) is equal to fraction occupied ($[AR]$) of total density $[R_T]$

Affinity: when $[A] = K_A$, fraction receptors bound ($[AR]/[R_T]$) is 50%

C.

HOWEVER: occupancy and response are not linearly linked

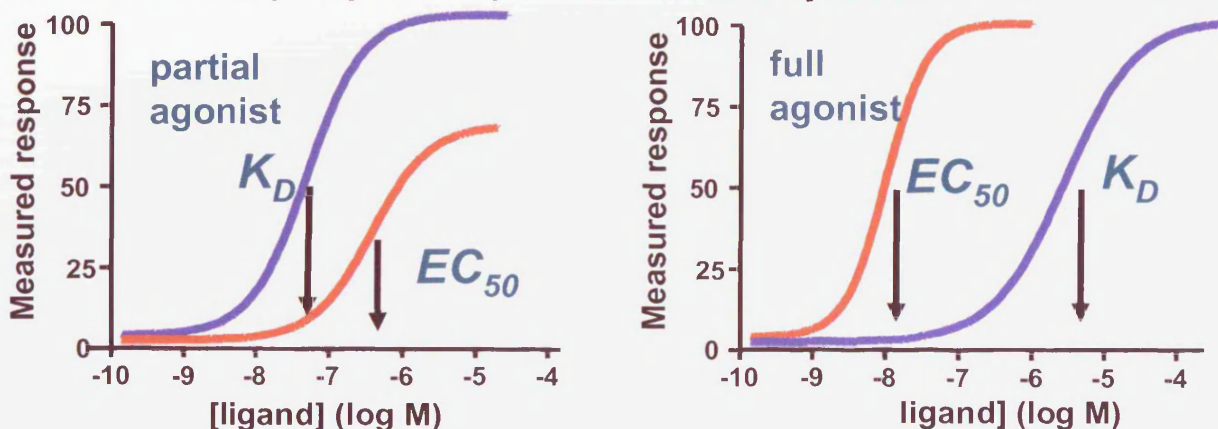


Fig. 1.3 Historical development of the concept of efficacy (I).

Schematic representation of the development of the concept of efficacy. Panel **A** depicts the derivation of the Langmuir isotherm to describe the binding of gas molecules to a solid surface. This equation was adapted by Clark (1937) to describe the binding of drug molecules to receptors (**B**). Later, the implicit assumption in this model, that occupancy was linearly related to response, was shown not to be true for all systems (**C**). For a full discussion, please see the text.

A.

Ariens (1954)

Introduces
proportionality factor

$$\frac{[E_A]}{[E_M]} = \alpha \frac{[AR]}{[R_T]} = \alpha \frac{[A]}{[A] + K_A}$$

Intrinsic activity: α

B.

Stephenson (1956)

receptor *stimulus* (S),

response (*f*) is a function of S

proportionality term 'efficacy': e

$$\frac{[E_A]}{[E_M]} = f(S) = \frac{e [A]}{[A] + K_A}$$

e = ability of drug to produce
stimulus and can be ≥ 0

C.

Furchgott (1966)

Introduces intrinsic efficacy: ϵ

$\epsilon = e/R_T$: ability of drug to produce
stimulus from single receptor

$$E = f(S) \quad f = \left(\frac{\epsilon_A \cdot R_T}{1 + K_A/[A]} \right)$$

Diagram labels:

- E : magnitude of effect
- $f(S)$: efficiency of transduction
- f : capacity to produce response
- ϵ_A : intrinsic efficacy
- R_T : receptor density
- $K_A/[A]$: affinity-related

Fig. 1.4 Historical development of the concept of efficacy (II).

The observation that tissue response was not a linear function of occupancy led to the inclusion of a proportionality factor into the equation determining efficacy (A). This model accounted for less than maximal activity at full occupancy, but not full-agonist tissue responses at less than full occupancy. Consequently, the model was refined by Stephenson to include a proportionality termed e (B). This was later refined into a form which described a measure of efficacy per unit of receptor (C). Full details are given in the text.

and its ability to induce a conformational change in the receptor that initiates a response (intrinsic activity component). An agonist capable of eliciting a maximal response would have an intrinsic activity value of 1, whereas a ligand that bound to the receptor but produced no measurable response, would have an intrinsic activity of 0. By assigning values between 0 and 1, this model accounted for the partial agonist behaviour of some agonists, which produced sub-maximal system responses despite maximal occupancy (intermediate intrinsic activities).

Implicit in Ariens' modification of receptor theory was that agonists possessing high intrinsic activity must occupy all available receptor binding sites in order to produce a maximal system response. Therefore, this model was unable to explain how full agonists can produce a similar maximal response at different fractional occupancies. Despite this deficiency, the term 'intrinsic activity' is still employed in modern pharmacology as an empirical measure of the maximal response to a test agonist, as a fraction of that to a full agonist acting at the same receptor type. One caveat to intrinsic activity, is that any ascribed value should not be taken as a universal characteristic of the agonist, given that the ligand may have different intrinsic activities dependent on the tissue/measured response/experimental conditions/etc. (Kenakin, 2004b). For this reason some investigators prefer the term *maximal agonist effect* (Jenkinson *et al.*, 1995).

As a result of observing the responses of guinea pig ileum to the application of a series of alkyltrimethylammonium ligands, Stephenson, (1956) introduced the concept of tissue response, R , as a function of stimulus, S , by proposing three modifications to earlier models (**Fig. 1.4B**): (1). a maximum response can be produced when only a small fraction of the receptors is occupied, (2). the relationship between occupation and response is not always linear, and (3). drugs have very different abilities to induce a response, termed *efficacy* (Dean, 1989). Here was the first attempt to correlate the efficiency of an agonist not only to produce a maximal tissue response, but also to relate the fraction of receptor sites occupied to produce the response. In Stephenson's model the response is a function of S and the proportionality term, efficacy, defines the ability of a drug to produce a response (Clarke and Bond, 1988). Stephenson also reported that the

muscarinic receptor antagonist atropine, at low concentrations was able to inhibit the response of partial, but not full agonists. This was the first empirical description of the concept of 'spare receptors' or 'receptor reserve'

Despite the advances made through Stephenson's analysis, efficacy as a concept was derived from both drug- and tissue-dependent variables, and was therefore limited in defining drug characteristics (Clarke and Bond, 1998). Furchgott, (1966) revised the Stephenson model to account for receptor density and introduced *intrinsic (relative) efficacy*, as a measure of efficacy per receptor unit (e/R_T). As can be seen from **Fig. 1.4C** this definition of a drug-mediated response is dependent on two tissue-specific variables and two variables related solely to the drug-receptor complex. Tissue factors are the density of receptors ($[R_T]$) and f , the efficiency of the tissue/pathway to convert the initial stimulus into a response. The drug-dependent variables are K_A , the equilibrium dissociation constant of the ligand-receptor complex, and ϵ , the intrinsic efficacy of the drug. Experimental evidence for different receptor reserves for agonists were inferred from the effects of receptor reduction on the agonist response by irreversibly inactivation of a proportion of the receptor population using alkylating agents. In this way, Furchgott was able to not only differentiate partial agonists from full agonists, but also to determine differences in intrinsic efficacy of agonists able to elicit the same maximal tissue response.

1.4.2 Contemporary pharmacology – the two-state model of agonism

All of the classical (occupational) pharmacological models described above are based on the tacit assumption that a homogenous population of receptors is essentially quiescent until a ligand, that possesses both affinity and efficacy, binds to a fraction of the receptors. Within the framework of this model a ligand with efficacy induces a conformational change in the receptor and its intrinsic efficacy is its ability to induce this conformation change. This view was challenged by the observation that GPCRs can sometimes elicit measurable tissue responses in the absence of ligand (Costa and Herz, 1989). This ligand-independent level of signalling has been termed 'constitutive activity' and its demonstration, in both recombinant and native cells, has necessitated the evolution

of the two-state receptor model of agonism (**Fig. 1.5**)

Central to this model is the predicted existence of two receptor conformations, **R** and **R***. **R** represents the receptor in its inactive or resting conformation, and **R*** the same receptor in a conformation that is active, i.e. able to couple productively to G-protein(s) and initiate a measurable response. **L** is an equilibrium constant that denotes the distribution of the two receptor conformations in the absence of ligand, and therefore indicates the degree (if any) of constitutive activity seen in a particular biological system (Leff, 1995). In most tissues **L** is sufficiently low to ensure most of the receptors are kept in the inactive conformation. However, in certain cells and under certain experimental conditions (e.g. over-expression of recombinant receptors, receptor mutagenesis) this energy barrier may be overcome. The subsequent increase in the probability of receptors spontaneously adopting the active conformation allows the experimenter to observe a significant receptor-induced response in the absence of ligand.

The influence of a ligand in this system is dictated by the ratio of its dissociation equilibrium constants, K_A and K_A^* (Leff, 1995; see **Fig. 1.5**). It is predicted that an agonist will have a higher affinity for the active conformation of the receptor and will therefore alter the equilibrium between the two receptor states to eventually increase downstream effector activity. A new class of ligand was defined as those that possess higher affinity for the inactive **R** conformation and therefore act to stabilize this receptor conformation. If there is sufficient measurable constitutive activity in the system, such a ligand may actually decrease a downstream system response and hence, these agonists have been termed 'inverse agonists'. In molecular terms the only true neutral antagonists are those that have an identical affinity for both the **R** and **R*** receptor conformations. As our ability to detect partial (inverse)-agonism increases, the existence of true neutral antagonists is likely to be rare (Daeffler and Landry, 2000). Within this model, by permitting ligands to have differential affinity for the two receptor states, a full spectrum of (inverse)-agonist activities is predicted. Under these circumstances efficacy is a property of the quantity of active state produced/stabilized by the agonist (Kenakin, 2001).

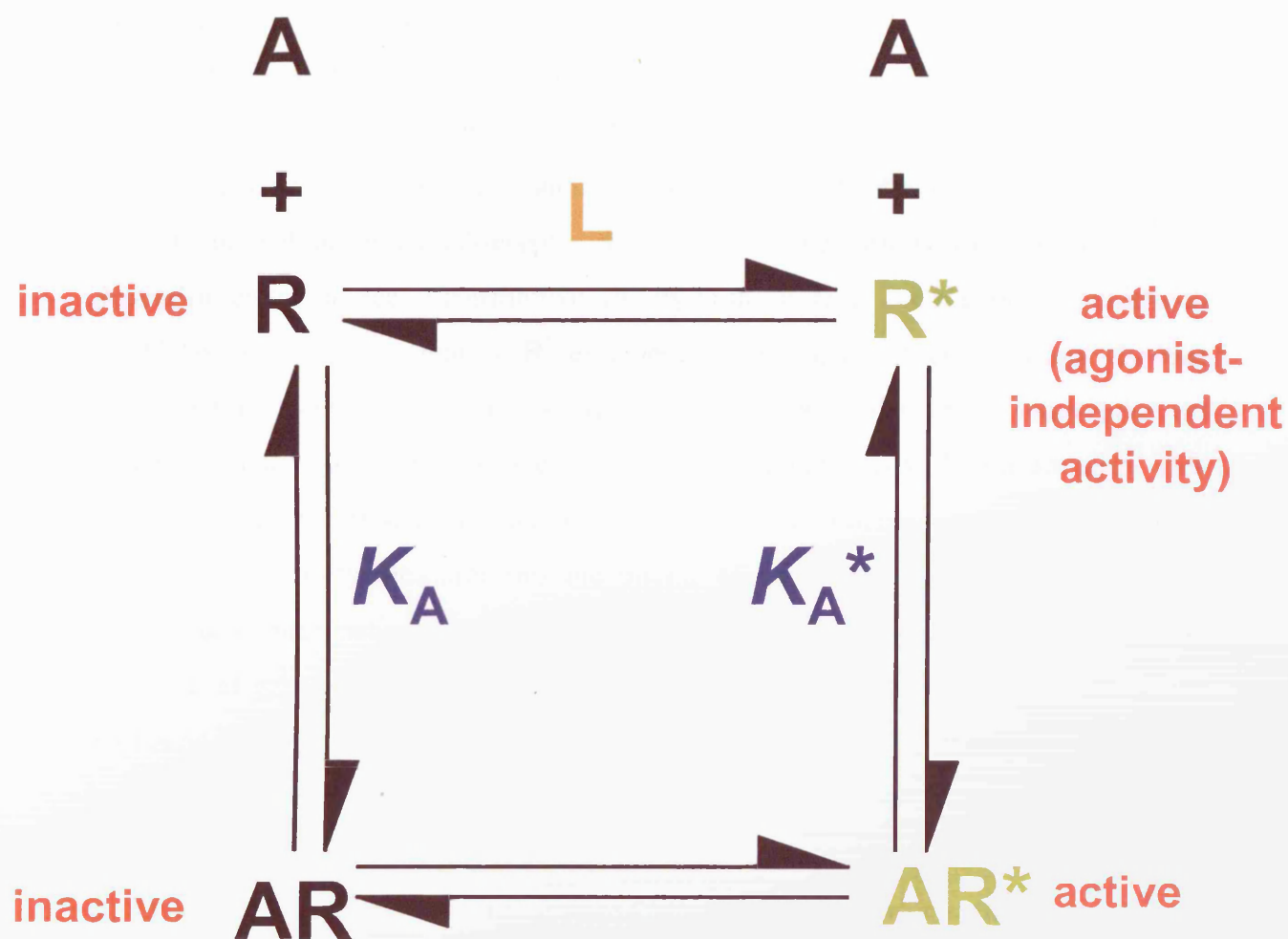


Fig. 1.5 Two-state model of receptor activation.

The two-state model is represented above, the receptor is predicted to exist in two states or conformations R and R^* . R^* represents the conformation of the receptor which is able to productively interact with G-proteins to initiate a downstream response. The distribution of the receptor conformations in the absence of any ligand is dictated by the equilibrium constant L . An (inverse) agonist acts to re-distribute the conformation of the receptors by possessing differential affinity for them, governed by K_A and K_A^* .

The prediction of ligand behaviour within the two-state model has been extensively modelled and compared to actual experimental data (Leff, 1995). One major consequence of accepting the two-state (or more complex models – see below) is that, unlike traditional receptor theory, affinity and efficacy are conceptually linked. The affinity ratio of $K_A : K_A^*$ determines the degree and direction of a ligand's efficacy. Another deviation from traditional occupational-receptor theory is that the affinity of a ligand is now dependent on the degree of constitutive activity in the system. The K_A for an agonist will be higher in a system that is R^* enriched, as it displays higher affinity for this conformation. Conversely, an inverse agonist will display higher affinity in a quiescent system, i.e. one in which most of receptors are found in the inactive R conformation. This system-dependence of affinity is not accommodated by traditional receptor theory, which predicts that affinity measurements are unique to each ligand-receptor pair. Another notable difference between traditional receptor theory and the two-state model is the variation of relative efficacy between cells or tissues. In a largely quiescent system a partial agonist may displace the receptor state equilibrium towards R^* , but only to a finite extent, and the degree of agonism (in terms of measurable system effects) appears partial. However, in a system with a substantial degree of constitutive activity the same (partial) agonist may achieve almost maximal displacement towards R^* , which enables it to elicit a similar maximal response to that of true full agonists. This type of behaviour would have been explained in traditional receptor theory as a demonstration of receptor-reserve (Leff, 1995). The detection of inverse-agonism is also system-dependent, as in a quiescent system it would not be possible to discriminate between an inverse agonist and a true neutral antagonist.

1.4.3 Agonist-specific receptor conformations

It has long been appreciated that GPCRs can couple to more than one subtype of $G\alpha$ (Eason *et al.*, 1992; Perez *et al.*, 1993; Arey *et al.*, 1997; Wellner-Kienitz *et al.*, 2003). This pleiotropic behaviour is frequently seen in heterologous expression systems, where relatively high levels of receptor increase the probability of multiple couplings between receptor and G-protein (Kenakin, 1997b). In this scenario there may be sufficient active receptors functionally to saturate the primary G-protein(s) activated, and coupling to other

G-protein(s) may follow leading to the receptor promiscuity (Kenakin, 1995a; Daeffler and Landry, 2000).

As can be seen from **Fig. 1.6**, the two-state model permits a receptor to couple to two (or more) G-proteins in a promiscuous fashion. Additionally the diversity of G-proteins activated can be qualitatively influenced by agonists in a manner termed 'strength of signal'. **Fig. 1.6** depicts a receptor that is predominantly coupled to G-protein **G₁**, and less efficiently coupled to **G₂**. An agonist of high efficacy is able to stabilize sufficient active receptor to enable coupling not only to **G₁**, but also (at higher agonist concentrations) to **G₂** (**Fig. 1.6A, B**). An agonist of lesser efficacy may only be able to activate or stabilize the receptor to such a degree that only coupling to the most efficiently coupled G-protein (**G₁**) can be detected (**Fig. 1.6C, D**). If the two different G-proteins are coupled to separate downstream effector pathways, diversity of signalling is achieved, on the basis of agonist-strength. When considering a divergence of signalling between agonists it must be appreciated that multiple pathways can be influenced by both $G\alpha$ -GTP and $\beta\gamma$ subunits, allowing crosstalk between pathways (Hill, 1998), thus, dual signalling alone is not evidence for the existence of promiscuous coupling at the level of the G-protein (Hermans, 2003).

However, data have accumulated in the past decade that cannot be reconciled with the existence of a single active receptor state and this has led to the concept of two (or more) active receptor conformations. These active receptor species are proposed to differ in their conformation such that the complement of G-proteins regulated by each active conformation is different. Agonists may have different affinity for these conformational states, and can therefore exhibit altered potency and intrinsic efficacy at two (or more) downstream effector pathways (Christopoulos and Kenakin, 2002) (**Fig. 1.7**). Traditionally, altered potency ranking orders (a pharmacological mainstay), measured in different cell-lines would have been interpreted as evidence of different receptor subtypes. 'Agonist-specific stimulus trafficking' does not only account for highly divergent signalling at a single receptor subtype, but also provides the potential for new therapeutic agents. These may be designed to target a specific receptor-effector pathway,

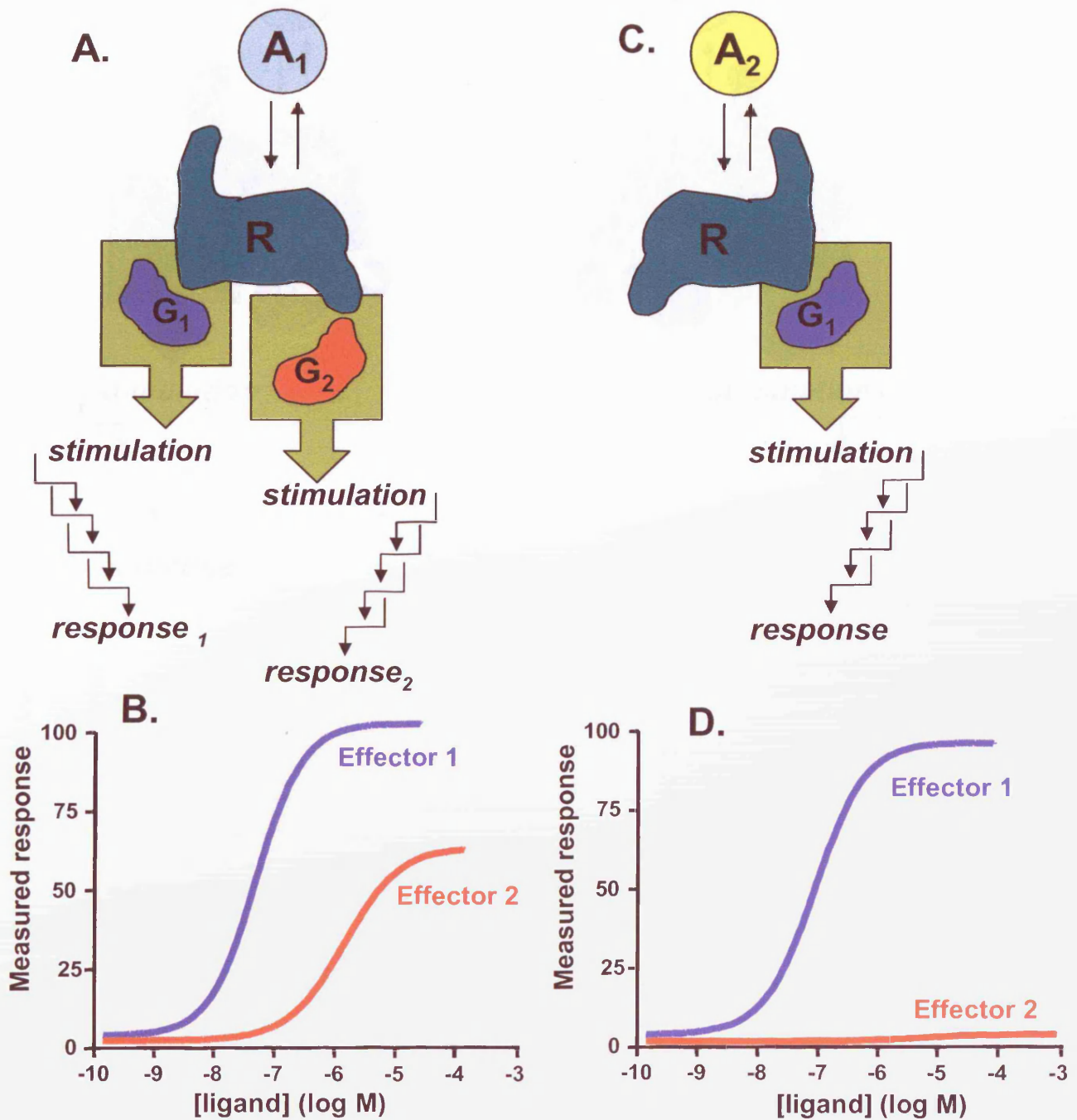


Fig. 1.6 Promiscuous coupling through agonist 'strength of signal'

A receptor (**R**) is coupled to two G-proteins (G_1 & G_2), the receptor is predominantly coupled to G_1 and more modestly coupled to G_2 . A_1 is a full agonist and is therefore able to stabilize sufficient active receptors to allow productive coupling to G_1 and to a lesser extent G_2 . A_1 exhibits full agonism through pathway 1 and partial agonism through pathway 2. A_2 is a partial agonist and is only able to initiate coupling to the most efficiently coupled G-protein, G_1 . A_2 therefore produces agonism through pathway 1, but is essentially quiescent through pathway 2.

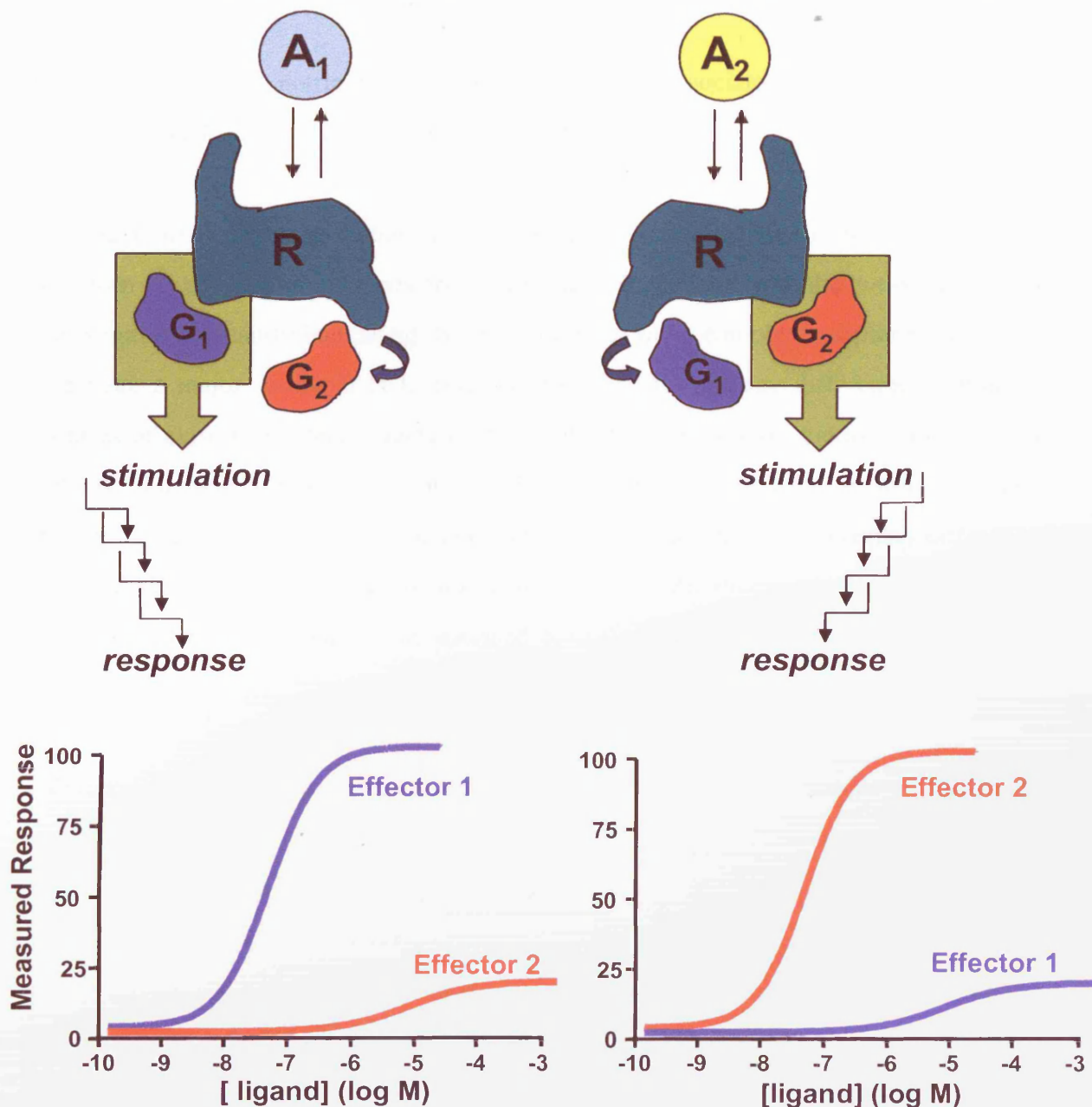


Fig. 1.7 Promiscuous coupling through agonist-directed trafficking

A receptor (R) is coupled to two G proteins (G_1 & G_2) each of links to a different downstream effector pathway, the measured response from which is quantified as a % of the max. response. Agonist A_1 elicits a conformational change in the receptor which produces preferential coupling to G_1 and modest coupling to G_2 , A_1 is therefore a full agonist through pathway 1 and a partial agonist through pathway 2. Agonist A_2 stabilizes a different conformation of the receptor, which allows preferential coupling to G_2 and is therefore a full agonist with respect to pathway 2 and only a partial agonist through pathway 1. Note the responses can have altered potency and intrinsic activity orders.

and thereby reduce detrimental side-effects that are associated with the activation of secondary, undesirable pathways (Kenakin and Onaran 2002).

Although the existence of distinct active receptor conformations is still largely theoretical, the diversity of receptor subtypes providing data irreconcilable with the two-state receptor paradigm is constantly increasing. Before looking at more complex receptor models to fit the data, a major principle is to rule out efficacy and potency differences within the context of existing models, caused by 'strength of signal' and/or downstream cross-talk effects. Literature examples that clearly suggest the need for a more complex pharmacological model usually demonstrate ligands that either: (1). Display differences in intrinsic activity (maximal agonist responses) or (2). reversals of relative potency ranking orders, which cannot be ascribed to different 'strengths of signal' (Kenakin, 2003).

Nevertheless, to date data which are interpreted in terms of agonist-specific stimulus trafficking are usually either modest in terms of potency/efficacy changes or measured at an output distal to the ligand-receptor interaction and is therefore open to the criticism of downstream cross-talk and/or amplification causing the apparent effect. Inferences of G-protein activation based on effectors, or subsequent signalling is difficult as effectors are almost always subject to regulation by more than one G protein or pathways downstream of multiple G protein activation (Manning, 2002).

The *Drosophila* octopamine/tyramine receptor stably expressed in Chinese hamster ovary (CHO) cells exhibits altered pharmacology when two independent downstream effectors are assayed in response to agonist stimulation (Robb *et al.*, 1994). Octopamine is two orders of magnitude weaker than tyramine, with respect to their abilities to inhibit forskolin stimulated cyclic AMP accumulation. However octopamine is slightly more potent in elevating cytosolic Ca^{2+} concentration. Although the reversals in potency are modest it is difficult to reconcile these data with the existence of a single-active R^* receptor species.

Chapter 1: Introduction

Agonist-specific receptor conformations have also been proposed for biogenic amines operating through the human α_{2A} -adrenoceptor stably expressed in HEL 92.1.7 cells (Kukkonen *et al.*, 2001). Within this system 19 agonists were tested, for their ability to augment forskolin-stimulated cyclic AMP levels and to increase intracellular Ca^{2+} levels. The agonists belonged to three structural classes: catecholamines, imidazolines and non-catecholamine, non-imidazolines. Although the intrinsic efficacies of the ligands were similar whichever pathway was measured there were strikingly different potencies. Catecholamines were several times less potent in decreasing cyclic AMP levels than in mobilizing Ca^{2+} , whereas the other two classes of amines had opposite potency orders. As all responses were essentially eliminated by pertussis toxin, the authors suggested that the different classes of amines stabilized active conformations of the α_{2A} -adrenoceptor that preferentially activate different members of the $\text{G}\alpha_{i/o}$ family.

The two studies outlined above, while providing data highly suggestive of distinct active receptor conformations, rely on the measurement of downstream responses and the activation of separate $\text{G}\alpha$ sub-populations in an agonist-receptor specific manner can only be inferred.

The simplest model to accommodate the concept of agonist-specific trafficking is to postulate the existence of a second active receptor species, R^{**} , as in the three-state model of receptor activation (Leff *et al.*, 1997; Scaramellini and Leff, 1997,2002; Leff and Scaramellini, 1998). Most of the pharmacological evidence to date, at least in terms of differential G-protein coupling, is satisfied by the inclusion of one additional receptor-active state. For simplicity the model assumes that each of the receptor-active conformations can couple to only one G-protein type, which in turn activates a distinct downstream effector pathway. The model, depicted in **Fig. 1.8A** predicts that agonist affinity now depends on three affinity constants: K_A the affinity for the uncoupled or resting R conformation; K_A^* , which determines the extent to which R^* is enriched, and finally K_A^{**} which will determine the proportion of receptors stabilized in the second active R^{**} conformation.

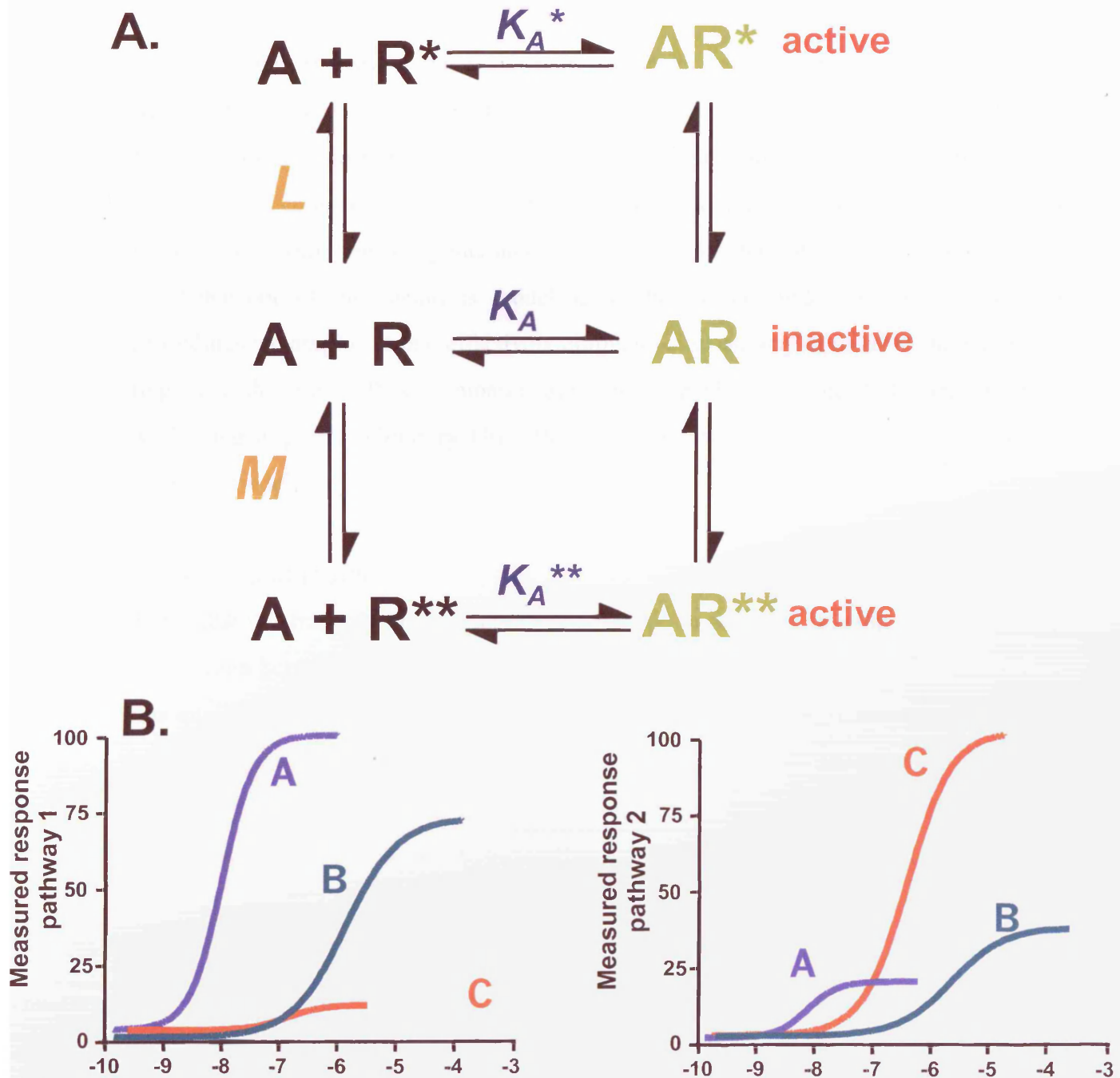


Fig. 1.8 The three state-model in the ‘intact-mode’

A. The affinity of the an agonist for a receptor conformation is determine by the values K_A , K_A^* and K_A^{**} , these will determine the extent to which the two active receptor conformations (R^* and R^{**}) are enriched. Both of these active conformation are predicted to activate different downstream effector pathways, via distinct G_α subunits.

B. The actions of three agonists (**A**, **B** and **C**) acting in the intact mode – as all the occupied receptor conformations are stabilized over the same concentration range, the potency for each pathway is identical. However, there are a finite number of receptors, therefore an agonist with high efficacy through pathway 1 has low efficacy through pathway 2. The agonists can therefore have different efficacy (intrinsic activity) orders (please see text).

The activation of distinct functional pathways from a single receptor subtype can be measured in two ways, which have important implications for the pharmacological behaviour of agonists within the context of the three-state model (Leff *et al.*, 1997). When a receptor is coupled to two distinct G-proteins, and the agonists effects are studied principally by simultaneously measuring the functional output of the respective pathways the behaviour of the ligand is modelled in the 'intact mode'. When experimental procedures are employed that effectively eliminate one pathway in order to study the first (e.g. with the use of PTx to monitor agonist-G α_s -mediated cyclic AMP accumulation whilst negating any inhibitory G α_i effects), the ligands behaviour is modelled in the 'isolated mode'.

1.4.3.1 Intact mode

Fig. 1.8A illustrates the three-state model operating through the intact mode in which the equilibrium between the individual receptor conformations is linked. An agonist perturbs this equilibrium by having differential affinities for the three receptor $R/R^*/R^{**}$ species, however as all the species are occupied to some degree ($AR/AR^*/AR^{**}$) over the same concentration range, the concentration-effect curve will be co-located. Therefore, in the intact mode the agonist potency orders for the two downstream responses are identical (Leff *et al.*, 1997). However, as the equilibria are linked and there are a limited number of receptors, an agonist that enriches one receptor species does so at the expense of another. The model therefore predicts that an agonist with high efficacy through one pathway will have modest efficacy through the other (unless a substantial degree of receptor reserve exists for either pathway). Therefore, intrinsic activity (but not potency) measurements for agonist activation of the two pathways may be reversed (**Fig. 1.8B**). An agonist with high affinity for one of the active conformations may appear to act as an inverse agonist when a different downstream pathway is measured. If a high degree of constitutive activity is introduced into either pathway, this may effect not only the magnitude, but also the direction of the agonist response (Leff *et al.*, 1997)

The 5-HT_{2C} receptor has been shown to be independently coupled, via two different G-proteins, to the activation of PLC-mediated inositol phosphate (IP) accumulation and

PLA₂-mediated arachidonic acid (AA) release when stably expressed in CHO-cell lines. These two pathways were measured simultaneously from the same cells, and the responses assessed relative to the reference agonists 5-HT (Berg *et al.*, 1998a,b). More importantly by expressing the receptor at relatively low levels (approx. 200 fmol mg⁻¹ protein) the authors could clearly demonstrate a lack of receptor reserve for either pathway, therefore permitting a test of the three-state 'intact mode' predictions. When IP accumulation was assessed the rank order of intrinsic activity was TFMPP=5HT>quipazine>bufotenin=DOI>LSD, conversely the efficacy order for AA release was bufotenin>5-HT=DOI>quipazine=TFMPP>LSD. Apart from LSD, which was a poor agonist in both pathways, the agonists displayed identical potency for each pathway but exhibit a reversal of pathway efficacy (measured as intrinsic activity).

1.4.3.2. Isolated Mode

The isolated mode (**Fig. 1.9A**) would be predicted to occur when independent assay systems are used to measure agonist effect on each downstream pathway or when the assay is conducted using broken cell preparations when structural elements which influence multiple G-protein coupling and pathway cross-talk may be impaired. In either case, the equilibrium between the active receptor conformations is no longer linked, and therefore the enrichment of R^* depends on K_A and K_A^* whilst the affinity for R^{**} depends solely on K_A and K_A^{**} . Since the affinity for the receptor species can vary, and there is no mutual depletion of one active conformation at the expense of another, both potency and efficacy orders can be different for agonists depending on the pathway being measured (**Fig. 1.9B**).

Two forms of neuropeptides – PACAP-27 and PACAP-28 are endogenous agonists at the pituitary adenylyl cyclase-activating polypeptide receptor (PACAP receptor). When transiently transfected into LLC PK1 cells the PACAP receptor has been demonstrated to couple to both adenylyl cyclase and phospholipase C, via two distinct G α G-protein subtypes (Spengler *et al.*, 1993). The two responses were measured independently from separate cell populations and therefore within the definitions of Leff *et al.*, (1997), these measurements can be modelled in the 'isolated mode'. PACAP-27 was shown to be

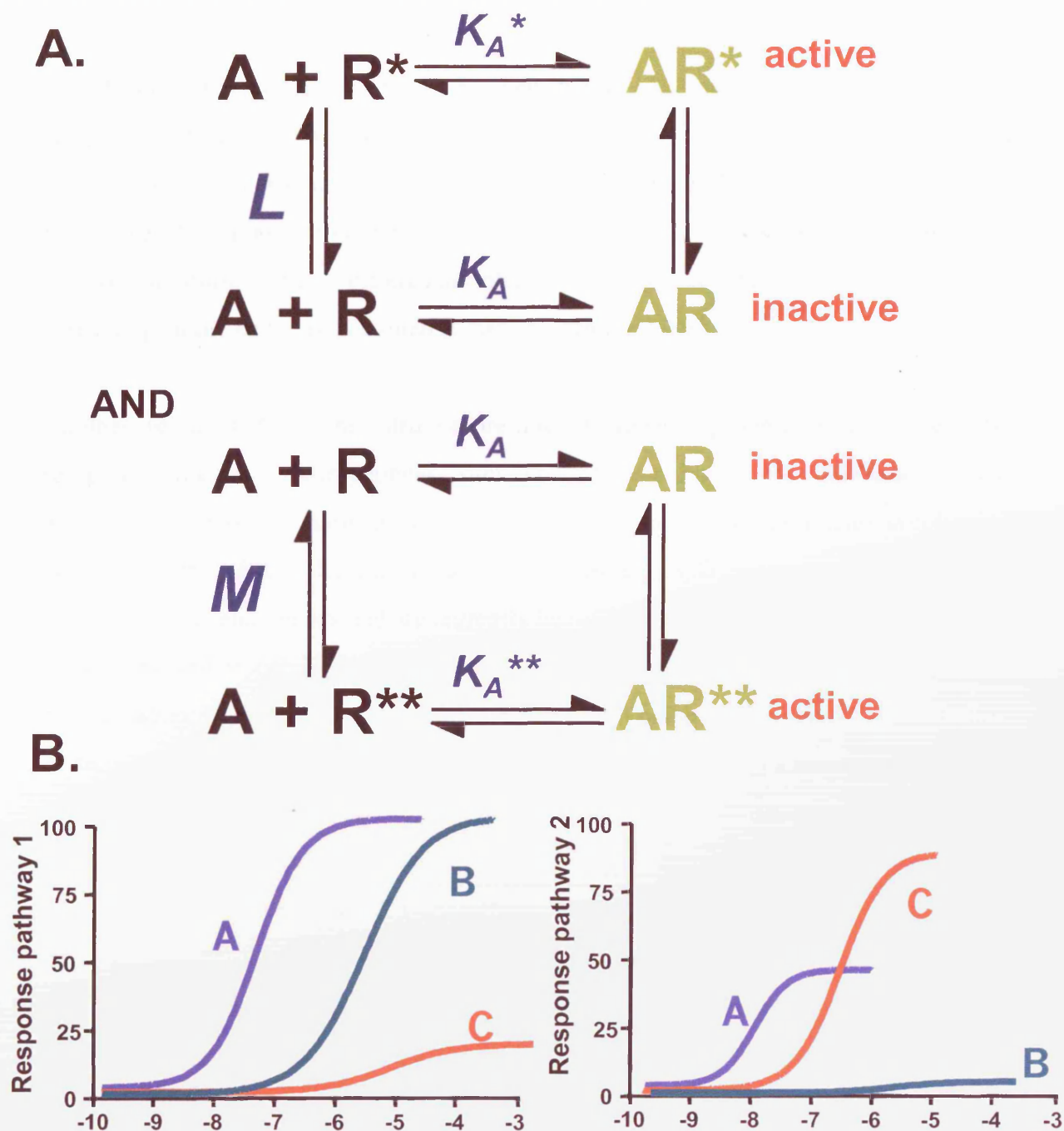


Fig. 1.9 Three state-model in the ‘isolated-mode’

A. The affinity of the an agonist for a receptor conformation is determined by the values K_A , K_A^* and K_A^{**} , these will determine the extent to which the two active receptor conformations (R^* and R^{**}) are enriched. As the pathways are measured from different cells, or in assays that eliminate one pathway, the R^* and R^{**} conformations are no longer linked.

B. The action of three agonists (**A**, **B** and **C**) acting in the isolated mode – the stabilization of the two active conformations R^* and R^{**} are now independent of one another and depend on K_A and K_A^* for the R^* pathway and K_A and K_A^{**} for the R^{**} pathway. As the affinity constant can vary between agonists they can exhibit different efficacy AND potency orders.

slightly more potent, than PACAP-28 when cyclic AMP accumulation was measured (EC_{50} 0.1 versus 0.4 nM, respectively). However, when PLC activation was quantified PACAP-28 was considerably more potent than PACAP-27 (15 versus 1000 nM, respectively). Although it is likely that the assay system possesses considerable receptor reserve, the results indicate differential intrinsic efficacy and potency for the two ligands depending on the pathway measured (Clarke and Bond, 1998).

Another recent study has revealed a more novel therapeutic property of agonist-specific receptor trafficking. Neuropeptides, such as bombesin have been implicated in the pathology of small cell lung cancer (SCLC), by acting at the bombesin/gastrin-releasing peptide receptor (B/GPCR). Substance P analogues, e.g. SpD, were originally classified as neuropeptide antagonists and are currently being assessed for their therapeutic value in the treatment of SCLC. However, it has been demonstrated that SpD may possess agonist activity when the modulation of different downstream pathways is monitored in addition to antagonising the Ca^{2+} mobilization elicited by Bombesin (MacKinnon *et al.*, 2001). Bombesin elicits promiscuous coupling of the B/GPCR by causing Ca^{2+} mobilization via $G\alpha_{q/11}$, the activation of the c-Jun N-terminal kinase (JNK) pathway via $G\alpha_{12}$ and to activation of the extracellular signal-regulated protein kinase (ERK) via an uncharacterized pathway. SpD was able to antagonize the bombesin-induced Ca^{2+} release but additionally cause ERK activation within the same concentration range (IC_{50} 3.7 EC_{50} 4.19 μ M, respectively). Furthermore, the activation of ERK by SpD was PTx-sensitive, whereas that of Bombesin was PTx-insensitive potentially implicating the recruitment of different classes of $G\alpha$ subunits. SpD was also shown to be an agonist with respect to JNK activation with a similar potency to that of ERK activation (EC_{50} 1.2 μ M). SpD therefore acts as 'biased' agonist by eliciting the activation of certain G-proteins whilst antagonising the activation of others.

1.4.4. The two/three state model – an oversimplification?

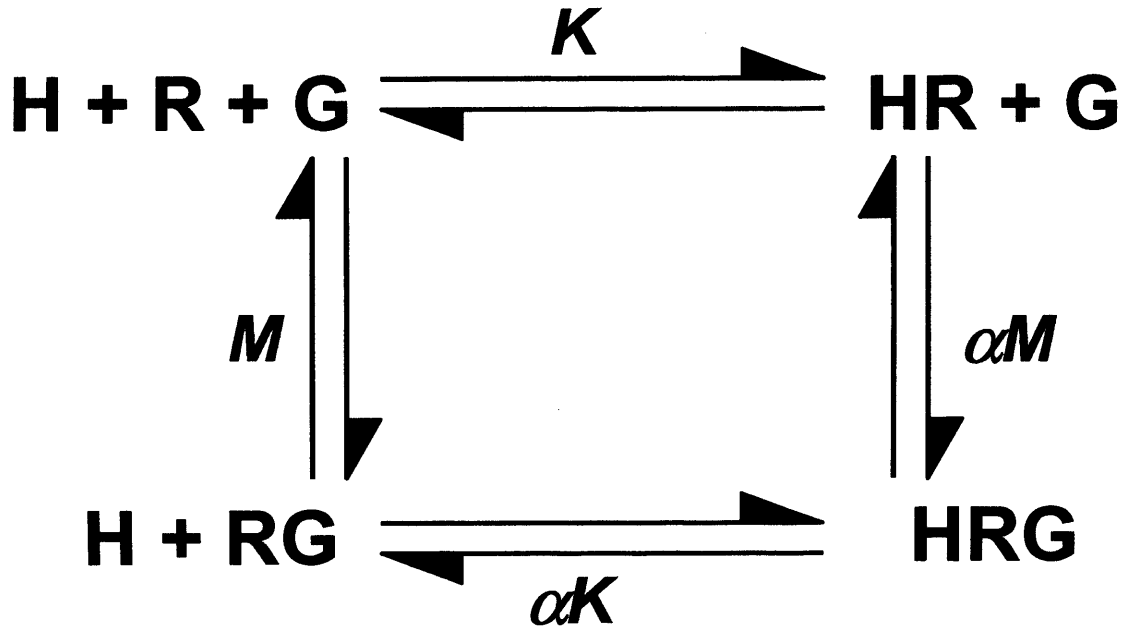
The two-state theory was originally developed to explain the behaviour of some ion channels (Kenakin, 1997c), which could be demonstrated to spontaneously open.

Although undeniably useful for predicting agonist and antagonists effects, both the two and three state models are likely to be oversimplifications of ligand-GPCR-G-protein interactions (Milligan, 2003a). Specifically, the models can be criticized due to the lack of appreciation of the effect of the G-protein on agonist efficacy and affinity (Strange, 1999), except to assume that an agonist has identical affinity for the receptor in the absence (R^*) and in the presence of G-protein (R^*G) (Leff and Scaramellini, 1998). The following section summarizes the influence of the G-protein on ligand-receptor efficacy and affinity.

1.4.4.1. The influence of the G-protein on efficacy and affinity

As described in section 1.1.2, many receptors exert their effects on intracellular biochemical pathways via the activation of heterotrimeric guanine nucleotide-binding proteins (Chidiac, 1998). The earliest model developed to explain the interactions between agonist, receptor and G-protein were the ternary complex model (De Lean *et al.*, 1980). The key features of the model are briefly summarized here, before considering why this model cannot accommodate some key agonist-binding pharmacological properties measured *in vitro*.

The **full** ternary complex model (TCM) is depicted in **Fig. 1.10**. The model was initially proposed to explain a number of pharmacological observed effects when comparing agonist and antagonist binding at the frog erythrocyte β -adrenoceptor. Competition curves for antagonists elicited slope factors of near unity whilst agonist competition curves were shallower which seemed to indicate at least two affinity-states of the receptor. The higher affinity state was only observable in the absence of guanine nucleotides, whilst the lower affinity state predominated in the presence of guanine nucleotides. Furthermore, the intrinsic activity of the agonist, with respect to cyclic AMP accumulation, correlated with the K_H/K_L of the affinity constants for the high (R_H) and low (R_L) affinity states of the receptor. The progression of pharmacological receptor theory is elegantly illustrated in this study, as the authors considered progressively more complex models until the simulated data from computer-generated models accurately predicted the observed effects under different the experimental conditions.



Adapted from Kenakin (2001).

Fig. 1.10 The ternary complex model

The ternary complex model predicts the formation of a high affinity complex, consisting of agonist (**H**, hormone), G-protein (**G**) and receptor (**R**). The binding of an agonist to inactive receptor leads to the isomerization of the receptor to the active state dependent on the efficacy of the agonist.

K – equilibrium constant for receptor and agonist

M – allosteric constant denoting the ratio of receptor in the active versus inactive state

α - factor defining the affinity of the ligand for the active versus inactive state and also the effect of ligand binding on receptor activation

The formation of a high-affinity complex consisting of agonist, receptor and G-protein (termed **X** in the original model) satisfied all of the binding data observed. Within this model α is the factor which controls the **R-G** interaction and is directly related to the efficacy of the ligand (Daeffler and Laundry, 2000). This efficacy-related constant defines the effect of ligand binding on the formation of the **RG** complex, and the relative affinity of the ligand for **R** and **RG**. The potency of the agonist is related to the affinity for **R** and **RG**, its effect on the interactions between **R** and **G** and also the concentration of G-protein in the particular system.

Non-hydrolysable guanyl nucleotides (e.g. GppNHp, GTP γ S) are able to decrease any spontaneous (agonist-independent) association of **R** and **G** (dictated by the equilibrium constant **M**), and therefore the observed affinity of agonist for receptor is decreased in their presence (experimentally observed as a rightward shift in the binding curve). The model predicts that the 'GTP-shift' (the ratio of high affinity binding (K_H) obtained in the absence to that obtained in the presence of GTP (K_L)), may be used as a biochemically-derived measure of the efficacy of the ligand within the particular assay system (Christopoulos and El-Fakahany, 1999). De Lean *et al.*, (1980) were able to demonstrate a direct relationship in the efficacy of agonists in a functional assay compared to the degree of the GTP-shift from binding data. However this receptor system appears to have been a fortuitous choice as some receptor systems show little correlation between K_H/K_L values and efficacy (Gardner *et al.* 1997,1998), or undetectable GTP shifts where they might be expected (Waelbroeck *et al.*, 1997). Therefore efficacy may be more complex than the quantity of active state produced/stabilized by the agonist e.g. GDP/GTP exchange rates.

The ternary complex model was used to describe pharmacological receptor effects until new experimental evidence exposed its limitations and forced its modification (Kenakin, 2004a). Specific mutations within GPCRs that increased the agonist-independent or constitutive activity towards effector pathways verified that the ternary complex model could not account for all of the properties of agonists and antagonists at these modified receptors. The replacement of the C-terminal portion of the third intracellular loop of the β_2 -adrenoceptor with the homologous region of the α_{1B} receptor was shown to cause

agonist-independent activation of adenylyl cyclase, and its effects on agonist binding properties led to the modification of the original ternary complex model (Samama *et al.*, 1993).

It is sometimes cited (Gether, 2000; Kenakin, 2004a) that the demonstration of agonist-independent activity initiated the modification of the ternary complex model. However as can be seen from **Fig. 1.10** the full ternary complex model includes a RG (or at the time RX) complex described as a “*spontaneously-formed, loose complex, which might be capable of interacting with the effector, resulting in an agonist-independent basal response*” (De Lean *et al.*, 1980). It was additional experimental observations (Samama *et al.*, 1993) in conjunction with this constitutive activity that were not accommodated by the ternary complex model’s predictions of agonist behaviour: **(1)**. An increased affinity for agonists (even in the absence of G-proteins) with the magnitude of increase positively correlated to the intrinsic activity of the ligand; **(2)**. No increase in the affinity of antagonists; **(3)**. Increased potency of agonists with respect to the stimulation of adenylyl cyclase activation and **(4)**. An increase in the intrinsic activity of partial agonists (Samama *et al.*, 1993). With extensive computer simulations it was shown that changes in the equilibrium constants which govern the distribution/formation of species, in the ternary complex model, could not accommodate all of these findings with constitutively active mutant.

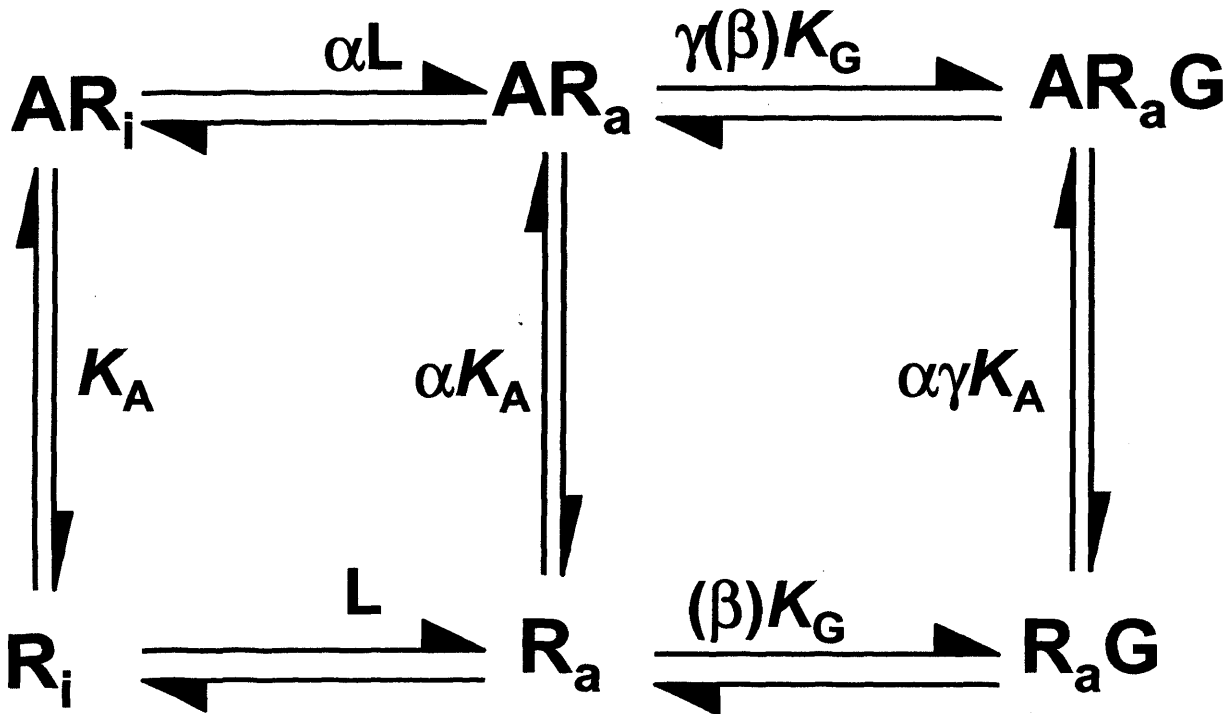
The logical adaptation of the ternary complex model was to include an explicit isomerization of the receptor (**R**) to an active state (**R***) which can now either spontaneously interact with G-protein or form a high affinity complex with agonist and G-protein (Samama *et al.*, 1993; Lefkowitz *et al.*, 1993). This extension is known as the extended ternary complex model (ETC) and is depicted in **Fig. 1.11**. Within this model it is only the active state of the receptor **R*** (**R_a**) that can couple to the G-protein to initiate a biochemical response (Strange, 1999). It was proposed that the creation of the chimeric β/α_{1B} -adrenoceptor had lowered the energy barrier (denoted **L**) so that receptor spends relatively more time in the **R*** state increasing the probability of a spontaneous association with G-protein. However, in order to accommodate all of the experimental

findings the authors suggested that the mutation may have also increased both K (M in the original model) and γ (α in the original model) which are an affinity constant and a coupling constant respectively, describing the formation of H^*RG . The ability of an agonist to elicit functional G-protein coupling now depends on its ability to facilitate the transition of R to R^* and also its ability to stabilize the ternary complex AR^*G (two distinct steps explained below).

The right-hand loop describes the equilibrium between R and R^* governed by L , whilst the left-hand loop describes the equilibrium between R^* and G governed by K_G (see Fig. 1.11). The main differences between the conventional and extended ternary complex models is that molecular efficacy in the extended model depends on the product of two constants α and γ (modifiers of affinity). These describe the extent to which the ligand binding influences the receptor isomerization to R^* and the stabilisation of the R^*G complex respectively (molecular efficacy in the TCM was described by a single constant α - dictating the formation of the ARG complex, see Fig. 1.10). Another distinct difference between the models is that in the absence of ligand, constitutive activity in the ETC model is determined by the affinity of the receptor for G-proteins ($(\beta)K_G$) and the fraction of receptors able to be productively interact with G-proteins (L) (Daeffler and Landry, 2000). Computer simulations predict that the constitutively activating mutations alter L , such that the receptor proportionately resides more frequently in the R^* conformation increasing the probability of spontaneous G-protein coupling. One caveat to the use of the constitutive active mutants is how physiologically relevant the mutationally induced R^* species is with respect to the active conformations stabilised by agonists (or spontaneously formed) in endogenous receptors (Strange, 1999).

1.4.4.2. Beyond the three-state model

A more thermodynamically complete extension of the two-state model is the cubic ternary complex model (CTC), which is outlined in Fig. 1.12. This extension incorporates all the features of the original model, but allows the G-protein to interact with inactive receptors and provides a complete description of the three-way equilibrium between ligand, receptor and G-proteins (Weiss *et al.*, 1996a,b,c). Whether experimental



Adapted from Kenakin (2001)

Fig. 1.11 The extended ternary complex (ETC) model

The extended ternary complex model includes an explicit isomerization of the receptor (R_i) to an active state (R_a or R^*), which can now either spontaneously interact with G-protein ($R_a G$), or form a high-affinity complex with agonist and G-protein ($AR_a G$). Affinity of the receptor for G-protein is modified by β and γ .

K_a – equilibrium constant for receptor and agonist

K_g – equilibrium constant for receptor and G-protein

L – allosteric constant denoting the ratio of receptor in the active versus inactive state

α - factor defining the affinity of the ligand for the active versus inactive state and also the effect of ligand binding on receptor activation

β - factor which defines differential affinity of the receptor for G-proteins

γ - factor that describes differential affinity of the receptor for G-proteins when the ligand is bound

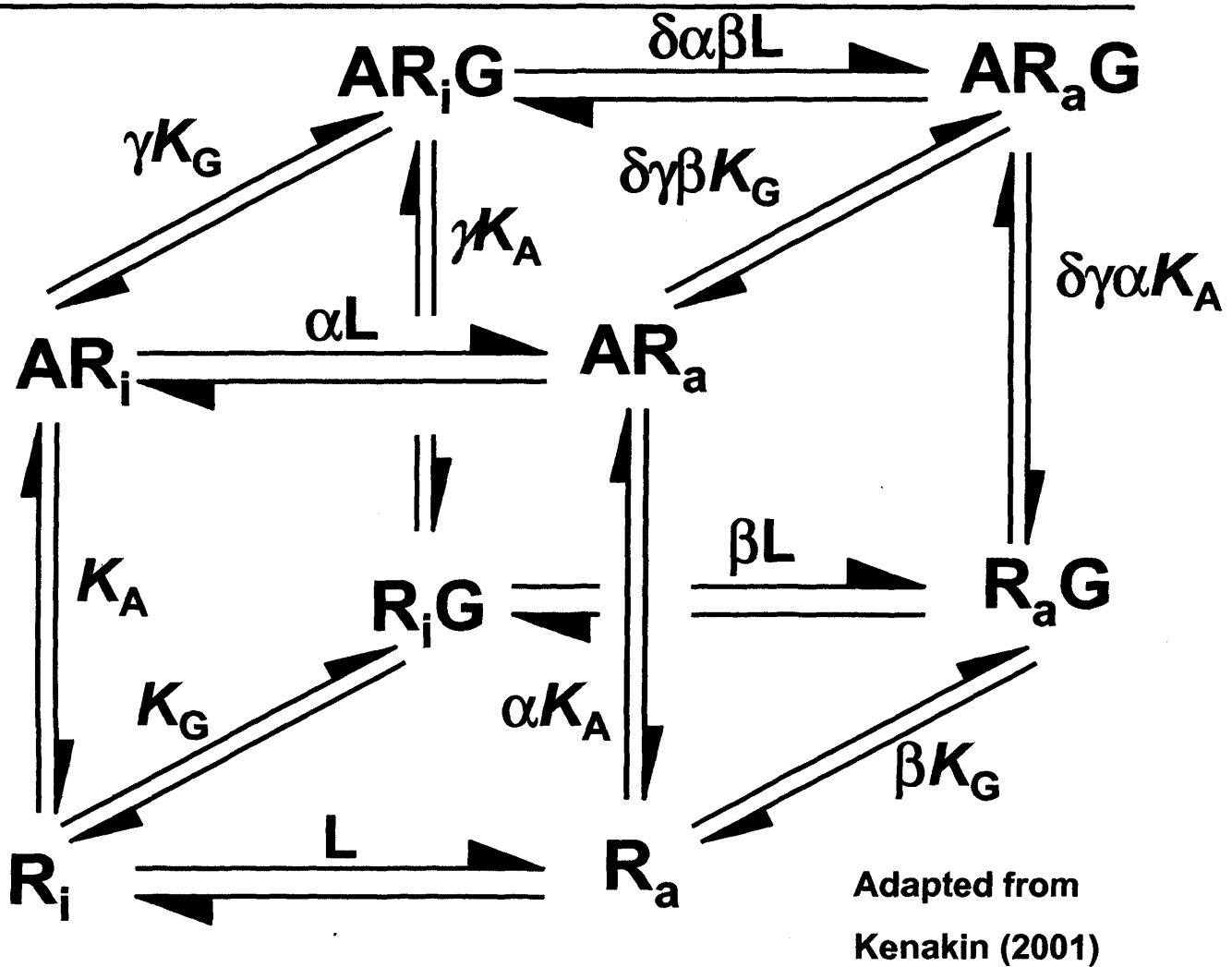


Fig. 1.12 The cubic ternary complex (CTC) model

The more thermodynamically complete cubic ternary complex model, which allows the formation of a non-signalling complex between the inactive receptor and G-protein. The affinity of the receptor for the G-protein is now additionally modified by δ .

K_a – equilibrium constant for receptor and agonist

K_g – equilibrium constant for receptor and G-protein

M – allosteric constant denoting the ratio of receptor in the active versus inactive state

α - factor defining the affinity of the ligand for the active versus inactive state and also the effect of ligand binding on receptor activation

β - factor which defines differential affinity of the receptor for G-proteins

γ - factor that describes differential affinity of the receptor for G-proteins when ligand is bound, here it describes the effect of the ligand on G-protein binding to the inactive-state

δ - factor which defines the synergy produced by simultaneous ligand binding on the interaction of the G-protein with the receptor

evidence justifies this high-level of complexity is open to debate (indeed most of the parameters cannot be experimentally determined) (Kenakin, 2002,a), however evidence for the interaction of an inactive receptor sequestering G-protein has recently been presented (Newman-Tancredi *et al.*, 1997; Bouaboula *et al.*, 1997; McLoughlin and Strange, 2000; Monczor *et al.*, 2003; Fitzsimons *et al.*, 2004).

Although the extended and cubic ternary complex models are often referred to as two-state models, many different conformations of the active receptor (R^*) may be stabilised by assuming ligand-specific micro-affinities through different values α , γ and δ (Kenakin, 2002). In this way the active receptor conformation (and therefore the G-protein complement activated) may be specific for each ligand (Kenakin, 1997c) resulting in unique agonist-receptor activation states. (This is quite different from R^*/R^{**} proposed in the three-state model used to explain agonist-trafficking, which pre-determines the existence of a finite number of active receptor species).

One constraint in both extended and cubic ternary complex models (also known as linkage theory) is that the receptor species present are pre-defined (Kenakin, 2004a), this has lead some investigators to develop receptor theory using a probabilistic approach (Kenakin 1997a; Onaran and Costa 1997; Kenakin and Onaran, 2002). These models assume that an unbound receptor oscillates between many (micro)conformations in the absence of ligand, some being energetically preferred over others. Ligand binding causes the redistribution of these microconformations into a new 'ensemble'. Therefore, the unique selective affinity of a ligand for the various receptor conformations will change the distribution of receptor species and initiate or inhibit a response. It has been suggested that this could form the basic mechanism of ligand efficacy and the molecular nature of ligands (Kenakin, 2001). These models are also more versatile than linkage theory as they accommodate ligand efficacies other than those described by G-protein binding. Agonist-specific differences in phosphorylation (Thomas *et al.*, 2000) and internalization (Keith *et al.*, 1996) have been described. 'Ensemble' theory predicts that there may be differences in agonist properties acting at GPCRs, other than those directly attributable to those mediated directly by G-proteins due to the different micro-conformations stabilised. As

we begin to investigate these G-protein-independent, agonist-specific differences, the acceptance of ‘*ensemble*’ theory, with its unlimited microconformations, may gain a widespread acceptance.

1.5. Constitutively active mutants – applications and role in pathology

The concept of constitutive activity and inverse agonism was reviewed in section 1.4.2, within this Thesis attempts have been made to characterise constitutively active M₁ and M₃ mACh receptors created by specific point mutations. The physiological relevance of constitutive activity remains to be established (Leurs *et al.*, 2000) as its detection, especially in native systems is experimentally difficult. Some endogenous receptors have been shown to display quite high levels of constitutive activity in tissues, including H₃ histamine, A₁ adenosine, M₂ muscarinic, δ -opioid receptors and $\beta_{1/2}$ adrenoceptors (Leurs *et al.*, 2000). Despite this, the majority of GPCRs exhibit low levels of agonist-independent activity, even when relatively over-expressed. Therefore in order to examine the pharmacology of ligands in systems with detectable levels of agonist-independent activity, the construction of constitutively active mutants (CAMs) has become routine. Although the use of CAMs may not reflect the endogenous active state (R^*), their study has made a significant contribution to our understanding of receptor function and drug action at a molecular level (Cotecchia *et al.*, 2003). The following section will briefly discuss the role of constitutive activity in the pathology of disease and the contribution that constitutive active mutants have made in drug discovery and in the delineation of the receptor activation process.

1.5.1. Constitutively active mutants – role in disease pathology

Constitutively active mutant GPCRs have been implicated in the pathology of several diseases (Leurs *et al.*, 2000). The first naturally occurring mutation was identified in rhodopsin, where mutation at Lys-296 in transmembrane domain 6 results in a severe form of autosomal dominant retinitis pigmentosa. This mutation is predicted to disrupt a salt bridge binding region located between transmembrane domains 3 and 7 that assists in constraining opsin in an inactive conformation. The resultant constitutive activation leads to consistent activation of photoreceptors, which may lead to their degeneration and

consequently the disease pathology (Cotecchia *et al.*, 2003). Other diseases in which a constitutive active mutant receptor has been shown to play a part in disease pathology include hyperthyroidism (thyroid stimulating hormone receptor), male precocious puberty (luteinizing hormone receptor), Jansen's metaphyseal chondrodysplasia (parathyroid hormone-related peptide receptor), gastric carcinoid tumours (cholecystokinin-B/gastrin receptor type-2) and congenital night blindness (rhodopsin) (Arvanitakis *et al.*, 1998, Seifert and Wenzel-Seifert, 2002). Although constitutively active mutants are currently implicated in the progression of a subset of rare genetic disorders, it is possible that as we increase our understanding of these receptors and the biochemical pathways constitutively activated, their prevalence in (patho)-physiology may be more common than initially suspected.

Agonist-independent activity can also occur *in vivo* by other means than the mutation of the GPCR itself. Auto-antibodies can possess agonist-like properties and have been associated with several human diseases including idiopathic dilated cardiomyopathy and Chagas disease (anti-M₂ muscarinic and β_1 -adrenergic), Graves disease (anti-TSH receptor) and various forms of malignant hypertension (anti-AT₁ receptor). Antibodies produced in Chagas disease behave as muscarinic receptor partial agonists and are able to induce phosphorylation of the M₂ receptor when expressed in recombinant cells (Borda and Sterinoborda, 1996). Studies with the β_2 adrenoceptor have demonstrated that antibodies corresponding to the second intracellular loop are able to induce or stabilise an active conformation of the receptor (Leurs *et al.*, 2000). From these studies, and the role of constitutive activity in some disease in general, it appears intuitive that the administration of inverse agonists, to diminish constitutive activity, would be advantageous compared to neutral antagonists. Despite this the potential importance of inverse agonists and whether they have inherent benefits as therapeutic agents remains to be established (Milligan, 2003a).

1.5.2. Constitutively active mutants – drug discovery

As well as the discussed therapeutic applications of inverse agonism in disease involving a CAM-receptor, they have a previously unappreciated application in the drug discovery

process (Ligt *et al.*, 2000). Because of the low proclivity of some GPCRs to form an active state, high-throughput screening using a quiescent receptor would be unable to distinguish inverse agonist from neutral antagonism. A recent study demonstrated the high-throughput capability of a constitutively active human calcitonin receptor (hCTR2) expressed in melanophores (Chen *et al.*, 1999). In addition to the detection of inverse agonists, partial agonists and inverse agonist display greater efficacy and potency in CAM-receptor systems, allowing the detection of potentially useful ligands that might not be detected in less sensitive primary screens (Kenakin, 2003).

1.5.3. Constitutively active mutants – receptor activation

If we accept that CAMs, at least in part, mimic the active state of the receptor then the position and processes affected by these mutations might also give some clues as to the major structural differences between the active and inactive conformations (Parnot *et al.*, 2002). Although some progress has been made on this respect, receptor activation can involve numerous and apparently unrelated residues in many parts of the GPCR structure (Cotecchia *et al.*, 2003). This probably reflects that receptor activation is a complex, highly regulated process, with a strong evolutionary pressure to maintain the receptor in inactive state (Milligan, 2003). This point is perfectly illustrated by replacement of an alanine residue in the third intracellular loop of the hamster α_{1B} -adrenoceptor, by any other amino acid, lead to varying degrees of agonist-independent activity (Kjelsberg *et al.*, 1992). Despite these caveats, mutations of residues leading to constitutive activity, which indirectly implicate areas important for receptor activation have been identified in most regions of GPCRs. These include the D/ERY motif found at the cytoplasmic end of TM3 (Scheer *et al.*, 1996), constraints between TM6 and TM7 (Han *et al.*, 1998), and TM3 and TM6 (Grobowski *et al.*, 1997; Greasley *et al.*, 2001). From these studies a common mechanism appears to be a switch in the cytoplasmic ends of TM3 and TM6 (therefore altering the conformation of the intracellular loops), evidence for this is discussed further in the appropriate Results Chapters.

1.6. Thesis aims

There are two main experimental objectives within this thesis:

(1). To investigate any agonist-specific differences in the complement of G α subtypes activated in response to M₁ and M₃ mediated receptor activation. Initially the global population of G α subtypes activated, in response to agonists, will be assessed using a filtration [³⁵S]-GTP γ S binding assay using membranes prepared from M₁ and M₃ mACh receptors stably expressed in Chinese hamster ovary cells. Some indication of the potential heterogeneity of G-protein activation will be made using membranes pre-treated with Pertussis toxin to assess the population of G α_i -like and G α_q -like agonist activation profiles. I will then employ a complementary immunoprecipitation technique to allow the direct determination of the potency and relative efficacy of G $\alpha_{q/11}$ and G α_{i1-3} agonist-mediated activation. It is important to understand whether agonist activation of a particular subtype leads to a uniform G-protein activation profile or whether agonists activate specific G α subtypes. This may lead to the development of compounds with a reduced side-effect profile and the appreciation that efficacy measurements taken from one particular functional assay are not necessarily indicative of an agonist's behaviour in another.

(2). The effects of a homologous mutation, in the M₁ and M₃ mACh receptor, proposed to reside at the junction of transmembrane domain 6 and the third extracellular loop, will be investigated. A homologous mutation in the M₅ receptor has reported to confer agonist-independent 'constitutive-activity' (Spalding *et al.*, 1995). The effects of the mutation on the G-protein coupling profile of agonists will be investigated using a variety of functional read-outs. Many antagonists have also been reported to actually have 'inverse-agonist' efficacy at constitutive activity receptors, and also that they may exert their effects in a more complex manner than simply stabilising the inactive receptor conformation, **R**. Therefore a range of structurally diverse antagonists will be employed to test for their inverse-agonist potential. All of the experiments will look for evidence of multiple active and inactive receptor conformations. It is hoped that not only will this study provide information on the receptor activation process, but as many muscarinic

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antagonists are used therapeutically it is important that we understand the effects of inverse agonists on downstream signalling pathways.

Chapter Two.

Materials and Methods

Chapter 2: Materials and Methods

The methods detailed in this section detail protocols conducted on a routine basis. Specialized procedures and assays run on a more restricted basis are reviewed in the appropriate Results Chapters. Methods detailed are also for final assay procedures, any optimization of assay steps is detailed in the Results sections.

2.1 Cell culture techniques

2.1.1 Chinese hamster ovary cells

Chinese hamster ovary (CHO) cells transfected with the cDNA encoding the human M₁ (CHO-m1) or human M₃ (CHO-m3) muscarinic acetylcholine receptors were originally obtained from Dr N. Buckley (then of the National Institute for Medical Research, Mill Hill, London). CHO cell clones were grown in minimum essential medium (α MEM) supplemented with 10% foetal calf serum, 100 IU/ml penicillin, 100 μ g ml⁻¹ streptomycin and 2.5 μ g ml⁻¹ fungizone. Cells were maintained at 37°C in 5% CO₂/humidified air. Cells were routinely split 1:10, using trypsin-EDTA to lift cells, three times weekly and were not used in assays beyond passage 35.

2.1.2 Human embryonic kidney cells

2.1.2.1 Cell culture

Human embryonic kidney 293 (HEK-293) cells were obtained from Dr A.B Tobin (Dept. of Cell Physiology and Pharmacology, University of Leicester, UK). HEK-293 cells were grown in α MEM supplemented with 10% foetal calf serum, 100 IU/ml of penicillin, 100 μ g ml⁻¹ streptomycin and 2.5 μ g ml⁻¹ fungizone. Cells were maintained at 37°C in 5% CO₂/humidified air. Cells were routinely split 1:10, using PBS containing 2 mM EDTA to lift cells, once weekly and were not used in assays beyond passage 35.

2.1.2.2 Cell adhesion

HEK-293 cells do not efficiently adhere to plastic, in assays where cells would be subjected to frequent wash steps this can lead to a significant loss of cells from the substratum. Therefore, when plating HEK-293 cells for specialized assays (e.g. [³H]-

Chapter 2: Materials and Methods

inositol phosphate accumulation, or receptor upregulation) poly-L-lysine was used to create a cell culture substratum. Briefly, 0.5 ml of a 0.01% sterile filtered poly-L-lysine solution was added to each well of a 24-well plate or 1 ml to each well of a 6-well plate. After 10 min the solution was aspirated and plates left to air dry for at least 30 min prior to plating.

2.1.3 Pertussis toxin treatment

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

2.2 Transfection procedures

The quantity of plasmid DNA (µg) added per well or flask varied with experimental design. Therefore, the amount of plasmid DNA added to cells is detailed in the appropriate Results section. The Table below serves as general guide to the transfection quantities used.

	24-well plate	6-well plate	175 cm ² flask
No. Cells (x 10 ⁵)	0.2-0.8	1-3	12-48
Complete volume of growth medium	1 ml	4 ml	25 ml
Volume serum-free medium in transfection mix	25 µl	100 µl	1000 µl
Volume GeneJuice™	0.4 – 2 µl	6.4 µl	20 – 80 µl
Plasmid DNA	0.1 – 0.5 µg	1.6 µg	5-20 µg
Experiments used for plate/flask type	(1) receptor upregulation (2) receptor expression levels	Receptor phosphorylation	Membrane preparations for large scale [³ H]-NMS saturation and

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	(3) [³ H]-inositol phosphates (4) IP ₃ mass		displacement experiments
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Cells were plated and grown to approx. 40-50% confluency before transfection and the medium replaced with fresh. The plasmid DNA was diluted to the appropriate concentration in ddH₂O. The ratio of DNA : GeneJuice™ (transfection reagent) was found to be optimal at 1 µg per 4 µl and this ratio was used throughout the experiments.

The transfection protocol was as follows: the appropriate volume of GeneJuice was added drop-wise to serum-free medium in a 7 ml Bijou bottle and incubated for 5 min at room temperature. The entire volume of GeneJuice/medium was added to the appropriate volume of diluted plasmid DNA in a fresh 7 ml Bijoux, gently mixed and incubated for a further 15 min. The mixture was then pipetted drop-wise over the cell monolayers and gently rocked to ensure even distribution. After 12-14 h the medium was replaced with fresh and experiments conducted after the time intervals indicated in the Results sections.

2.3 Cell membrane preparation

The CHO-cell or HEK-cell clones were grown to 90-100% confluency and briefly washed with ice-cold 'lifting buffer' (2x 10 mL; 10mM HEPES, 0.9% NaCl, 0.2% EDTA pH 7.4). All subsequent steps were conducted at 0-4°C to negate the use of protease inhibitors. Ten mL of lifting buffer was added and left for approx. 20 min for the cells to detach from the plastic. The cells were then removed from the flask into a 30 mL centrifuge tube and the flask washed with a further 5 mL lifting buffer and this was also added to each tube. The cell suspension was centrifuged at 250 xg for 5 min to allow a pellet to form. The supernatant was aspirated and 10 ml per 175 cm³ flask of wash buffer 1 (10 mM HEPES, 10 mM EDTA, pH 7.4) was added to the pellet. This was homogenized using a Polytron (20,000 r.p.m., 5 x 10 s bursts) and subsequently centrifuged at 50,000 xg at 4°C (Sovrall RC5). The supernatant was discarded and the pellet re-homogenized and centrifuged as described in wash buffer 2 (10 mM HEPES, 0.1 mM EDTA, pH 7.4). The final pellet was suspended in wash buffer 2 at a concentration

of 3-5 mg ml⁻¹. Protein concentration was determined by the appropriate method (see section 2.10), using BSA as a standard and then kept at -80°C until required.

2.4 [³H]-NMS radioligand binding to CHO or HEK cells

2.4.1 [³H]-NMS saturation binding

2.4.1.1 [³H]-NMS saturation binding to membrane preparations

Cell membranes were prepared as previously described. A range of concentrations of [³H]-NMS was used (0.07-3 nM) to construct saturation binding curves, as described by Lambert *et al* (1989). Non-specific binding was determined in the presence of 1 µM atropine. CHO/HEK-cell membranes (approx. 20 µg) were incubated at room temperature for 60 min, to ensure equilibrium was reached in assay buffer (10 mM HEPES, 1 mM MgCl₂, pH 7.4). Reactions were terminated by rapid vacuum filtration through Whatman GF/B filters followed by 4 washes with ice-cold assay buffer. Filters were removed and placed in scintillation vials and 5 ml scintillant was added. Radioactivity was left to extract for >12 h before quantifying by liquid scintillation spectrometry. Aliquots of the stock [³H]-NMS solutions were also taken to determine precisely how much radioactivity was added to each tube.

2.4.1.2 [³H]-NMS saturation binding to plated whole cells

(Transfected) HEK/CHO cells were grown to near confluency in 24-well plates, medium was gently aspirated and cells washed three times in 1 ml Krebs-Henseleit buffer (KHB) (118.6 mM NaCl, 4.7mM KCl, 1.3 mM CaCl₂, 10 mM HEPES, 11.7 mM D-Glucose, 1.2 mM MgSO₄, 1.2 mM KH₂PO₄, 4.2 mM NaHCO₃, pH 7.4 after saturating with O₂/CO₂ (95:5) at 37°C). A range of concentrations of [³H]-NMS was used (0.07-3 nM) to construct saturation binding curves, as described by Lambert *et al* (1989). Non-specific binding was determined in the presence of 1 µM atropine. Plated cells were incubated at 37°C for 60 min, to ensure equilibrium was reached in KHB. After the incubation period the plate was placed on ice and washed with the following protocol using ice-cold KHB:

2 x 1 mL rapid washes, 1 x 1 mL 15 min wash and 1 x 1 mL rapid wash. 250 µl of 0.1 M NaOH was added to each well and incubated for 20–30 min. The cells were then transferred to 5 ml scintillation vials and the wells washed and lifted with a further 250 µl 0.1 M NaOH. Five ml scintillant was added to each well and radioactivity was left to extract for >12 h before quantifying by liquid scintillation spectrometry. Aliquots of the stock [^3H]-NMS solutions are also taken to determine precisely how much radioactivity was added to each tube.

For some experiments only an estimation of the maximal receptor density was required. In this case cells were treated as detailed above, but incubated in the presence of 4 nM [^3H]-NMS to ensure saturation binding was achieved. NSB was always determined in the presence of 1 µM atropine.

2.4.1.3 [^3H]-NMS saturation binding to plated whole cells pre-treated with antagonists

Twenty-four hours prior to radioligand binding cell monolayers were incubated with the appropriate concentration of antagonist made up in medium. (Transfected) HEK/CHO cells were then grown to near confluency in 24-well plates, medium was gently aspirated and cells washed five times in 1 ml Krebs-Henseleit buffer to ensure that all traces of antagonist were removed with the following protocol: 3 x 1 mL rapid wash; 1 x 1 mL 15 min wash and 1 x 1 mL rapid wash. Plated cells were incubated at 37°C for 60 min, to ensure saturation was reached, in KHB with 4 nM [^3H]-NMS (NSB was determined in the presence of 1 µM atropine. After the incubation period the plate was placed on ice and washed with the following protocol using ice-cold KHB: 2 x 1 mL rapid washes, 1 x 1 mL 15 min wash and 1 x 1 mL rapid wash. 250 µl of 0.1 M NaOH was added to each well and incubated for 20–30 min. The cells were then transferred to 5 ml scintillation vials and the wells washed and lifted with a further 250 µl 0.1 M NaOH. 5 ml scintillant was added to each well and radioactivity was left to extract for >12 h before quantifying by liquid scintillation spectrometry. Aliquots of the stock [^3H]-NMS solution are also taken to determine precisely how much radioactivity was added to each tube.

2.4.1.4. [^3H]-NMS displacement binding

To obtain affinity estimates for agonists and antagonists [^3H]-NMS displacement experiments were performed. [^3H]-NMS was used at an approx. K_D concentration (between 200-400 pM) with the actual concentration used in each experiment being quantified to ensure the correct value was used in subsequent affinity calculations. [^3H]-NMS was incubated in the presence of the appropriate concentration of unlabelled ligand and membrane concentration in binding buffer (10 mM HEPES, 1 mM MgCl_2 , pH 7.4) for 60 min at room temperature. The absolute quantity of membrane added was adjusted so that [^3H]-NMS binding did not exceed more than 10% of the total radioactivity added. Total binding was determined in the absence of ligands and non-specific binding in the presence of 1 μM atropine. Reactions were terminated by rapid vacuum filtration through Whatman GF/B filters followed by 4 washes with ice-cold assay buffer. Filters were removed and placed in scintillation vials and 5 ml scintillant was added. Radioactivity was left to extract for >12 h before quantifying by liquid scintillation spectrometry. Aliquots of the stock [^3H]-NMS solution are also taken to determine precisely how much radioactivity was added to each tube.

2.4.1.5. Effects of GTP on [^3H]-NMS displacement binding

To evaluate the effects of guanosine 5'-triphosphate (GTP), GTP was added to the binding buffer at a final concentration of 100 μM .

2.5 [^{35}S]-Guanosine 5'-[γ -thio]triphosphate radioligand binding in stably transfected CHO, or transiently transfected HEK cells

2.5.1 Filtration assay of [^{35}S]-GTP γS binding

Prior to experimentation the cells were harvested and prepared as detailed in section 2.3 ('Cell membrane preparation'). The protocol of Dowling *et al* (2004) was followed. Briefly, 100 μL of [^{35}S]-GTP γS (200-400 pM final concentration), 100 μL GDP (1 μM final concentration), and 100 μL test-ligand (appropriate final concentration) were added to a 5 mL tube with assay buffer added to a final volume of 900 μL . In some tubes non-specific binding was determined with the addition of 100 μL GTP γS (10 μM final

concentration). Assay mixtures and membranes preparations were brought to 30°C and the assay initiated with the addition of 100 µL of membranes (25-50 µg protein per tube). Assays were run for 45 min at 30°C. Towards the end of the incubation period glass fibre filters were wetted with ice-cold assay buffer. Reactions were terminated by rapid vacuum filtration through Whatman GF/B filters followed by 4 washes with ice-cold assay buffer. Filters were removed and placed in scintillation vials and 5 ml scintillant was added. Radioactivity was left to extract for >12 h before quantifying by liquid scintillation spectrometry. Aliquots of the stock [³⁵S]-GTPγS solution are also taken to determine precisely how much radioactivity was added to each tube.

2.5.2 Scintillation proximity assay (SPA) to measure [³⁵S]-GTPγS binding mediated by CHO-cells expressing the human mACh receptor

Prior to experimentation the cells were harvested and prepared as detailed in section 2.3 ('Cell membrane preparation'). 20 µL of test drug (at an appropriate concentration) was mixed in a final volume of 200 µL, containing 20 µL GDP (1 µM final), 100 µL of membrane preparation (25-50 µg protein per well final) and 10 µL of [³⁵S]-GTPγS (200-400 pM final). Basal values were determined by the replacement of drug with assay buffer (10 mM HEPES, 100 mM NaCl, 10 mM MgCl₂ pH 7.4) and NSB in the presence of 20 µL GTPγS (10 µM final). The reaction was carried out in 96-well opti-plates and initiated by the addition of [³⁵S]-GTPγS. Immediately after the addition of [³⁵S]-GTPγS, 50 µl of WGA-SPA beads were added (0.88 mg ml⁻¹ final) and incubated for 30 min at 30°C. The assay was terminated by centrifugation at 5000 xg for 6 min and radioactivity was quantifying by liquid scintillation spectrometry.

2.5.3 Immunoprecipitation of [³⁵S]-GTPγS-bound G proteins with antisera to specific Gα proteins

Prior to experimentation the cells were harvested and prepared as detailed in section 2.3 ('Cell membrane preparations'). The protocol of Dowling *et al* (2004) was followed. Membrane aliquots were diluted to concentration of 75 µg/50 µL in assay buffer. 50 µl of membranes were incubated in the presence of 10 µL [³⁵S]-GTPγS (10 nM final), 10 µL test drug (at an appropriate concentration), 10 µL GDP (1 µM final) and 20 µL assay

buffer. Basal levels were determined by the substitution of test drug for assay buffer and NSB estimated by the addition of 10 μ L GTP (100 μ M final). Incubations were initiated by the addition of membrane and incubated for 2 min at 37°C. Reactions were rapidly terminated by the addition 1000 μ L of ice-cold assay buffer and placing the Eppendorf tube on ice. Membranes were separated by centrifugation at 20,000 $\times$ g for 6 min (Eppendorf 5417 R) all subsequent steps were carried out at 0-4°C. The supernatant was removed and the pellet solubilized by the addition of 50 μ L ice-cold solubilization buffer containing SDS (100 mM Tris, 200 mM NaCl, 1mM EDTA, 1.25% Igpal CA 630, 0.2% SDS, pH 7.4) and repeated vortex-mixing. Once the membranes had been successfully solubilized 50 μ L of solubilization buffer (minus SDS) was added to each tube.

The solubilized membranes were pre-cleared with normal rabbit serum (1:100 dilution) and 30 μ L protein A beads (protein A sepharose bead suspension 3% w/v in TE buffer) by rolling for 1 hour at 4°C. The protein beads and any insoluble material was collected by centrifugation at 20,000 $\times$ g for 6 min. 100 μ L of the supernatant was transferred to a fresh Eppendorf tube containing the appropriate G α -specific antiserum (1:100 dilution). Samples were vortex-mixed and rolled overnight at 4°C. 70 μ L of protein A solution was then added and samples rolled as before for 90 min. Protein A beads were then pelleted by centrifugation as described before. The supernatant was removed by aspiration and the beads washed in 1000 μ L of solubilization buffer (minus SDS) and centrifuged as before. This process was repeated so the beads received four washes in total. After the final wash the sepharose beads were mixed with 1.1 ml of scintillation fluid and vortex-mixed. Radioactivity was left to extract for >12 h before quantifying by liquid scintillation spectrometry. Aliquots of the stock [³⁵S]-GTP γ S solution are also taken to determine precisely how much radioactivity was added to each tube.

2.6 G-protein western blotting

Western blotting samples were prepared from cells harvested and prepared as detailed in section 2.3 ('Cell membrane preparations'). Additionally western blotting was also performed on transfected cell monolayers harvested from 6-well plates. An appropriate volume of protein (between 25-50 μ g protein in 20-30 μ L) was mixed with equal volume

of sample buffer (100 mM Tris-HCl, pH 6.8, 200 mM dithiothreitol, 4% SDS, 0.1% bromophenol blue, 20% glycerol) and boiled for 5 min. Samples were subjected to SDS-PAGE by loading into 5% stacking gel, 12% running gel and running at 180 mV for 30-60 min (running buffer: 25 mM Tris-HCl, 150 mM glycine, 0.1% SDS). Molecular weight of proteins was determined by running known standards. Semi-dry apparatus was used to transfer the proteins to nitrocellulose ($0.65 \text{ mA cm}^2 \text{ gel}^{-1}$ for 2 hours: blotting buffer; 48 mM Tris-HCl, 39 mM glycine, 0.037% SDS, 20% methanol). Transferred proteins were blocked overnight in a 10% powdered milk solution in TBS-Tween. Membranes were then incubated in antibody (1:1000) in 4 ml TBS-Tween for 60 min at room temperature with gently agitation. Membranes were washed 4 x 5 min in excess TBS-Tween before incubation with secondary peroxidase conjugated antibody at 1:1000 dilution in PBS-Tween. The membranes were washed as before but with the addition of a final 20 min wash in TBS-Tween. The ECL reagent kit from Amersham was used (according to the manufacturers instructions) to develop the blot.

2.7 [^3H]-inositol phosphate accumulation

Transfected HEK-monolayers (cultured in a 24-well plate) were grown to 70-80% confluency. The growth medium was removed and the cells labelled with 1 $\mu\text{Ci/ml}$ (0.074 MBq/ml) of D-*myo*-[2- ^3H] inositol in 0.5 mL of fresh medium for 24 h. All subsequent steps were carried out in a waterbath at 37°C except where indicated. The loading medium was removed and cells gently washed three times in 1 mL KHB. The final wash solution was removed and the antagonist and/or lithium chloride (10 mM final concentration) as per the protocol developed in section 4.3. After 10 min incubation agonist or vehicle control was added to each well and the incubations continued (as per section 4.3). The reaction was rapidly terminated with aspiration of the stimulating solution and the addition of 0.5 mL ice-cold trichloroacetic acid (0.5 M TCA). The plates were then transferred to ice and left to extract for 20 min before each sample was removed to a 1.5 mL centrifuge tube containing 10mM EDTA (100 μL). This was followed by the addition of 1,1,2 – trichloro-trifluoroethane/ tri-*n*-octylamine (1:1 v/v, 500 μL). Tubes were then vortexed several times over a 15 min period, before centrifugation for 2 min at 13,000 rpm. 450 μL of the top layer was transferred to fresh

Eppendorf tubes containing 125 μ L 60 mM NaHCO_3 . Tubes were stored at 4°C until ion-exchange chromatography.

Inositol phosphates were separated according to their charge by anion exchange chromatography over a 1 ml depth of Dowex 1 – X8 (200-400 mesh, formate form; Sigma-Aldrich Ltd), as described previously (Challiss *et al.*, 1992). Columns were first regenerated with regeneration buffer (2M ammonium formate [$\text{CH}_2\text{O}_2\text{NH}_3$] / 0.1M formic acid [CO_2H_2]; 10 ml), followed by excess deionised water. Samples were then washed onto columns with approximately 5 ml water. Glycerophosphoinositol phosphates were then removed from each sample by washing with 60 mM ammonium formate/ sodium tetraborate ($\text{Na}_2\text{B}_4\text{O}_7$) (10 ml). [^3H]- InsP_{1-3} were then eluted into 20 ml scintillation vials, with 0.75 M ammonium formate / 0.1 M formic acid (10 ml). Radioactivity in a 5 ml sample of the elute was determined by scintillation counting.

2.8 Assessment of receptor phosphorylation

The protocol of Tobin, 1997 was followed; briefly cells were grown to 80-90% confluency in 6-well cell culture plates. The media was removed and cells gently washed twice in phosphate free Krebs/HEPES buffer. The last wash was aspirated and replaced with 500 μ L fresh phosphate Krebs/HEPES buffer containing [^{32}P]-orthophosphate (50 $\mu\text{Ci/mL}$) and incubated for 60 min at 37°C. The cells were then challenged with the appropriate concentration of agonist for a further 10 min, note that for inverse-agonist experiment ligands were added simultaneously with the [^{32}P]-orthophosphate. The Krebs/HEPES buffer was removed and replaced with 0.5 mL ice-cold solubilisation buffer (10 mM Tris-HCl, 10mM EDTA, 500mM NaCl, 1% NP-40, 0.1% SDS, 0.5% deoxyxholate, 0.2 mM sodium vanadate, 2mM disodium nitrophenyl phosphate) and the plate placed on ice for 30 min. The solubilised slurry was transferred to a 1.5 mL microcentrifuge tube and pre-cleared by centrifugation (14,000 rpm 5 min). 450 μ L of pre-cleared solution was transferred to fresh centrifuge tubes containing 1 μ L of M_3 antibody (kind gift from Prof. A.B Tobin) and incubated on ice for 1 hr. 100 μ L of protein A solution was added to each sample and rotated for 1 hr at 4°C. The protein A solution was pelleted by a brief centrifugation (14,000 rpm 1 min), the supernatant was aspirated

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and the beads washed in TE buffer (10mM Tris-HCl, 10mM EDTA, pH 7.4). This procedure was repeated so that the beads were washed 4 times in total. The final pellet was re-suspended in 20µL of 2X SDS PAGE sample buffer and the sample heated for 2 min at 85°C. A duplicate plate enabled the receptor density of each well to be calculated, so that an equal number of receptors could be loaded onto an 8% SDS-PAGE gel to resolve the proteins. After separation the gel was stained with 0.2% Coomassie blue in 50% methanol/10% acetic acid to visualise the immunoprecipitated antibody to confirm equal loading. After de-staining with 50% methanol/10% acetic acid, the gel was dried and an autoradiograph obtained.

2.9 Protein determination

For all experiments protein was determined by a modification of the method of Lowry *et al* (1951). Protein standards were prepared using a bovine serum albumin (BSA) solution (1 mg ml⁻¹) which was further diluted to give an appropriate range of protein concentrations (0–250 µg ml⁻¹). 500 µL of standards across an appropriate range were aliquoted into test tubes, in duplicate. Protein samples were diluted in 0.1 M NaOH to give a final volume of 500 µL. To each tube 2.5 mL of ‘solution A’ was added (50 mL 2% Na₂CO₃ in 0.1 M NaOH and 0.5 mL 1% CuSO₄ and 0.5 mL 2% Na⁺ K⁺-tartrate). After a 10 min incubation 100 µL of Folin-Ciocalteu’s phenol reagent (diluted 1:3 in water) was added to each tube and vortex mixed. After 20 min the absorbance was measured at 750 nm and the protein concentration was determined relative to the protein standards.

2.10 Materials

List of reagents and suppliers

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

Acetic acid

Agarose

Ammonium formate

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Ampicillin sodium salt
Atropine sulphate
Bovine serum albumin (BSA)
Bromophenol blue
Carbamylcholine chloride (Carbachol)
Coomassie Blue
4-Diphenylacetoxy-N-methylpiperidine methiodide (4-DAMP)
Disodium nitrophenyl phosphate
Dithiothreitol (DTT)
Dowex formate
Ethidium bromide
Ethylenediaminetetra-acetic acid (EDTA)
Ethanol
Folin-Ciocalteu's phenol reagent
Formic acid
Glycine
Guanosine-5'-diphosphate (GDP)
Guanosine-5'triphosphate (GTP)
Guanosine-5'-O-(thiophosphate) tetralithium salt (GTP γ S)
HEPES
Igepal
Isopropanol
Isopropyl-beta-D-thiogalactopyranoside (IPTG)
LB Agar Tablets
Methacholine chloride
Methanol
Methoctramine tetrahydrochloride
Nonidet-P40
Normal rabbit serum
Oxotremorine methiodide (Oxotremorine-M)
Oxotremorine sesquifumarate salt (Oxotremorine)

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Pertussis toxin

Poly-L-lysine solution

Pilocarpine dihydrochloride

Pirenzepine dihydrochloride

Sodium tetraborate

Sodium vanadate

1,1,2-Trichlorotrifluoroethane (Freon)

Tri-*n*-octylamine

Tris-base

Tris-HCl

X-gal

From Fisher Scientific Equipment, Loughborough, Leics, England

Calcium chloride (CaCl_2)

Cupric sulphate

D-glucose

Hydrochloric acid (HCl)

Magnesium chloride hexahydrate

Magnesium sulphate (MgSO_4)

Potassium chloride (KCl)

Potassium dihydrogen orthophosphate (KH_2PO_4)

Potassium hydroxide (KOH)

Potassium sodium tartrate

Sodium Chloride (NaCl)

Sodium carbonate (Na_2CO_3)

Sodium dodecyl sulphate (SDS)

Sodium hydrogen carbonate (NaHCO_3)

Sodium hydroxide (NaOH)

Sodium succinate hexahydrate

Trichloroacetic acid (TCA)

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From (Invitrogen Incorporation (Incorp. GIBCO), Paisley, Scotland.

1000 bp Molecular Weight Markers

Alpha MEM medium

Foetal calf serum

Fungizone

Penicillin

Phosphate buffered saline (PBS)

Protein Standards

S.O.C media

Streptomycin

Trypsin-EDTA

From Amersham Biosciences UK Limited

D-*myo*-[2-³H] Inositol

ECL Western Blotting detection Kit

Guanosine 5'-[γ-³⁵S] thiophosphate triethylammonium salt ([³⁵S]-GTPγS)

L-[N-methyl-³H]Scopolamine methyl chloride ([³H]-NMS)

[³²P]-orthophosphate

Peroxidase conjugated anti-rabbit secondary antibody

Protein A sepharose

From EMD Biosciences (Incorp. Novagen), Nottingham, England.

GeneJuice

Mobius 1000 plasmid kit

NovaBlue strain singles competent cells

NovaTaq DNA polymerase Kit

SpinPrep gel DNA kit

SpinPrep PCR Clean-up Kit

SpinPrep Plasmid Kit

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

Anti G α_{i-3} rabbit polyclonal IgG (Cat# sc-1726)

From Roche Molecular Biochemicals, Lewes, East Sussex, England.

Alkaline phosphatase

Bam H1, *Eco* R1, *Acc*I, *Xho* I and *Ssp* BI restriction enzymes

From Stratagene, La Jolla, California, USA.

QuikChange site-directed mutagenesis kit

Chapter Three.

Evaluation of the G-protein coupling profile of CHO-cells stably expressing the M₁ and M₃ human muscarinic acetylcholine receptors

3.1. Introduction

3.1.1. Mechanisms of GPCR multiple effector pathway activation

It was originally proposed that GPCRs were essentially monogamous, coupling to either a single GTP-binding protein, or to closely related members of a single sub-family. However, for many GPCRs it has become generally accepted that simultaneous coupling to unrelated G-protein family members can be readily observed (Hermans, 2003; Rashid *et al.*, 2004). Multiple or 'promiscuous' G-protein coupling has been reported for receptors expressed both recombinantly (Palmer *et al.*, 1995; Calandra *et al.*, 1999; Selkirk *et al.*, 2001; Wellner-Kienitz *et al.*, 2003) and endogenously (Allgeier *et al.*, 1997; Kilts *et al.*, 2000; Jin *et al.*, 2001; Krsmanovic *et al.*, 2003) in cell-lines.

The demonstration of multiple effector coupling elicited by a single agonist is not in itself direct evidence of multiple G α subtype coupling. **Fig. 3.1.** depicts three ways in which multiple effector pathways may be activated by a receptor(s) interaction with a single G-protein α subtype, which are briefly summarized below:

(1). For most GPCRs, multiple receptor subtypes have been identified which may have diverse G-protein-coupling profiles (Martin *et al.*, 1998; Sidhu and Niznik, 2000; Sailer *et al.*, 2001). This diversity of receptor subtypes is further complicated by possibility of differential G-protein coupling due to the presence of receptor splice variants (Vanetti *et al.*, 1993; Alexandre *et al.*, 2002; Pindon *et al.*, 2002; Germano *et al.*, 2004) and/or RNA editing of receptor subtypes (Niswender, 1998; Fitzgerald *et al.*, 1999; Price and Sanders-Bush, 2000; Berg *et al.*, 2001). Therefore, differential effector activation could be mediated by agonist binding at different receptor subtypes and/or by post-translational modifications (**Fig. 3.1A**).

(2). Although the G α subunit is thought of as the principal signalling unit there is substantial evidence for diverse signalling mediated by G $\beta\gamma$ subunit-effector interactions (see section 1.1.2.3.). The $\beta\gamma$ subunit may operate in a synergistic or antagonistic manner to the G α subunit in modulating effector activity (Hermans, 2003). Alternatively the $\beta\gamma$

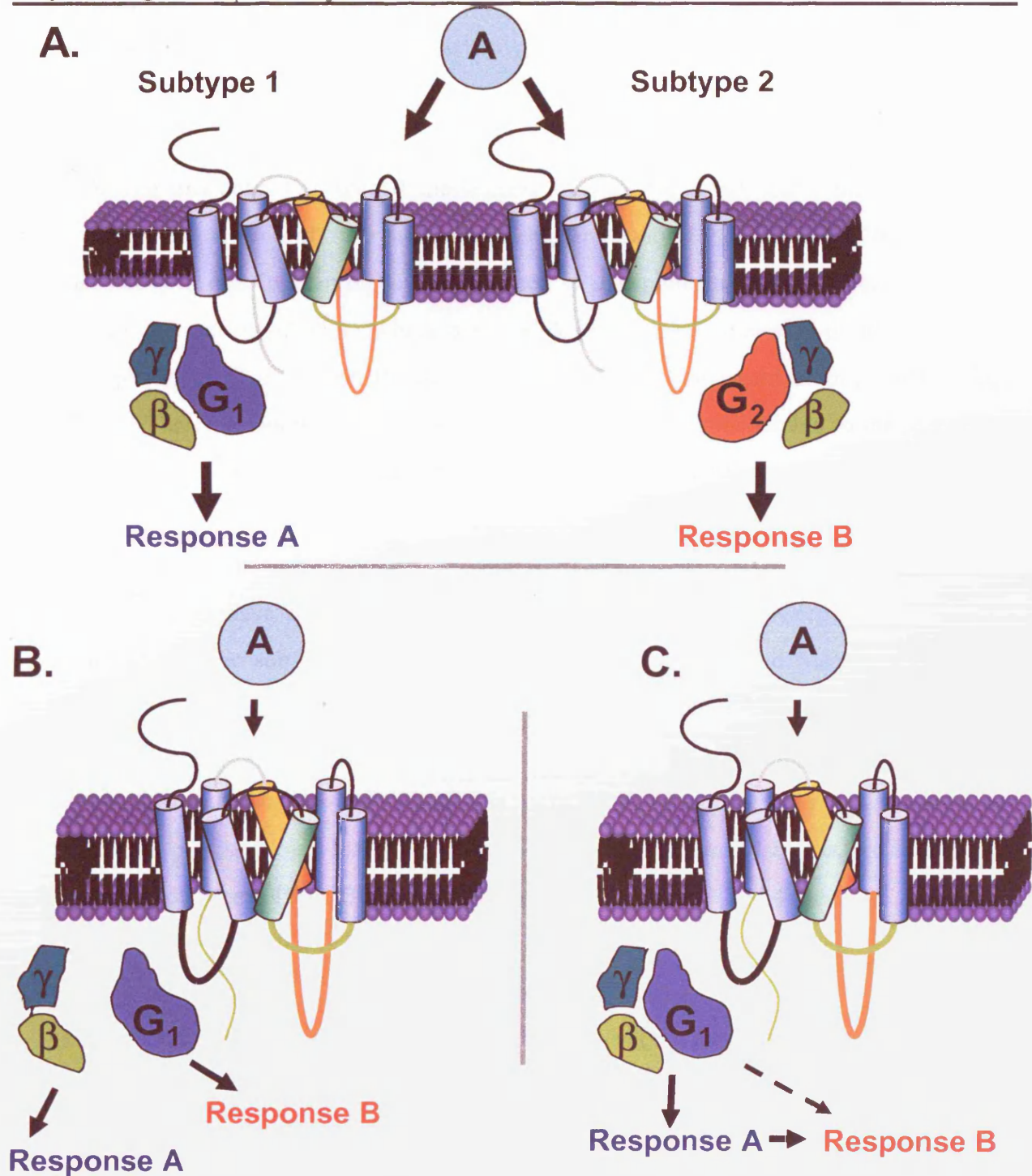


Fig. 3.1. Mechanisms of multiple effector coupling at G-protein coupled receptors (I).

G₁ and G₂ represent two distinct Gα subtypes which couple to different downstream effector-pathways, the activation of which maybe assessed from the measurement of **Response A** and **Response B**.

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subunit may modulate the activity of an unrelated downstream effector pathway to provide another level of signalling diversity, which is not necessarily initiated by multiple subtypes G α subunit activation.

(3). The production of second messengers (e.g. cyclic AMP, Ca²⁺) may themselves modulate the activity of other second messenger pathways (Dumont *et al.*, 2002; Noda *et al.*, 2004). A classic example is the activation of phospholipase A₂ which can be caused by an increase in [Ca²⁺]_i which may be downstream of direct G-protein mediated phospholipase C activation (Schievella *et al.*, 1995; Hirabayashi *et al.*, 1999). (**Fig. 3.1C**). Therefore complexity in cell signalling may also be attributable to the secondary modulation of intracellular effectors (Dumont *et al.*, 2002; Hermans, 2003)

When agonist-mediated effector activation displays overt diversity and the above considerations have been addressed, the possibility that a single receptor subtype may activate multiple subtypes of G α subunits needs to be considered. Many GPCRs have been shown to couple to multiple different G α subtypes (Eason *et al.*, 1992; Glass and Felder, 1997; Bonhaus *et al.*, 1998). One example is the dual coupling of some receptors to both G α_i and G α_s , the latter interaction normally being evident after pre-incubation of cells with pertussis toxin to eliminate receptor-mediated activation of the G α_i component (Eason and Liggett, 1995; Bonhaus *et al.*, 1998; Heubach *et al.*, 2004; Szucs *et al.*, 2004).

One increasingly observed pharmacological phenomenon is that the complement of G α subunits activated by the same receptor subtype can vary in an agonist-dependent manner (Kenakin, 1995a). Often the diversity of the individual G α subtypes activated is related to the efficacy of the agonist. Consider that a single receptor is coupled to two different G α subtypes, one G α with efficient coupling and the other G α subtype with relatively poor coupling. Agonists of high efficacy may stabilize a sufficient proportion of receptors in the active state to initiate coupling to both G α subtypes to initiate two downstream responses. However, an agonist of lower efficacy may only be able to modulate the activity of the most efficiently coupled G-protein and therefore only affect the activity of one downstream pathway (**Fig. 3.2A**). This has been termed ‘agonist-strength of signal’

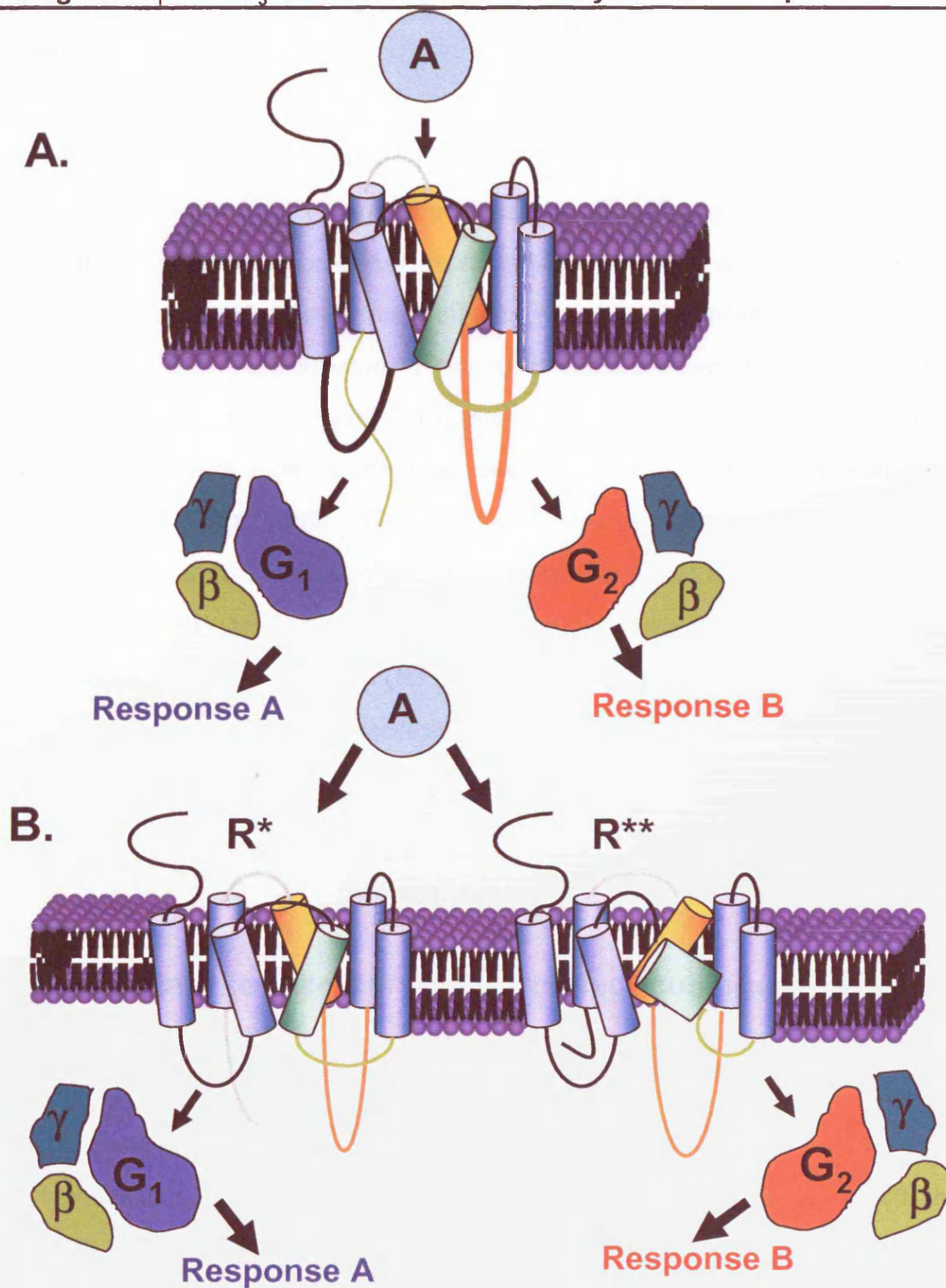


Fig. 3.2. Mechanisms of multiple effector coupling at G-protein coupled receptors (II).

G_1 and G_2 represent two distinct G- α subtypes which couple to different downstream effector-pathways, the activation of which maybe assessed from the measurement of **Response A** and **Response B**.

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(Kenakin, 1995a) and has been thoroughly reviewed previously (in Section 1.4.3. of the Introduction).

Diverse G-protein coupling profiles have also been experimentally determined where agonist-specific differences cannot be attributed to the efficacy of the agonist. Usually two (or more) agonists demonstrate reversed potency, efficacy or intrinsic activity when two different independent downstream effector pathways are measured (Spengler *et al.*, 1993; Robb *et al.*, 1994; Berg *et al.*, 1998a,b). Because the two-state model allows only for the quantity and not the quality of G-protein activation to vary between agonists, a second active conformation has been proposed (Leff *et al.*, 1997a,b; Scaramellini and Leff, 1998, 2002) (**Fig. 3.2B**). This second active receptor conformation (termed **R^{**}**) is proposed to have a different G-protein coupling profile to the other active conformation (**R^{*}**). Agonists can therefore alter the equilibrium between the two active receptor conformations and the resting state (**R**) by possessing differential, preferential affinity for one or the other active receptor species. In this way agonists may have altered potency and efficacy depending on the pathway measured. This has been termed ‘*agonist-specific receptor trafficking*’ (Kenakin, 1995b, 1997a) and this too has been reviewed previously (in section 1.4.3. of the Introduction).

3.1.2. Muscarinic receptors and promiscuous coupling

The human M₁ and M₃ muscarinic acetylcholine (mACh) receptors predominantly couple to the phospholipase C pathway via the activation of G $\alpha_{q/11}$ -proteins (Eglen *et al.*, 1994a,b). However, a considerable body of experimental evidence suggests that, at least when recombinant mACh receptors are expressed in surrogate cell-systems, both the M₁ and the M₃ receptors couple to multiple G-proteins from distinct families. Some of this experimental evidence is briefly summarized below:

Total [³⁵S]-GTP γ S has been used to assess the coupling properties the M₁–M₄ receptors stably expressed in CHO cells (Lazareno *et al.*, 1993a). Agonist-mediated [³⁵S]-GTP γ S binding above basal at M₂ and M₄ receptors was essentially abolished by pre-incubation with pertussis toxin. However, a significant proportion of [³⁵S]-GTP γ S binding remained

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subsequent to PTx-treatment in membranes expressing the M₁ and M₃ receptors, indicating coupling to both PTx-sensitive and -insensitive G-proteins.

A statistically significant proportion of the high-affinity (GTP-sensitive) binding sites, seen with [³H]-NMS displacement by carbachol at the M₃ mACh receptor expressed in CHO-cells was determined to be PTx-sensitive (Burford *et al.*, 1995b). This was taken as evidence of interactions of the M₃ receptor with Gα_{i/o} subunits, although this result was not reproduced in a lower expressing M₃ mACh receptor-expressing cell-line. A more distal examination of the same cell-lines showed that both M₁ and M₃ mACh receptors were capable of eliciting an accumulation of cyclic AMP, which exhibited different potency and kinetics to IP₃ production in the same cells, and may be indicative of a direct activation of Gα_s subunits (Burford *et al.*, 1995a). Akam *et al.*, (2001) analysed [³⁵S]-GTPγS binding followed by immunoprecipitation to demonstrate that both M₁ and M₃ mACh receptors coupled to both Gα_{q/11} and Gα_{i/o}.

Using subtype-specific immunoprecipitation of Gα subunits photolabelled with [α-³²P]-GTP-azidoanilide, Offermanns *et al.* (1994) were able to show selective carbachol-mediated activation of Gα_{q/11}, Gα_{i1}, and Gα_{i3}, but not Gα_{i2} or Gα_s. The effective activation of the Gα_i subunits only occurred at relatively high carbachol concentrations (EC₅₀ approx. 10-20 μM). Although these experiments were carried out in HEK-cells (as opposed to CHO-cells in the study above) the lack of Gα_s activation by M₁ and M₃ mACh receptors was taken as evidence of cyclic AMP accumulation occurring as a downstream consequence of PLC activation, as others have also suggested (Felder *et al.*, 1989). The coupling properties of M₁ and M₃ mACh receptors are further complicated by the observation that the potency and efficacy of agonists demonstrate a cell-type-dependency (Richards and Van Giersbergen 1995a,b).

3.1.3. [³⁵S]-GTPγS binding: evaluation of techniques

All of the experimental data reported in this Chapter examining the functional coupling of M₁ and M₃ mACh receptors was obtained using one of two different [³⁵S]-GTPγS binding

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methodologies. It is therefore appropriate briefly to discuss the [³⁵S]-GTPγS binding assay with regard to its advantages over other functional assays.

All [³⁵S]-GTPγS binding assays exploit the fundamental property of the G-protein cycle, that is the activated receptor catalyses the exchange of GDP for GTP on the G-protein α subunit (Manning, 2002). In the assay the endogenous GTP is replaced by GTPγS, within which the γ-thiophosphate bond is hydrolytically stable and therefore relatively resistant to the intrinsic GTPase activity of the α subunit (Manning, 2002; Harrison and Traynor, 2003). Since the activated G-protein is prevented from reforming as a heterotrimer the [³⁵S]-GTPγS labelled Gα subunits can accumulate and by counting the amount of [³⁵S]-label incorporated, quantitative information about the extent of GDP/GTP exchange stimulated following GPCR activation can be assessed (Harrison and Traynor, 2003).

Traditional [³⁵S]-GTPγS binding assays generally employ a membrane preparation expressing the receptor of interest. Agonist-stimulated activation of G-protein can then be monitored with addition of [³⁵S]-GTPγS in the binding medium. [³⁵S]-GTPγS bound to Gα subunits can then separated from free [³⁵S]-GTPγS by vacuum filtration to yield information on the potency and intrinsic activity of the agonist of interest (Dowling *et al.*, 2004). The reliance on a manual filtration step is rather restrictive on the ‘throughput’ that can be achieved using this methodology. Increasingly investigators are employing small polyvinyltoluene beads, which contain scintillant, to provide a homogeneous assay. The scintillation proximity bead assay (SPA) also allows the assay to be adapted to a 96-well plate format, which greatly expands the number of samples that can be handled. (for exact methodology please see section 2.5.2.).

The total [³⁵S]-GTPγS binding assay measures a functional consequence of agonist-receptor occupancy at one of the earliest-mediated events (Harrison and Traynor, 2003). However, a single activated receptor can catalyse the GDP/GTP exchange on more than one Gα subunit (Selley *et al.*, 1997; Lorenzen *et al.*, 2002) so even the total [³⁵S]-GTPγS binding assay is subject to some degree of amplification. Despite this the degree of receptor reserve is likely to be significantly less than other more downstream read-outs

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(Traynor and Nahorski, 1995; Alt *et al.*, 1998; Umland *et al.*, 2001). In this regard the total [³⁵S]-GTPγS binding assay is more useful for assessing the efficacy differences of agonists than more distal downstream effectors, which are confounded by non-linearity in signal, cross-amplification and desensitization (Albert and Robillard, 2002; Hur and Kim, 2002; Milligan, 2003b).

The total [³⁵S]-GTPγS binding assay measures the global activation of a potentially heterogeneous G-protein population, although with the use of certain toxins (e.g. PTx) inferences about the main subtypes of G-protein activated in response to agonist can be made. Additionally the assay has been primarily used to estimate agonist efficacy in systems in which the receptor is coupled to Gα_{i/o} subtypes (Traynor and Nahorski, 1995; Szekeres and Traynor, 1997). Gα_{i/o} proteins are generally more highly expressed than other subunits and exhibit higher rates of nucleotide exchange (Harrison and Traynor, 2003). It can therefore be difficult to experimentally dissect agonist-mediated [³⁵S]-GTPγS binding for receptors predominantly coupled to Gα_{q/11} from the background noise (receptor independent) associated with GTP/GDP exchange on Gα_{i/o} subunits (Milligan, 2003b).

Recent reports have highlighted three different experimental modifications of traditional total [³⁵S]-GTPγS binding, which can be exploited to dissect the identity of individual Gα subtypes from the agonist-mediated activation of the global Gα population (Harrison and Traynor *et al.*, 2003; Milligan, 2003b, DeLapp, 2004):

(1). Co-expression of G-proteins and receptors in Sf9 insect cell-lines (Windh and Manning, 2002a,b; Massotte 2003). The G-proteins expressed endogenously within these cell-lines do not significantly interact with the transfected recombinant receptors and therefore the isolated interactions between receptors and transfected Gα/βγ subunits can be studied. This approach has successfully revealed agonist-specific activation of individual Gα subtypes of the Dopamine (D_{2L}) receptor (Cordeaux *et al.*, 2001; Gazi *et al* 2003). However as both agonist potency and efficacy can be influenced by receptor-to-G-

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protein stoichiometry (Newman-Tancredi *et al.*, 1997) it is important to rule out agonist specific differences solely based on variable G-protein/receptor expression levels.

(2). The construction of a GPCR and a G-protein α subunit fusion protein with subsequent expression in a host cell, ensures a defined 1:1 stoichiometry between receptor and an individual G α (Milligan, 2000) and allows the direct assessment of agonist activation of specific G α subtypes to be made. Agonist-specific conformations for the μ -opioid (Massotte *et al.*, 2002) and the β_2 -adrenoreceptor (Wenzel-Seifert and Seifert, 2000; Seifert *et al.*, 2001) have been inferred from their respective receptor-G α fusion proteins. However, studies have shown that receptors catalyse the activation of more than one G α protein (Traynor and Nahorski, 1995; Selley *et al.*, 1997) and additionally that partial agonists are unable to activate as many G α as full agonists (Selley *et al.*, 1998). Therefore, although the pharmacology of ligands appears to be faithfully reproduced, the physiological relevance of such receptor-G α fusion proteins may be questioned.

(3). The inclusion of an additional antibody-capture step, to further dissect G α subclass activation, is becoming increasingly common (Newman-Tancredi *et al.*, 1999, 2002; DeLapp *et al.*, 1999; Selkirk *et al.*, 2001). The immunoprecipitation of the G α -[³⁵S]-GTP γ S complexes subsequent to agonist-stimulation and then quantifying the degree of radioactivity incorporated (for method see 2.5.3.) has allowed the detection of novel pharmacological properties, such as protean agonism (Newman-Tancredi *et al.* 2003). This method has been successfully employed to look at specific (inverse)-agonist-mediated G α subtype modulation at dopamine (Newman-Tancredi *et al.*, 1999), 5-HT_{2C} (Cussac *et al.*, 2002) 5-HT_{1A} (Newman-Tancredi *et al.*, 2002) and 5-HT_{1B} (Newman-Tancredi *et al.*, 2003) receptors. As has been previously reported, assessment of [³⁵S]-GTP γ S binding solely to G_{q/11} α , results in an improved signal-to-noise ratio, such that concentration response curves to even partial agonists can be obtained (DeLapp *et al.*, 1999).

3.1.4. Experimental objectives

As discussed above M₁ and M₃ mACh receptors have been convincingly demonstrated to couple to both PTx-sensitive and -insensitive G-proteins. However, the body of literature

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remains confused as to the precise nature of the individual G-proteins activated in each particular cell-line. The aim of this Chapter was initially to characterize the increase in total [³⁵S]-GTPγS binding in response to a range of structurally diverse agonists, at the M₁ and M₃ mACh receptor stably expressed in CHO-cells, and to evaluate the extent of PTx sensitivity of the response. An immunoprecipitation method was then used to try to determine the potency and efficacy of Gα_{q/11} or Gα_{i1-3} subunit activation in response to the same set of agonists.

3.2. Methods

For details of the methodologies used in this Chapter please refer to **Chapter 2 – Materials and Methods**:

2.1.3. Pertussis toxin treatment

2.3. Cell membrane preparation

2.4.1.1. [³H]-NMS Saturation binding to membrane preparation

2.4.2.1. [³H]-NMS Displacement binding

2.5.1. Filtration assay of [³⁵S]-GTPγS binding

2.5.2. Scintillation proximity assay (SPA) to measure [³⁵S]-GTPγS binding mediated by CHO-cells expressing the human mACh receptor.

2.5.3. Immunoprecipitation of [³⁵S]-GTPγS-bound G proteins with antisera to specific Gα proteins

2.6. G-protein western blotting

3.3. Data analysis

All data are shown as means ± S.E.M. for at least three separate experiments performed in duplicate except where indicated.

[³H]-NMS binding for each individual experiment was analysed by non-linear regression using a commercially available programme (GraphPad Prism version 3.0/4.0; GraphPad Software, San Diego, CA, USA) to give B_{max} and K_D estimates. Displacement binding data were fitted to sigmoidal (variable slope) curves using the same analysis package, IC₅₀ values were converted to pK_i values using the method of Cheng and Prusoff (1973).

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All concentration response curves, from either total or G α -specific [³⁵S]-GTP γ S binding data were fitted using the sigmoidal (variable slope) curve-fitting function of the same programme. All data were compared for a one- and two-site fit and only the most statistically significant value is quoted (*F*-test, GraphPad Prism).

3.4. Results

3.4.1. Preliminary characterization of the CHO-m1 and CHO-m3 cell lines

3.4.1.1. Saturation binding

Representative [³H]-NMS saturation binding curves for the M₁ and M₃ mACh receptor-expressing CHO cell lines are shown in **Figs. 3.3A** and **3B**, respectively. Over at least ten separate experiments, performed in duplicate the mean K_D value for [³H]-NMS binding and receptor expression levels were:

M₁:	K _D	0.14 nM (log K _D -9.85 ± 0.10) (<i>n</i> = 12)
	B _{max}	1.86 ± 0.13 pmol mg ⁻¹ protein
M₃:	K _D	0.16 nM (log K _D -9.79 ± 0.09) (<i>n</i> = 10)
	B _{max}	1.64 ± 0.22 pmol mg ⁻¹ protein

3.4.1.2. Displacement binding

Appropriate antagonists, based on their published affinities, were chosen at each receptor type for [³H]-NMS displacement experiments (**Fig. 3.4**). For the M₁ mACh receptor pK_i values were: atropine, 9.16 ± 0.05; pirenzepine, 8.03 ± 0.14. For the M₃ mACh receptor pK_i values were: atropine, 9.12 ± 0.16; darifenacin, 9.33 ± 0.07. All values are in broad agreement with published pK_i for the antagonists at the receptor subtypes (Caulfield and Birdsall, 1998). All slope factors were not significantly different from unity indicating homogeneous receptor populations of each subtype.

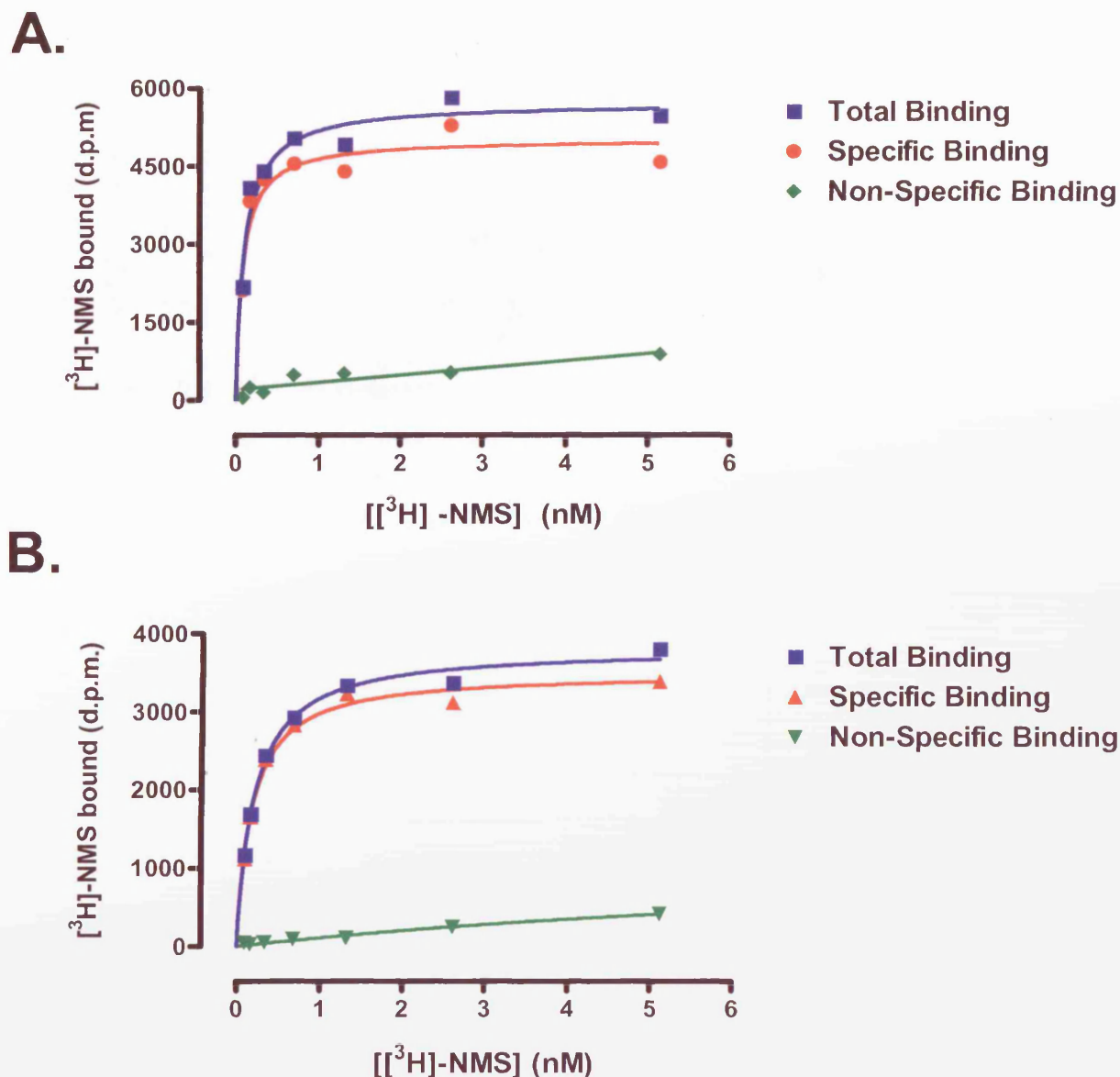


Fig. 3.3 [³H]-NMS binding to human muscarinic receptors expressed in CHO-cell membrane preparations

CHO-cell membranes, expressing A. M₁ and B. M₃, were incubated in the presence or absence of atropine for 60 min to obtain saturation binding isotherms. All experiments were performed in duplicate and the graphs above are representative of between 10-12 separate experiments. Receptor densities and dissociation constants for NMS are summarized in the main text.

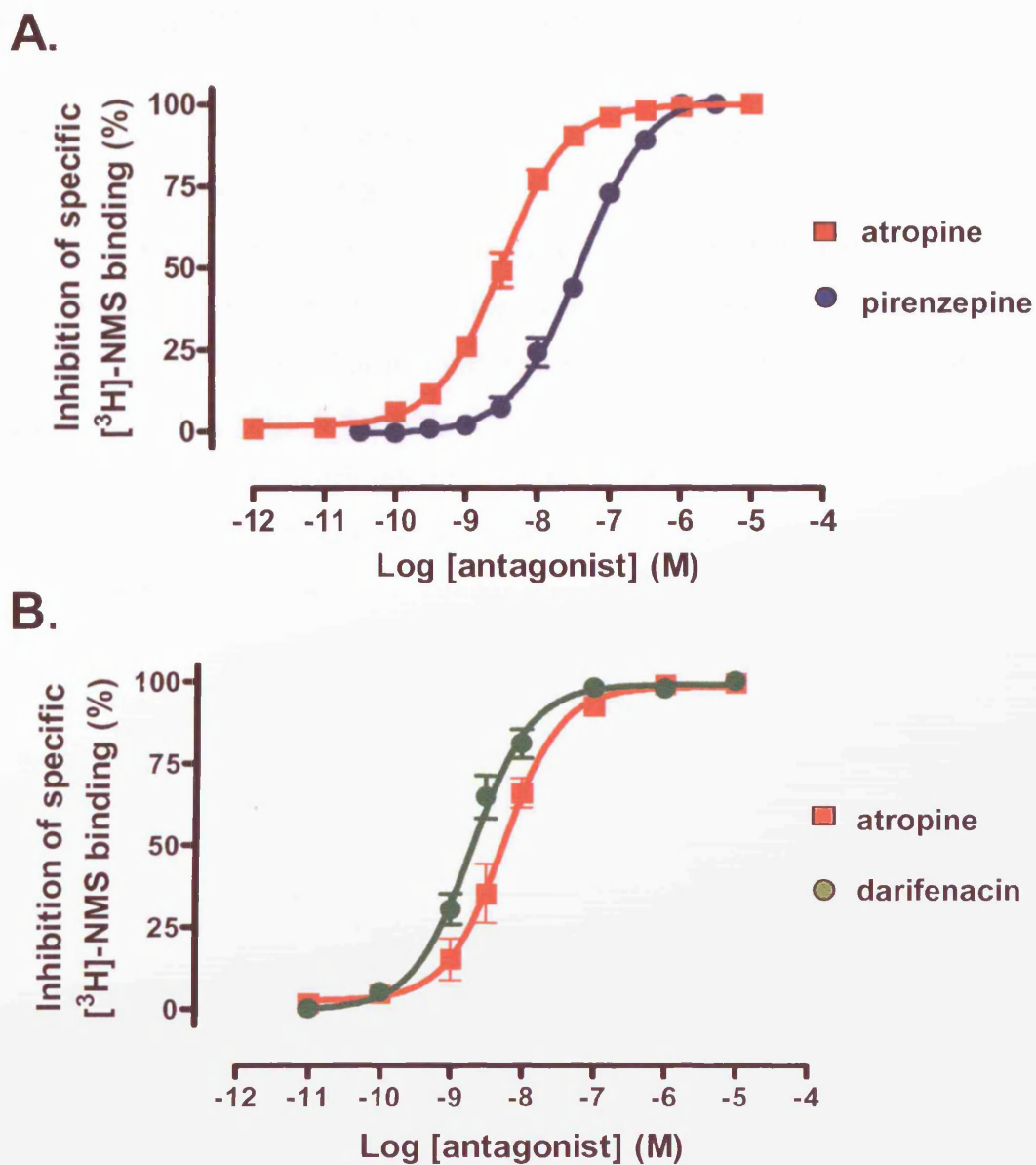


Fig. 3.4. Antagonist displacement of [³H]-NMS binding in M₁ and M₃ receptor-expressing CHO-cell membrane preparations

Inhibition binding experiments on M₁ (A) or M₃ (B) mACh stably expressing CHO-cells membrane preparations, in the presence of 300-500 pM [³H]-NMS. Data shown represent the means $\pm$ S.E.M. from at least three experiments performed in duplicate. Summary data for the antagonists are given in the text.

3.4.2. Total [³⁵S]-GTPγS binding in membranes prepared from CHO-m1 and CHO-m3 cells: Assay validation and optimization

3.4.2.1. Guanosine 5'-diphosphate (GDP) concentration

As discussed in section 3.1.3. individual families of Gα subunits have different GDP/GTP exchange rates and therefore the concentration of GDP employed in total [³⁵S]-GTPγS binding assays affects both the potency and efficacy of agonists. Initial experiments therefore looked at two different GDP concentrations on agonist-mediated [³⁵S]-GTPγS binding. **Fig. 3.5** clearly demonstrates that the inclusion of GDP at 1 μM provides an improved signal-to-noise compared to 10 μM at M₁ mACh receptor expressing CHO cells. Similar data was obtained for M₃ mACh receptor (data not shown) and therefore this concentration was employed in all further [³⁵S]-GTPγS binding assays.

3.4.2.2. Establishing the validity of the scintillation proximity assay (SPA)

The number of ligands and mACh receptor subtypes to be assessed for G-protein coupling meant that use of the traditional filtration method for [³⁵S]-GTPγS binding would be time-consuming. A scintillation proximity assay (SPA) allows for a greater throughput, however, it was important to establish that the 'miniaturization' of the assay did not affect the pharmacological profiles derived for the agonists which were to be tested, especially as data from both SPA and traditional filtration assays might need to be integrated and compared.

It has been previously shown that the SPA-binding technique yields similar results with respect to generating rank-orders and affinities of ligands for recombinant α adrenoceptors subtypes (Gobel *et al.*, 1999). Initial experiments concentrated on establishing whether the potency and efficacy of a full agonist, methacholine, and the affinity of the relatively M₁ mACh receptor-selective antagonist, pirenzepine, were comparable across both assay formats. Full concentration-response curves for methacholine, in CHO-m1 cell membranes prepared from control and PTx pre-treated cells are shown in **Fig. 3.6**. The absolute stimulations of [³⁵S]-GTPγS binding above basal were found to be greater in filtration assays. This is probably due, in part, to the

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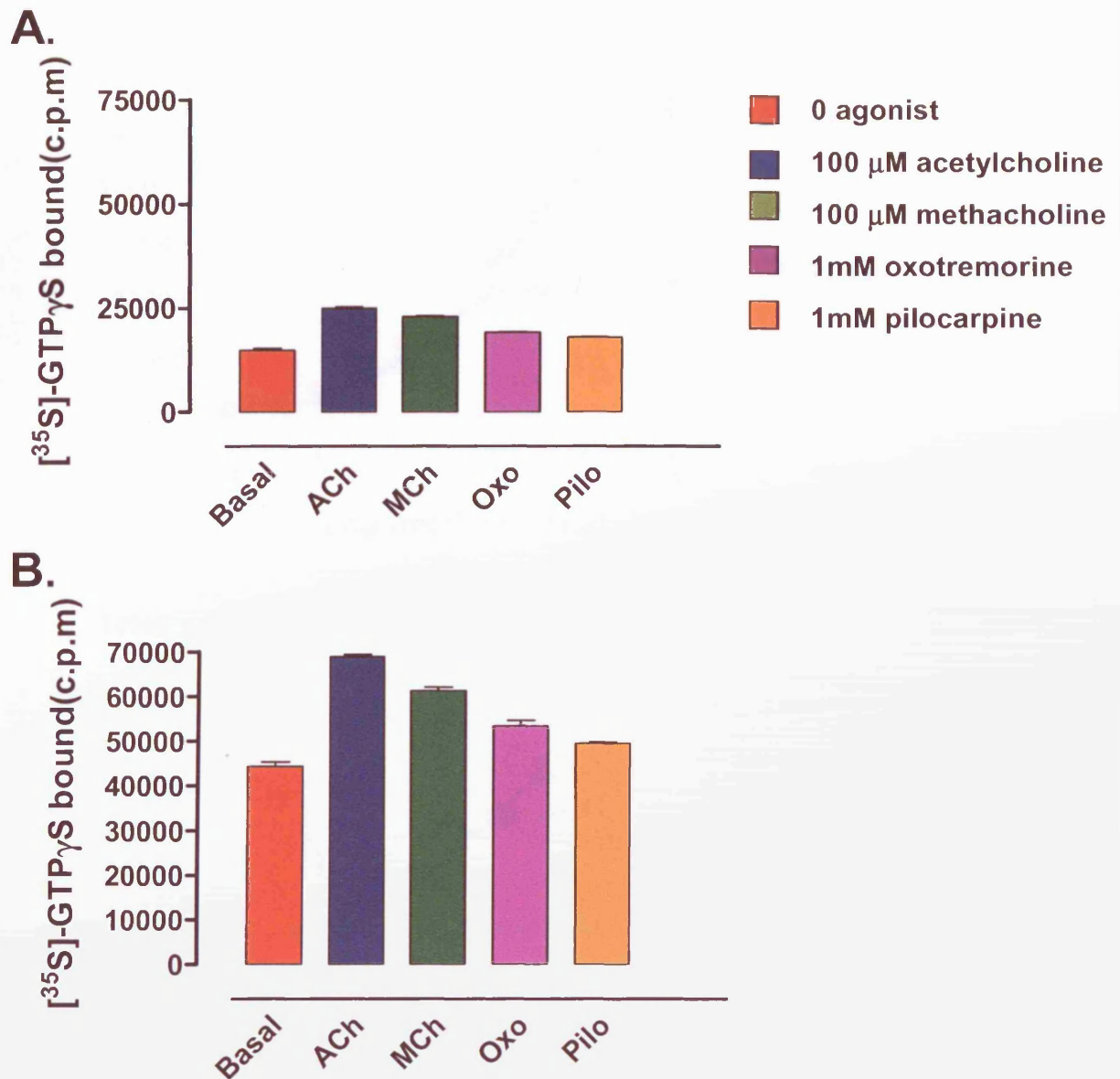


Fig. 3.5 Effects of GDP concentration on agonist-mediated total [35S]-GTP γ S binding in CHO-m1 cell membranes

Membranes prepared from Chinese hamster ovary cells, recombinantly expressing the human M₁ muscarinic acetylcholine receptor were incubated in the presence of either 10 μ M (A) 1 μ M (B) GDP, 300 pM [35S]-GTP γ S and the indicated concentrations of agonists for 30 minutes at 30°C. Data shown are representative of a least four experiments performed in duplicate.

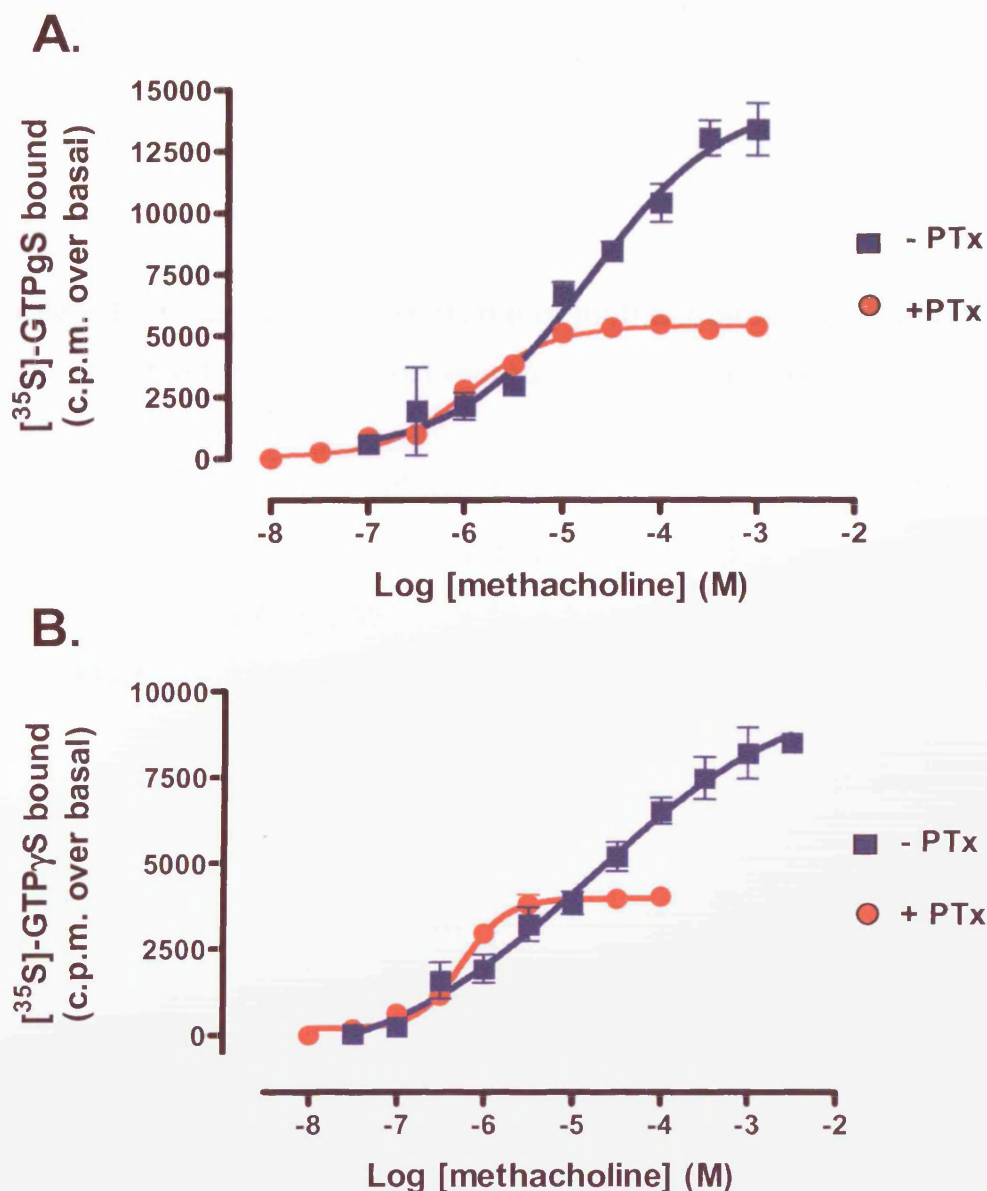


Fig. 3.6. Effects of pertussis toxin upon methacholine-stimulated total [³⁵S]-GTPγS binding in CHO-m1 cell membranes

Effects of increasing concentrations of methacholine on receptor-mediated total [³⁵S]-GTPγS binding in CHO-m1 cell membranes from control cells (■), or cells pre-treated (●) with pertussis toxin (100 ng ml⁻¹ 20-24 h). Data in panel in **A** are obtained using a filtration-based method, whereas those in **B** from using a scintillation proximity assay. Data are shown as means $\pm$ S.E.M. for 3-5 experiments carried out in duplicate.

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reduced efficiency of counting in the SPA assay and the increased non-specific binding to the bead-complex. However, the potencies of methacholine, for both $\pm$ PTx pre-treated membrane preparations, were not significantly different between assay formats. **Table 3.1.** summarizes the pharmacological data obtained from both assay techniques.

Table 3.1. Pharmacological profile of methacholine-stimulated [³⁵S]-GTP γ S binding in CHO-m1 membrane preparations

	filtration		SPA	
	- PTx	+ PTx	- PTx	+ PTx
pEC ₅₀	4.73 $\pm$ 0.05	6.00 $\pm$ 0.04	4.87 $\pm$ 0.04	6.04 $\pm$ 0.11
slope factor	0.69 $\pm$ 0.04	1.07 $\pm$ 0.05	0.51 $\pm$ 0.03	1.20 $\pm$ 0.09

Despite the different methods of quantifying agonist-stimulated [³⁵S]-GTP γ S binding, there was no significant difference in potency or slope factor for methacholine obtained from the assay methodologies ($p > 0.05$, Student's *t*-test). Additionally, the residual binding after PTx treatment, as a percentage of the maximal binding seen without PTx treatment, was similar for both assays (filtration, 40 $\pm$ 2%; SPA, 38 $\pm$ 2 %). We were therefore confident that despite the smaller signal-to-noise ratio obtained with the SPA beads, the potency and efficacy of ligands could be accurately determined and the values obtained would be directly comparable to those obtained in filtration binding assays.

As a further test of comparability between filtration and SPA methods, methacholine concentration-response curves were constructed in the absence or presence of three concentrations of pirenzepine (10-100 nM) and these data used for subsequent Schild analysis. **Fig. 3.7.** illustrates one such plot for the M₁ mACh receptor, showing parallel rightward shifts in the methacholine concentration-response curves in the presence of increasing concentrations of the antagonist. The corresponding Schild-plot has a slope of near unity, consistent with competitive antagonism (Arunlakshana and Schild, 1959), and yielding a pA₂ value of 8.29. This is in good agreement with values obtained from

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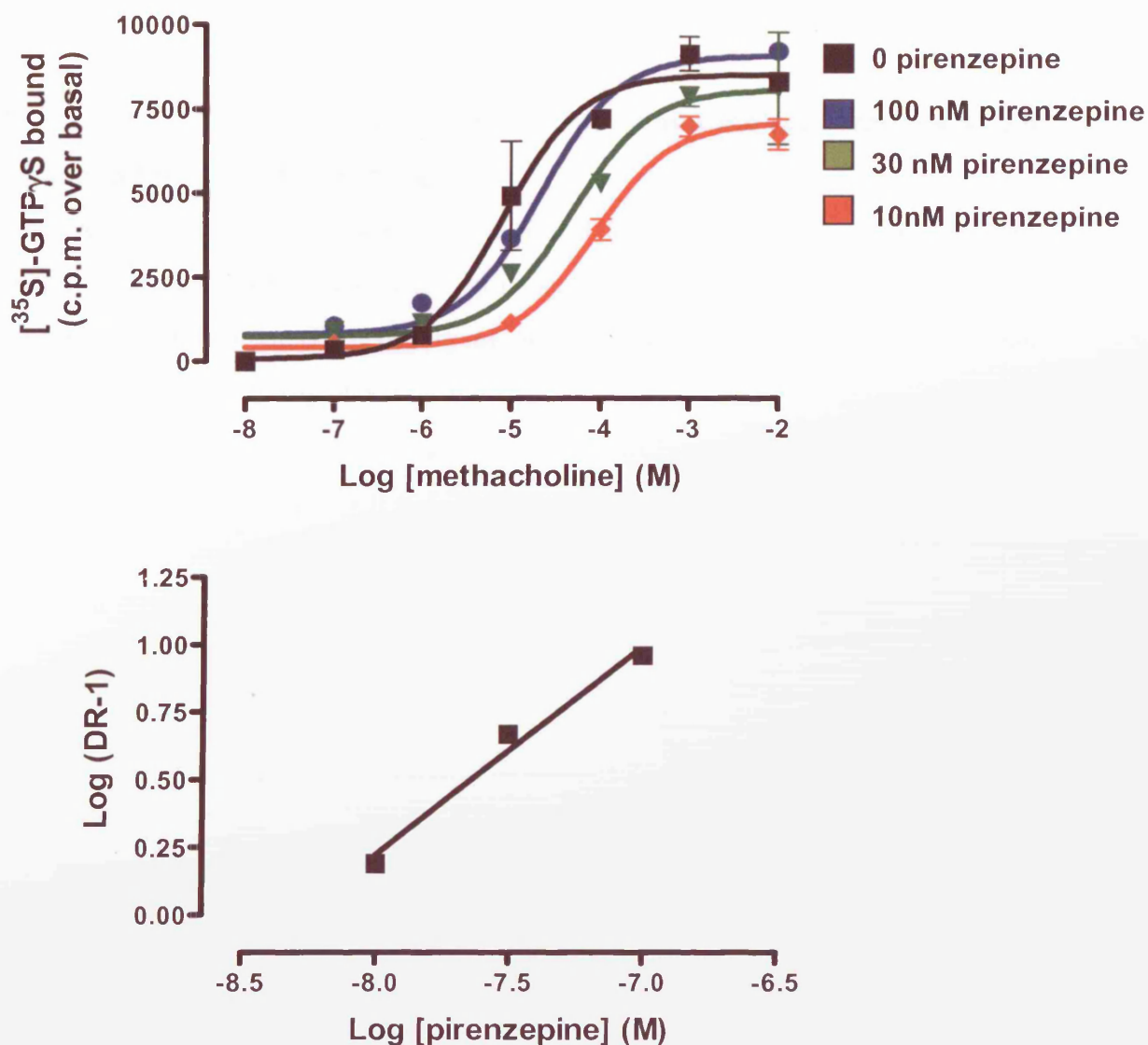


Fig. 3.7. Inhibition of methacholine-stimulated $[^3\text{S}]\text{-GTP}\gamma\text{S}$ binding by pirenzepine in CHO-m1 cell membranes using SPA beads

Membranes were pre-incubated with methacholine $\pm$ pirenzepine for 30 min and the assay initiated by the addition of $[^3\text{S}]\text{-GTP}\gamma\text{S}$. Dose-ratios obtained for the curves in the presence of pirenzepine have been used to construct Schild plots (inset). Derived pK_b values for pirenzepine are given in the text. Data shown are representative of a two experiments performed in duplicate.

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filtration binding assays (**Fig. 3.4.**) and is in the range for those reported in the literature (Caulfield and Birdsall, 1998). Therefore, in addition to agonists, the pharmacological properties of antagonists are maintained despite the changes in assay format.

3.4.3. Total [³⁵S]-GTPγS binding in CHO-cell membrane preparations stably expressing the M₁ and M₃ receptor

Total [³⁵S]-GTPγS binding was initially assessed at the M₁ and M₃ mACh receptor subtypes comparing control membranes to those prepared from cells that had been pre-treated with pertussis toxin (PTx, 100 ng ml⁻¹ for 20-24 h) prior to membrane preparation. PTx specifically ADP-ribosylates a cysteine residue near to the C-terminus of the Gα_{i/o} sub-family of G-proteins and thereby prevents their receptor-elicited activation (Mumby, 1999). Therefore, by using PTx it is possible to dissect the proportions and potency of non-PTx-sensitive (*e.g.* Gα_{q/11} type) G-proteins from the PTx-sensitive Gα_{i/o} type activated in response to agonist-mediated receptor stimulation.

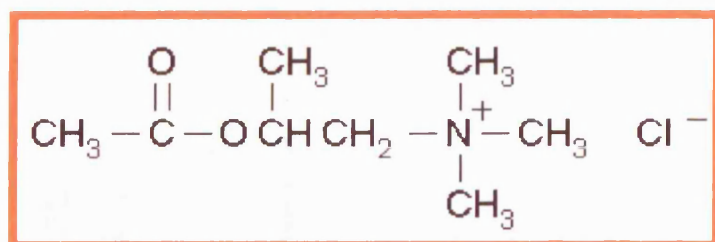
Five commercially available agonists were chosen for assessment due to their structural diversity (**Fig. 3.8.**) and the reported differences in potency, efficacy and affinity of these agents in eliciting downstream responses in recombinant mACh receptor-expressing systems (Lazareno *et al.*, 1993; Richards and Van Giersbergen, 1995a,b).

All assays were carried out under the optimal conditions established in section **3.4.2.** Concentration-response curves were only analyzed using a two-site fit when this proved to be statistically superior (*F* value <0.05).

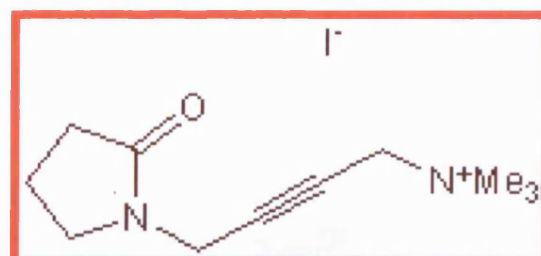
3.4.3.1. Agonist-mediated total [³⁵S]-GTPγS binding at CHO-cell membrane preparations expressing M₁ mACh receptors

Concentration-response curves for the five agonists, in the presence and absence of pertussis toxin, in CHO-cell membrane preparations expressing the M₁ receptor are shown in **Figs. 3.9-3.12.** No agonist-mediated binding was seen in untransfected CHO-K1 cells (data not shown). In addition, at the recombinant M₁ mACh receptor atropine (10 μM) completely prevented agonist-mediated [³⁵S]-GTPγS binding (data not shown).

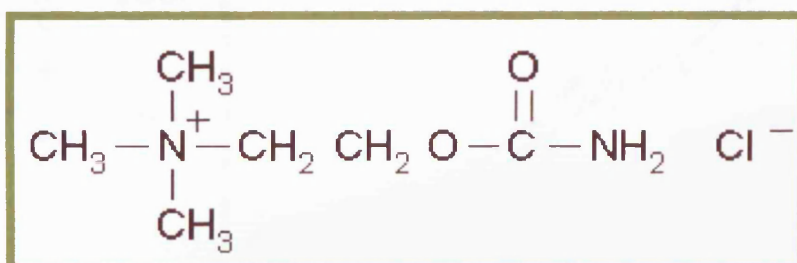
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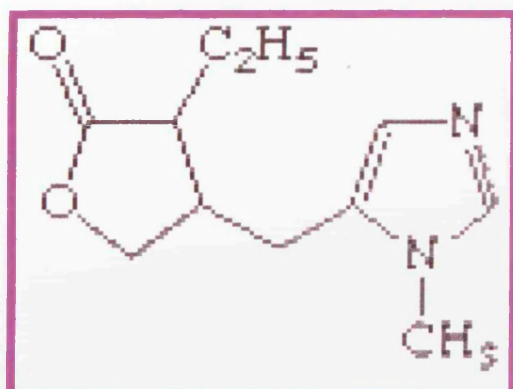
methacholine



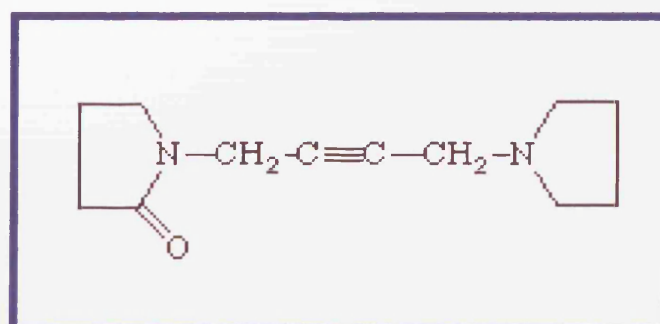
Oxotremorine-M



carbachol



pilocarpine



oxotremorine

Fig. 3.8. Chemical structures of the agonists employed in [³⁵S]-GTPγS binding experiments.

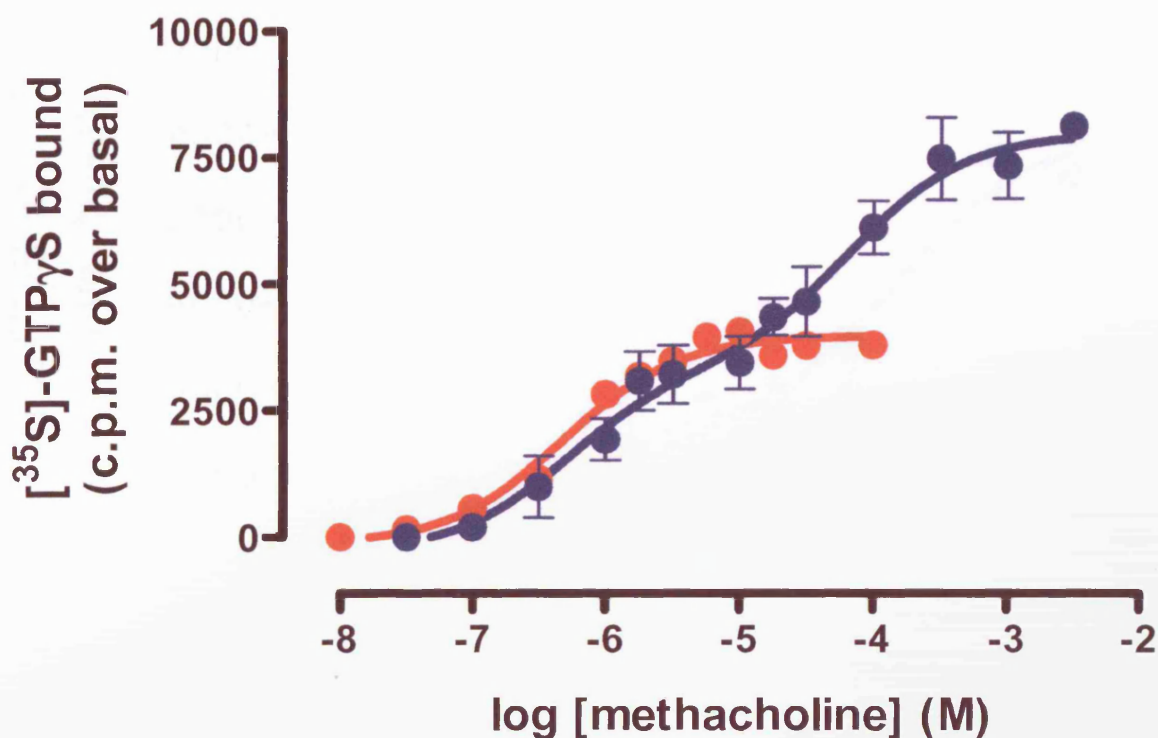


Fig. 3.9. Effect of pertussis toxin (100 ng ml⁻¹) on methacholine-stimulated total [³⁵S]-GTPγS binding in CHO-m1 cell membranes.

Effects of increasing concentrations of methacholine on M₁ mACh receptor-mediated [³⁵S]-GTPγS binding in membranes prepared from control (●), or pertussis toxin (100 ng ml⁻¹ 20-24 h) pre-treated (●) CHO-m1 cells. Basal values (determined in the absence of agonist) and non-specific binding (determined in the presence of 10 μM GTPγS) have been subtracted. Data are shown as means ± S.E.M. for 4 experiments carried out in duplicate. Data were fitted to a two-site model only if statistically significant ($p < 0.05$). $E_{\max}$, pEC_{50L} , pEC_{50H} and slope factors are summarized in **Table 3.2**

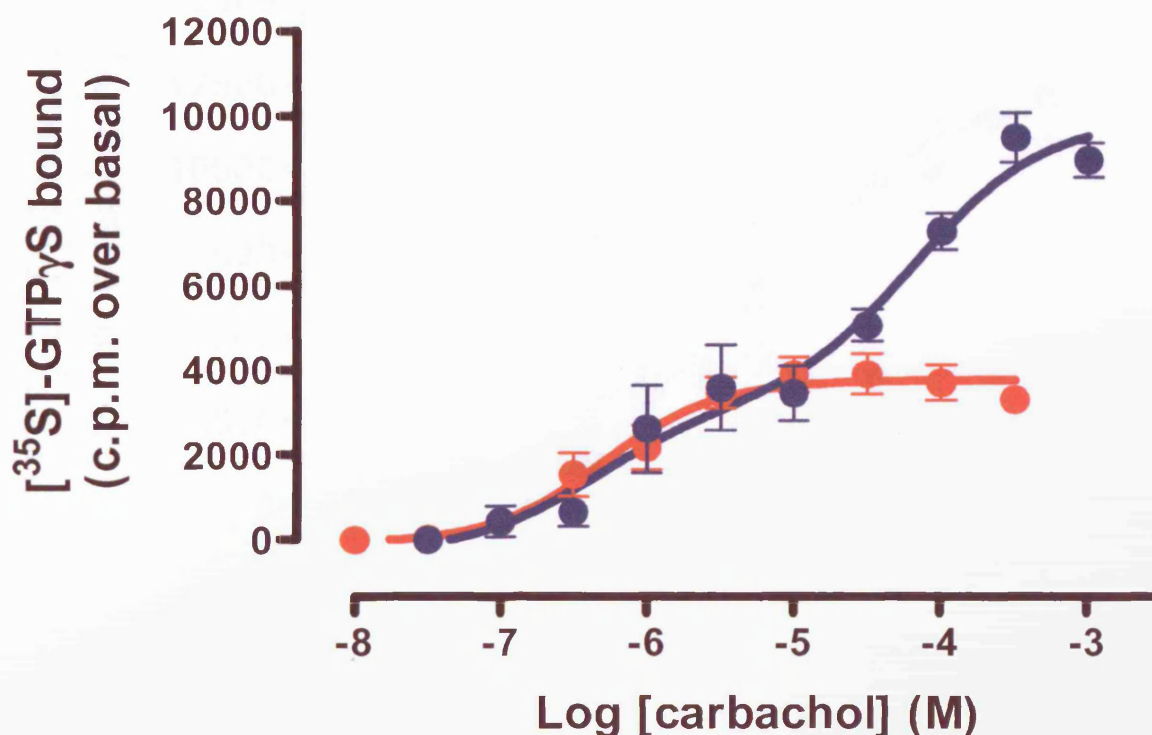


Fig. 3.10. Effect of pertussis toxin (100 ng ml⁻¹) on carbachol-stimulated total [³⁵S]-GTPγS binding in CHO-m1 cell membranes.

Effects of increasing concentrations of carbachol on M₁ mACh receptor-mediated [³⁵S]-GTPγS binding in membranes prepared from control (●), or pertussis toxin (100 ng ml⁻¹ 20–24 h) pre-treated (⊕) CHO-m1 cells. Basal values (determined in the absence of agonist) and non-specific binding (determined in the presence of 10 μM GTPγS) have been subtracted. Data are shown as means ± S.E.M. for 5 experiments carried out in duplicate. Data were fitted to a two-site model only if statistically significant ($p < 0.05$). $E_{\max}$, pEC_{50L} , pEC_{50H} and slope factors are summarized in **Table 3.2**.

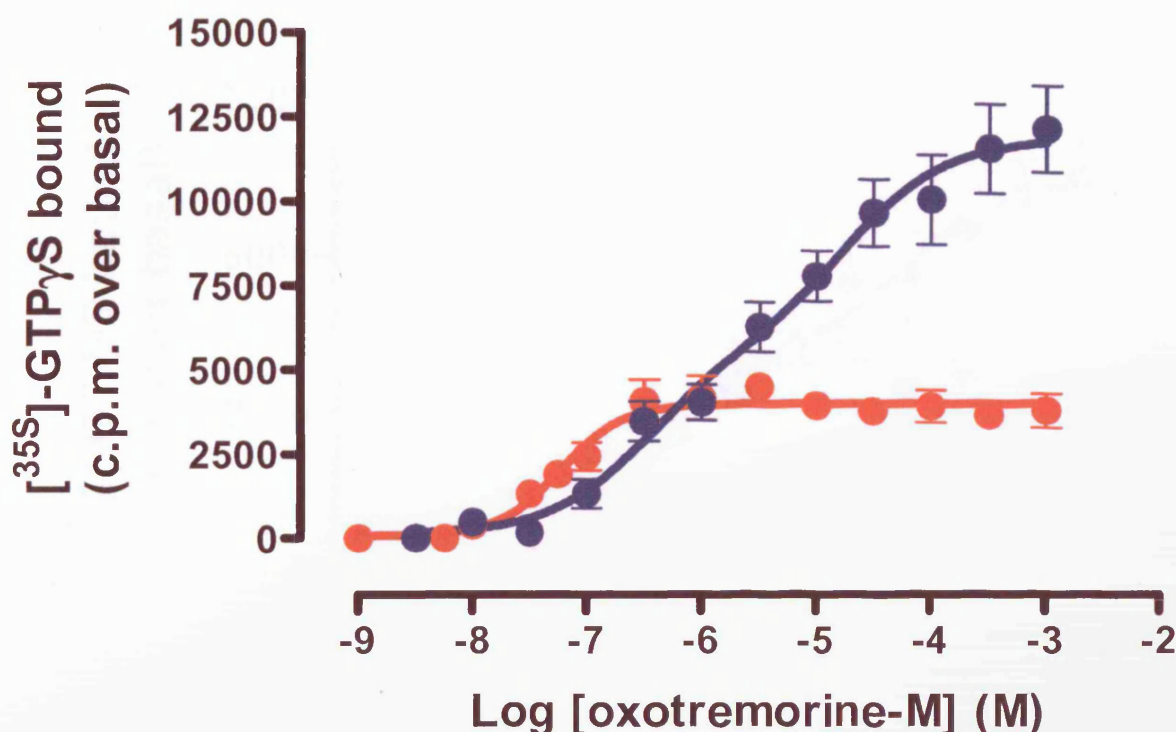


Fig. 3.11. Effect of pertussis toxin (100 ng ml⁻¹) on oxotremorine-M-stimulated total [³⁵S]-GTPγS binding in CHO-m1 cell membranes.

Effects of increasing concentrations of oxotremorine-M on M₁ mACh receptor-mediated [³⁵S]-GTPγS binding in membranes prepared from control (●), or pertussis toxin (100 ng ml⁻¹ 20-24 h) pre-treated (●) CHO-m1 cells. Basal values (determined in the absence of agonist) and non-specific binding (determined in the presence of 10 μM GTPγS) have been subtracted. Data are shown as means ± S.E.M. for 3-5 experiments carried out in duplicate. Data were fitted to a two-site model only if statistically significant ($p < 0.05$). $E_{\max}$, pEC_{50L} , pEC_{50H} and slope factors are summarized in **Table 3.2**.

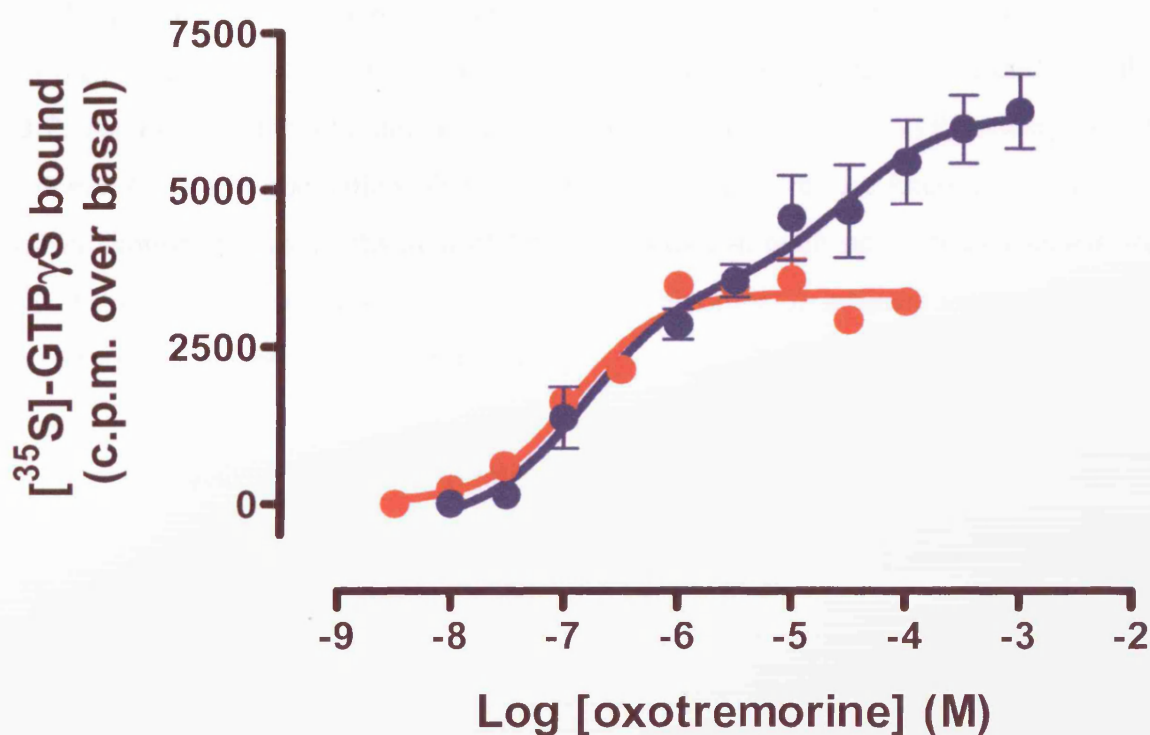


Fig. 3.12. Effect of pertussis toxin (100 ng ml⁻¹) on oxotremorine-stimulated total [³⁵S]-GTPγS binding in CHO-m1 cell membranes.

Effects of increasing concentrations of oxotremorine on M₁ mACh receptor-mediated [³⁵S]-GTPγS binding in membranes prepared from control (●), or pertussis toxin (100 ng ml⁻¹ 20-24 h) pre-treated (●) CHO-m1 cells. Basal values (determined in the absence of agonist) and non-specific binding (determined in the presence of 10 μM GTPγS) have been subtracted. Data are shown as means ± S.E.M. for 4 experiments carried out in duplicate. Data were fitted to a two-site model only if statistically significant ($p < 0.05$). $E_{\max}$, pEC_{50L} , pEC_{50H} and slope factors are summarized in **Table 3.2**.

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Concentration-response curves obtained for agonist-mediated stimulation of [³⁵S]-GTPγS binding in the absence of PTx-treatment had slope factors less than unity. Under these conditions a two-site fit was statistically superior to a one-site fit ($p < 0.05$, F -test) and therefore high (K_H) and low (K_L) potency values could be obtained. In the case of methacholine these high and low values differed by almost a hundred-fold, yielding a K_H of 6.23 ± 0.28 and a K_L of 4.33 ± 0.05 . Data for all agonists are summarized in **Table 3.2**. Total [³⁵S]-GTPγS binding measures agonist-stimulated GDP/GTP exchange for the 'global' G-protein population. Therefore, the bi-phasic curves are likely to be indicative of an agonist-mediated activation of heterogeneous G-protein populations characterized by strikingly different potencies. The overall rank-order of intrinsic activity (maximal activity) for the agonists was as follows:

methacholine > carbachol > oxotremorine > oxotremorine-M

The partial agonist pilocarpine elicited only modest increases in total [³⁵S]-GTPγS binding above basal and therefore a reliable estimation of its potency was unattainable. Agonists were also able to elicit robust increases in total [³⁵S]-GTPγS binding above basal in membranes prepared from PTx-treated CHO-cells. This confirms previous observations that recombinant M₁ mACh receptors can couple to both Gα_{i/o}-like and Gα_{q/11}-like classes of G-proteins (Lazareno and Birdsall, 1993; Burford *et al.*, 1995a). Slope factors, after PTx treatment, were not significantly different from unity ($p > 0.05$). This may indicate that after the inactivation of receptor-mediated activation of Gα_{i/o}-proteins, agonists now activated the remaining G-proteins with a uniform potency, or this could reflect the activation of a homogenous Gα-protein population. All of the pharmacological data for agonists are summarized in **Table 3.2**.

In addition to the increase in the slope factor the concentration-curves for agonists, after PTx-treatment membrane preparations, was significantly shifted to the right with a corresponding increase in potency. The pEC₅₀ for all agonist-stimulated total [³⁵S]-GTPγS binding, at PTx-treated membranes was not statistically different to the pEC₅₀ of the K_H obtained from non PTx-treated membranes (e.g. methacholine, 6.23 ± 0.28 and 6.26 ± 0.06 for pEC₅₀ K_H and PTx-treatment, respectively, $p < 0.05$, Student's t -test). It is

TABLE 3.2 Stimulation of [³⁵S]-GTPγS binding by agonists at CHO-m1 cell membrane preparations

Ligand	No Pertussis Toxin				With Pertussis Toxin	
	pEC ₅₀ K _H	pEC ₅₀ K _L [*]	Slope ⁺ factor	F-Test ⁺⁺ 2 site fit	pEC ₅₀ ^{**}	Slope Factor
Methacholine	6.23 ± 0.28	4.33 ± 0.05	0.52 ± 0.02	0.0002	6.26 ± 0.06	1.20 ± 0.15
Carbachol	6.27 ± 0.15	4.10 ± 0.14	0.50 ± 0.07	0.0019	6.31 ± 0.10	1.34 ± 0.10
Oxotremorine M	6.66 ± 0.30	4.85 ± 0.21	0.40 ± 0.12	0.026	7.29 ± 0.11	1.37 ± 0.17
Oxotremorine	6.80 ± 0.28	3.83 ± 0.14	0.62 ± 0.20	0.0373	7.00 ± 0.06	1.15 ± 0.19

Agonist potencies were determined by [³⁵S]-GTPγS binding at CHO-m1 cells membranes pre-treated or not pre-treated with pertussis toxin. Data shown are means ± S.E.M of at least three independent experiments performed in triplicate

⁺ Slope factor obtained when fitting data to one-site fit, ⁺⁺ F-Test value for two-site fit

^{*} All pEC₅₀ values for pEC₅₀ K_L were significantly different ($p < 0.05$, paired Student's *t*-test) from those obtained for the pEC₅₀ K_H

^{**} All pEC₅₀ values were not significantly different ($p > 0.05$, unpaired Student's *t*-test) from those obtained from the high potency estimate in the absence of pertussis toxin

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therefore tempting to suggest that the potency estimated from K_H reflects preferentially activation of G $\alpha_{q/11}$ and the activation of PTx-sensitive G-protein occurs at higher agonist concentrations and is reflected in the K_L potency estimation.

3.4.3.2. Agonist-mediated total [³⁵S]-GTP γ S binding at CHO-cell membrane preparations expressing M₃ mACh receptors

Concentration-response curves for the five agonists, in the presence and absence of pertussis toxin, in CHO-cell membrane preparations expressing the M₃ mACh receptor are shown in **Figs. 3.13 – 3.15**. No agonist-mediated binding was seen in untransfected CHO-K1 cell-lines (data not shown). In addition, in CHO-m3 membrane preparations atropine (10 μ M) completely inhibited agonist-mediated [³⁵S]-GTP γ S binding (data not shown).

Concentration-response curves obtained for agonist-mediated increases in total [³⁵S]-GTP γ S binding, in the absence and presence of PTx-treatment, are significantly smaller in magnitude than those obtained CHO-m1 membranes. This difference is also seen using the filtration methodology (data not shown, and see Akam *et al.*, 2001) and again provides evidence that the SPA methodology produces accurate agonist efficacy and potency measurements. This decrease in E_{max} (despite matched expression levels) could reflect a poorer coupling efficacy of the M₃ mACh receptor to the respective G-proteins in the CHO cell-line. In support of this concept oxotremorine, which was a partial agonist at the M₁ mACh receptor, was unable to elicit a significant stimulation above basal in CHO-m3 cell membrane preparations. As expected pilocarpine, which was a poor partial agonist at the M₁ mACh receptor, was similarly of poor efficacy in the M₃ mACh receptor membrane preparations and consequently no concentration-response data were obtained.

Slope factors for Concentration-response curves for all remaining agonists, in the absence of PTx-treatment, were significantly less than unity (0.60 ± 0.12 , 0.47 ± 0.15 , 0.58 ± 0.09 for methacholine, carbachol and oxotremorine-M, respectively: means $\pm$ S.E.M). Despite these low slope factors a single-site curve fit was statistically superior to a two-site fit ($p <$

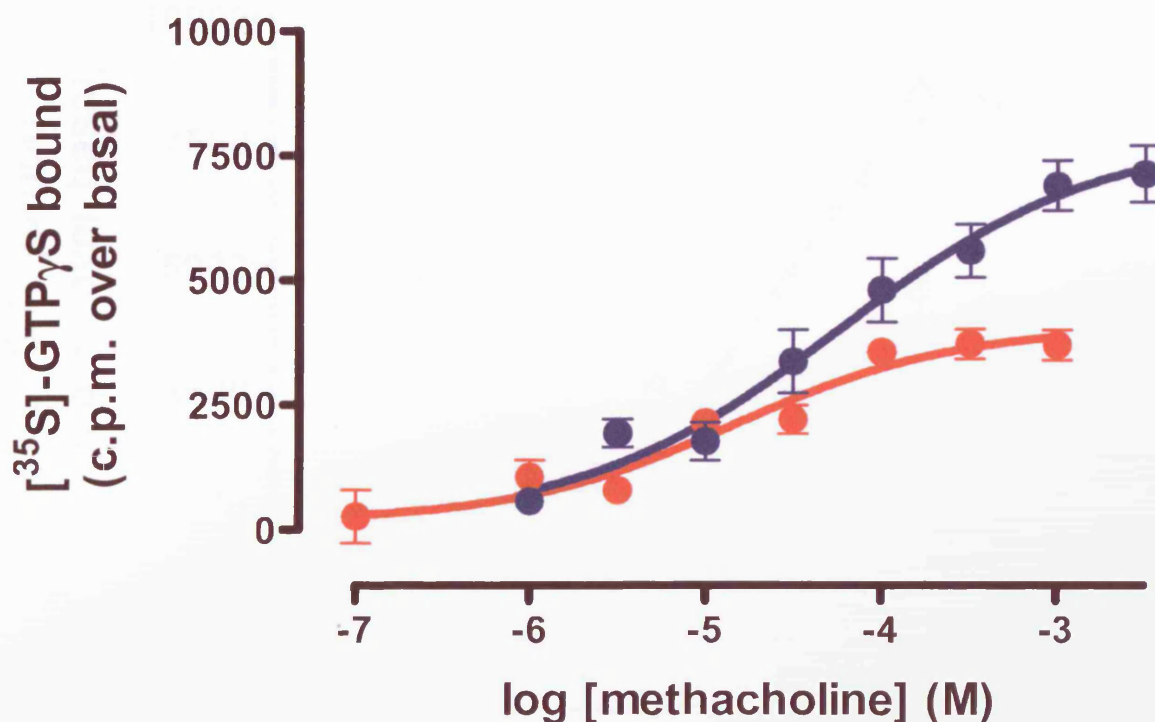


Fig. 3.13. Effect of pertussis toxin (100 ng ml⁻¹) on methacholine-stimulated total [³⁵S]-GTPγS binding in CHO-m3 cell membranes.

Effects of increasing concentrations of methacholine on M₃ mACh receptor-mediated [³⁵S]-GTPγS binding in membranes prepared from control (●), or pertussis toxin (100 ng ml⁻¹ 20-24 h) pre-treated (●) CHO-m3 cells. Basal values (determined in the absence of agonist) and non-specific binding (determined in the presence of 10 μM GTPγS) have been subtracted. Data are shown as means ± S.E.M. for 4 experiments carried out in duplicate. Data were fitted to a two-site model only if statistically significant ($p < 0.05$). $E_{\max}$, pEC_{50L} , pEC_{50H} and slope factors are summarized in **Table 3.3**.

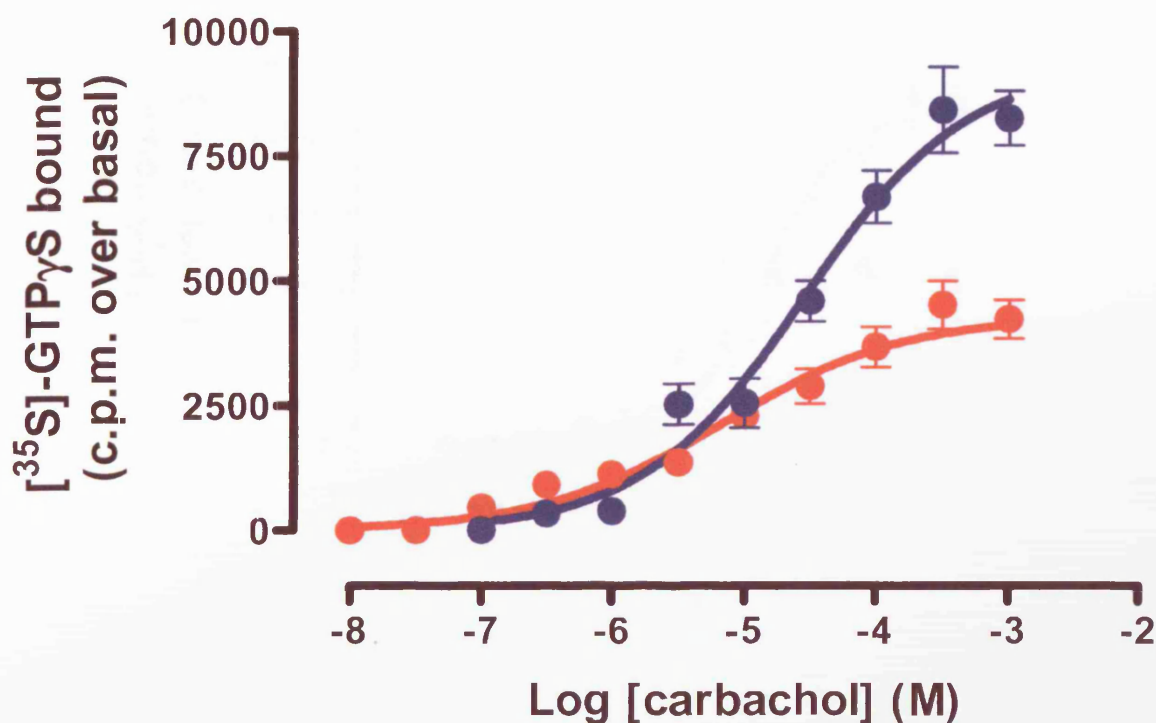


Fig. 3.14. Effect of pertussis toxin (100 ng ml⁻¹) on carbachol-stimulated total [³⁵S]-GTPγS binding in CHO-m3 cell membranes.

Effects of increasing concentrations of methacholine on M₃ mACh receptor-mediated [³⁵S]-GTPγS binding in membranes prepared from control (●), or pertussis toxin (100 ng ml⁻¹ 20-24 h) pre-treated (●) CHO-m3 cells. Basal values (determined in the absence of agonist) and non-specific binding (determined in the presence of 10 μM GTPγS) have been subtracted. Data are shown as means ± S.E.M. for 4 experiments carried out in duplicate. Data were fitted to a two-site model only if statistically significant ($p < 0.05$). $E_{\max}$, pEC_{50L} , pEC_{50H} and slope factors are summarized in **Table 3.3**.

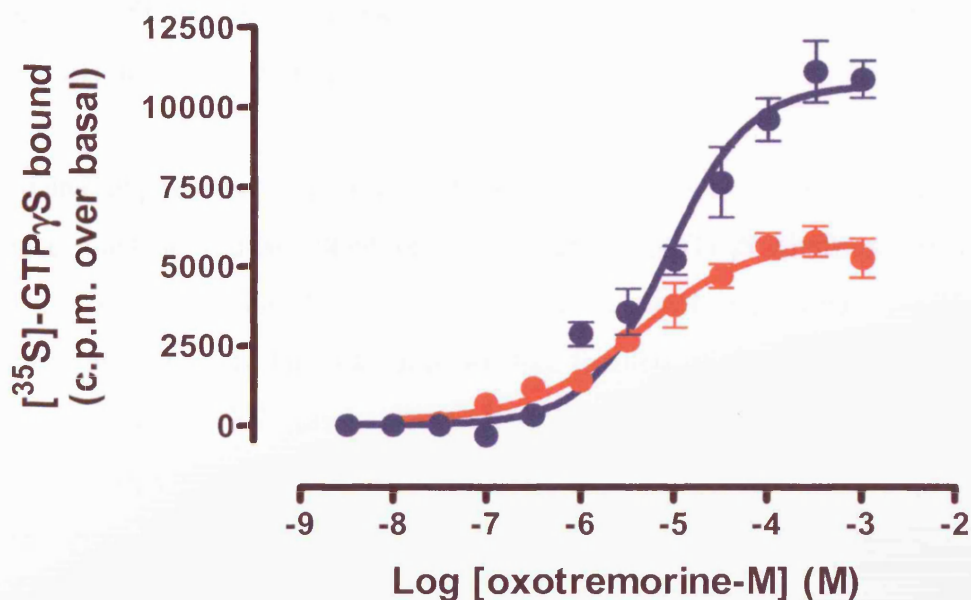


Fig. 3.15. Effect of pertussis toxin (100 ng ml⁻¹) on oxotremorine-M-stimulated total [³⁵S]-GTPγS binding in CHO-m3 cell membranes.

Effects of increasing concentrations of methacholine on M₃ mACh receptor-mediated [³⁵S]-GTPγS binding in membranes prepared from control (●), or pertussis toxin (100 ng ml⁻¹ 20-24 h) pre-treated (●) CHO-m3 cells. Basal values (determined in the absence of agonist) and non-specific binding (determined in the presence of 10 μM GTPγS) have been subtracted. Data are shown as means ± S.E.M. for 4 experiments carried out in duplicate. Data were fitted to a two-site model only if statistically significant ($p < 0.05$). $E_{\max}$, pEC_{50L} , pEC_{50H} and slope factors are summarized in **Table 3.3**

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0.05, *F*-test). This could be indicative of the poorer signal:noise ratio typically seen in CHO-m3 membrane preparations with the relatively large error in potency determination may preclude the meaningful determination of distinct potency sites. However, an alternative possibility is that agonists mediate [³⁵S]-GTPγS binding to a heterogeneous population of G-proteins, but does so with potencies that are too similar to allow accurate determination of their composites.

The latter hypothesis is strengthened by the observation that following PTx-treatment agonists mediate robust, albeit reduced, total [³⁵S]-GTPγS binding above basal. This indicates that, like the M₁, the M₃ mACh receptor expressed in CHO cells can productively couple to Gα_q-like and Gα_i-like families of G-proteins. The concentration-response curves in PTx pre-treated CHO-m3 membranes are left-shifted and although there is a corresponding increase in potency this is not statistically significant (pEC₅₀ values: methacholine, 4.50 ± 0.12 and 5.00 ± 0.40; carbachol, 4.54 ± 0.40 and 5.17 ± 0.40; oxotremorine-M, 4.95 ± 0.18 and 5.44 ± 0.08 for control and PTx-treated membranes, respectively; *p*>0.05, Student's *t*-test). The lack of statistically significant differences in the potency may also reflect, in part, the larger error in potency determination due to the diminished signal-to-noise ratio. All data are summarized in **Table 3.3**.

3.4.4. Specific immunoprecipitation of Gα subunits in membranes prepared from CHO-m1 and CHO-m3 clones

In order to determine more precisely which Gα subunits the M₁ and M₃ receptors couple to, we employed an immunoprecipitation strategy that allows the resolution of individual Gα subunits (Manning, 2002). This methodology relies on the development of specific antisera raised against individual Gα protein subtypes. After the assay incubation period the membranes are solubilized and probed with Gα-specific antibodies and the Gα-[³⁵S]-GTPγS-antibody complex can be isolated and quantified. This not only allows for the assessment of the diversity of Gα activation following agonist stimulation, but also produces a much improved signal:noise ratio for Gα_{q/11} proteins due to their isolation from background noise of basal Gα_{i/o} guanine nucleotide exchange (Harrison and

**TABLE 3.3 Stimulation of [³⁵S]-GTPγS binding by agonists at CHO-m3 cell
membrane preparations**

Ligand	No Pertussis Toxin		With Pertussis Toxin	
	pEC ₅₀	Slope Factor	pEC ₅₀	Slope Factor
Methacholine	4.5 ± 0.12	0.60 ± 0.12	5.00 ± 0.41	0.71 ± 0.21
Carbachol	4.54 ± 0.40	0.47 ± 0.15	5.17 ± 0.40	0.71 ± 0.09
Oxotremorine M	4.95 ± 0.18	0.58 ± 0.09	5.44 ± 0.08	0.75 ± 0.05

Agonist potencies were determined by [³⁵S]-GTPγS binding at CHO-m1 cells membranes pre-treated or not pre-treated with pertussis toxin. Data shown are means ± S.E.M of at least three independent experiments performed in triplicate

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Traynor, 2003; Milligan, 2003b). This technique has been validated in both recombinant systems (Alberts *et al.*, 1999) and for endogenous receptors (Panchalingam and Undie, 2000). One caveat to the employment of the immunoprecipitation step is that it is inherently poorly suited for 'scale-up' to more medium or high throughput screening strategies (Milligan, 2003b).

All assays were run under the optimal conditions established in section 4.3.2. Concentration-response curves were only analyzed using a two-site model if statistically superior (F value <0.05).

It has been appreciated for some time that different concentrations of GDP are required to optimize maximal [³⁵S]-GTPγS binding to different classes of Gα subtypes (Lorenzen *et al.*, 1993; Breivogel *et al.*, 1998). In this study the activation of Gα_{q/11} and Gα_{i1-3} was assessed through the use of Gα-specific antibodies. The optimal activation of these Gα subtypes, by mACh receptors has been shown also to require different concentrations of GDP (Hilf *et al.*, 1989; Offermanns *et al.*, 1994; Akam *et al.*, 2001). Additionally, it is known that the concentration of GDP can affect the efficacy of agonists, in particular partial agonists (Wenzel-Seifert and Seifert, 2000), and that variations in efficacy will be most important in conditions of poor receptor-G-protein-coupling (Pauwels *et al.*, 1997a, b). Therefore changing GDP concentration with each subtype Gα-[³⁵S]-GTPγS complex isolated may bias agonist efficacy and potency measurements (Pauwels *et al.*, 1997b). Therefore, to equitably assess agonist efficacy and potency differences the same GDP concentration (1 μM) was employed when quantifying both Gα_{q/11} and Gα_{i/o} activation.

3.4.4.1. Antibody specificity

Two antibodies were employed throughout the immunoprecipitation studies: an 'in-house' rabbit polyclonal antibody raised against the common C-terminus of Gα_q and Gα₁₁ ((C)LQLNLKEYNLV), and a commercially available antibody raised against the common C-terminus of Gα_{i1}, Gα_{i2} and Gα_{i3}. **Fig. 3.16.** shows representative immunoblots for the two antibodies to demonstrate their specificity. **Fig. 3.16A**, lanes 1&2 and 3&4 show samples from two independent CHO-m1 membrane preparations

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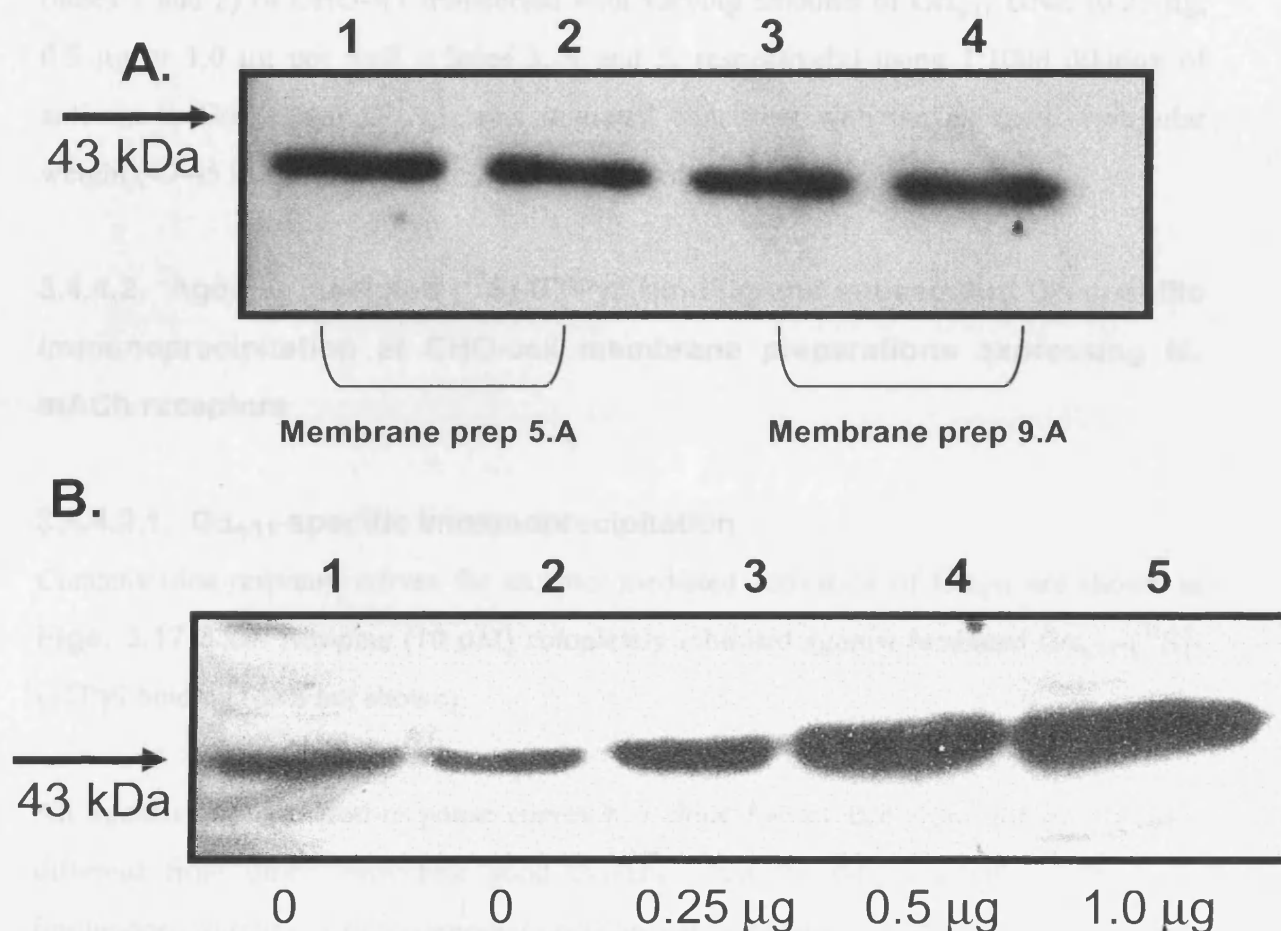


Fig. 3.16. Immunoblots prepared from CHO-cell membranes showing relative antibody specificity.

Blot **A**. Lanes 1&2 and 3&4 are duplicate lanes from two separate membranes preparations using 1:1000 dilution of antisera to Gα_{i1-3}. Blot **B**. untransfected CHO-K1 (Lanes 1 and 2) or CHO-K1 transfected with Gα_{q11} (0.25 μg, 0.5 μg or 1.0 μg per well – Lanes 3,4 and 5, respectively) using 1:1000 dilution of antisera to Gα_{q11}. All Gα proteins migrated consistently with the expected molecular weight (40-45 kDa) compared to M_r standards. No other bands, apart from those illustrated were visible on the gels, indicating relative specificity. Immunoblots are representative of a least three separate membrane preparations.

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using a 1:1000 dilution of the antibody raised against G α_{i1-3} . **Fig. 3.16B** are CHO-K1 (lanes 1 and 2) or CHO-K1 transfected with varying amounts of G $\alpha_{q/11}$ DNA (0.25 μ g, 0.5 μ g or 1.0 μ g per well – lanes 3, 4 and 5, respectively) using 1:1000 dilution of antisera to G $\alpha_{q/11}$. All G α proteins migrated consistent with the expected molecular weight (40-45 kDa) determined using M_r standards.

3.4.4.2. Agonist-mediated [³⁵S]-GTP γ S binding and subsequent G α -specific immunoprecipitation at CHO-cell membrane preparations expressing M₁ mACh receptors

3.4.4.2.1. G $\alpha_{q/11}$ -specific immunoprecipitation

Concentration-response curves for receptor-mediated activation of G $\alpha_{q/11}$ are shown in **Figs. 3.17-3.20**. Atropine (10 μ M) completely inhibited agonist-mediated G $\alpha_{q/11}$ -[³⁵S]-GTP γ S binding (data not shown).

All agonist concentration-response curves had slope factors that were not significantly different from unity, providing good evidence that the G $\alpha_{q/11}$ -specific antibody is immunoprecipitating a homogenous G-protein sub-population. Additionally, the signal-to-noise ratio achieved by effectively isolating the GDP/GTP exchange on G $\alpha_{q/11}$ subunits improves so that full concentration-response curves to the partial agonist pilocarpine can be obtained, due in part to removing the relatively high background GDP/GTP exchange on the G α_i subunits

With the exception of carbachol, the pEC₅₀ values for G $\alpha_{q/11}$ -specific [³⁵S]-GTP γ S binding were not statistically different from those obtained from either PTx-treated or K_H non-PTx treated membranes (e.g. methacholine: pEC₅₀ G $\alpha_{q/11}$ 6.21 $\pm$ 0.13, pEC₅₀ PTx-treated 6.26 $\pm$ 0.06 and pEC₅₀ K_H 6.23 $\pm$ 0.28; one-way ANOVA followed by Bonferroni's multiple-comparison; $p < 0.05$). This provides further support for the hypothesis that the pEC₅₀ K_H reflects preferential G $\alpha_{q/11}$ activation at low agonist concentrations. All data are summarized in **Table 3.4**. The rank order of agonists with respect to maximal G $\alpha_{q/11}$ activation (intrinsic activity) was:

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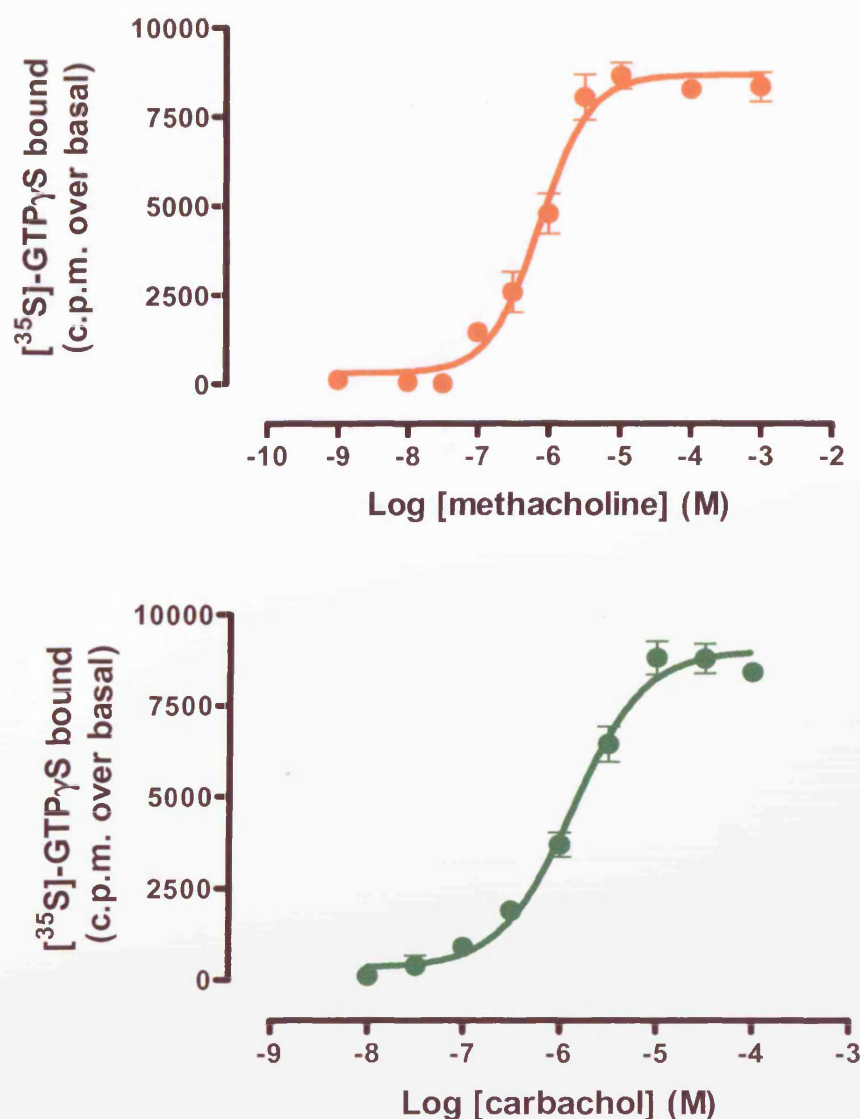


Fig. 3.17. Concentration-response data for agonist-stimulated [³⁵S]-GTPγS binding to Gα_{q/11} in CHO-m1 cell membranes.

Concentration-response curves for methacholine (upper panel) or carbachol (lower panel) - stimulated [³⁵S]-GTPγS binding to Gα_{q/11} in CHO-m1 cell membranes. Basal values (determined in the absence of agonist) and non-specific binding (determined in the presence of 10 μM GTPγS) have been subtracted. Data are shown as means ± S.E.M. for 3-6 experiments carried out in duplicate. E_{max}, pEC₅₀, and slope factors are summarized in **Table 3.4**

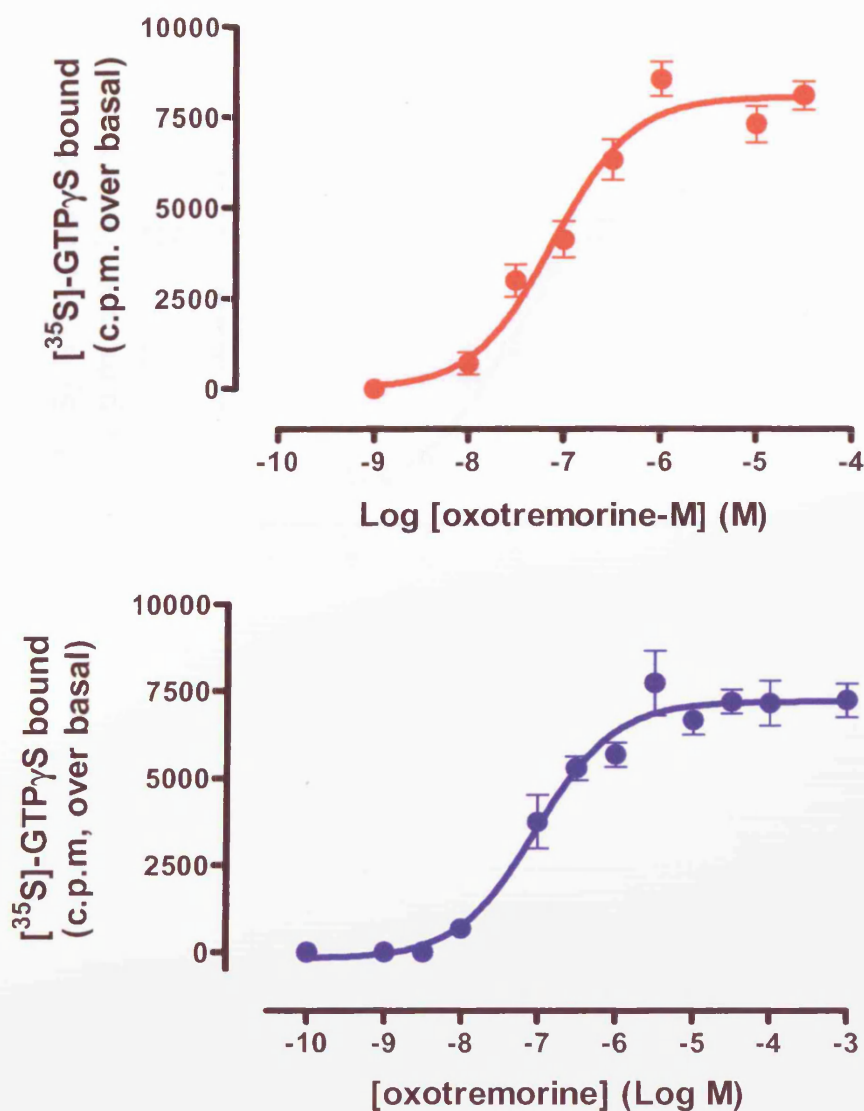


Fig. 3.18. Concentration-response data for agonist-stimulated [³⁵S]-GTP_γS binding to G_α_{q/11} in CHO-m1 cell membranes.

Concentration-response curves for oxotremorine-M (upper panel) or oxotremorine (lower panel) -stimulated [³⁵S]-GTP_γS binding to G_α_{q/11} in CHO-m1 cell membranes. Basal values (determined in the absence of agonist) and non-specific binding (determined in the presence of 10 μM GTP_γS) have been subtracted. Data are shown as means $\pm$ S.E.M. for 3-6 experiments carried out in duplicate. E_{max}, pEC₅₀, and slope factors are summarized in Table 3.4.

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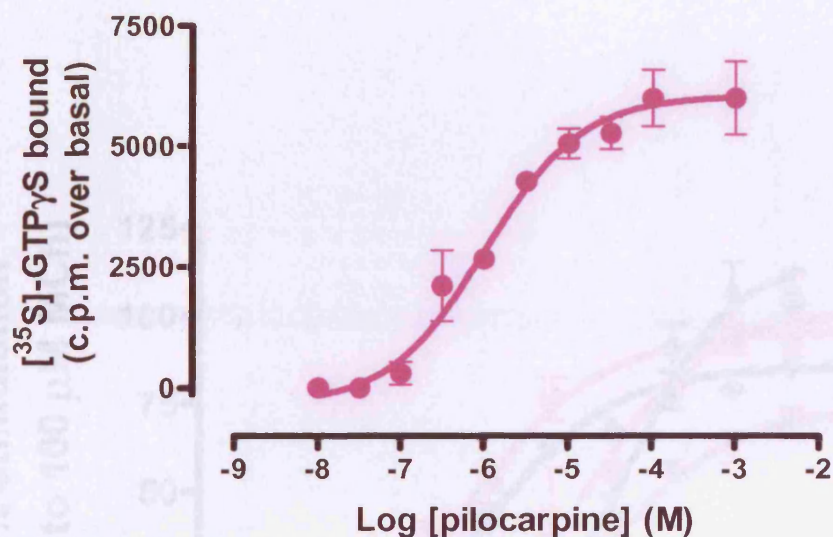


Fig. 3.19. Concentration-response data for agonist-stimulated [³⁵S]-GTPγS binding to Gα_{q/11} in CHO-m1 cell membranes.

Concentration-response curve for pilocarpine-stimulated [³⁵S]-GTPγS binding to Gα_{q/11} in CHO-m1 cell membranes. Basal values (determined in the absence of agonist) and non-specific binding (determined in the presence of 10 μM GTPγS) have been subtracted. Data are shown as means ± S.E.M. for 4 experiments carried out in duplicate. E_{max}, pEC₅₀, and slope factors are summarized in **Table 3.4**.

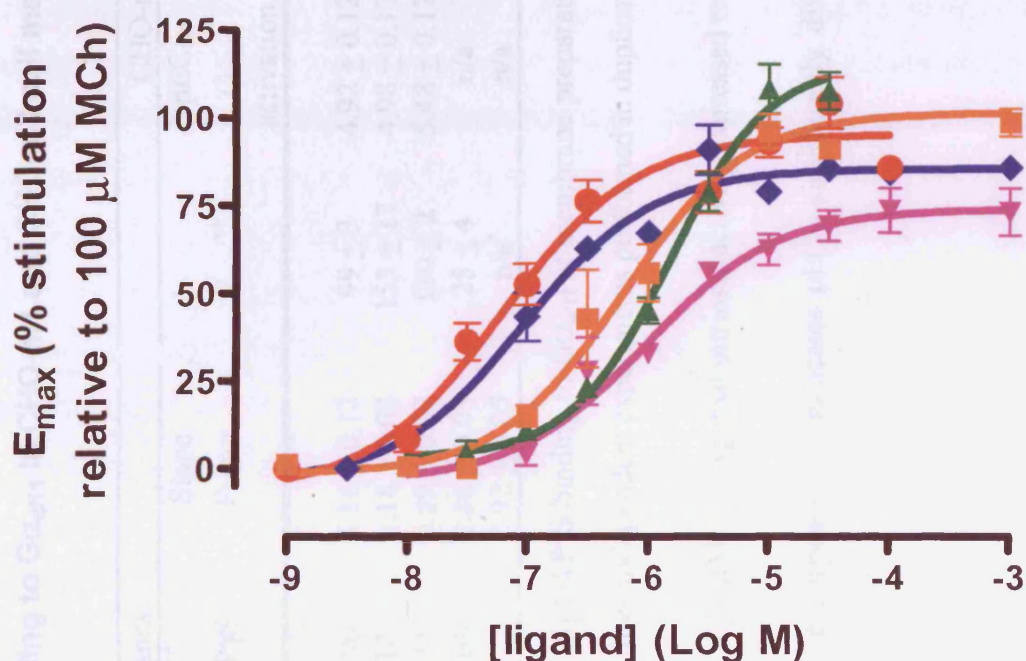


Fig. 3.20. Intrinsic activities of agonist-stimulated [³⁵S]-GTPγS binding to Gα_{q/11} in CHO-m1 cell membranes.

Intrinsic activities of carbachol (▲), oxotremorine-M (●), oxotremorine (◆), and pilocarpine (▼) as a percentage of the [³⁵S]-GTPγS binding to Gα_{q/11} in CHO-m1 cell membranes stimulated by 100 μM MCh (■). Basal values (determined in the absence of agonist) and non-specific binding (determined in the presence of 10 μM GTPγS) have been subtracted. Data are shown as means ± S.E.M. for 3-6 experiments carried out in duplicate. E_{max}, pEC₅₀, and slope factors are summarized in **Table 3.4**.

Table 3.4. Agonist Stimulated [³⁵S]-GTPγS binding to Gα_{q/11} in CHO-m1 and CHO-m3 cell membrane preparations

Ligand	CHO-m1 cell membranes				CHO-m3 cell membranes			
	E _{max} ⁺	pEC ₅₀ G _{q/11} activation	pEC ₅₀ ⁺⁺⁺ Total GTPγS PTx	Slope Factor	E _{max} ⁺⁺	pEC ₅₀ G _{q/11} activation	pEC ₅₀ ⁺⁺⁺ Total GTPγS PTx	Slope Factor
Methacholine	97 ± 1	6.21 ± 0.13	6.26 ± 0.06	1.16 ± 0.12	99 ± 3	4.92 ± 0.12	5.00 ± 0.41	1.02 ± 0.02
Carbachol	104 ± 6	5.85 ± 0.02	6.31 ± 0.10*	1.18 ± 0.08	153 ± 17	4.98 ± 0.17	5.17 ± 0.40	0.90 ± 0.05
Oxotremorine M	95 ± 2	7.14 ± 0.09	7.29 ± 0.11	0.99 ± 0.02	110 ± 2	5.48 ± 0.12	5.44 ± 0.08	0.90 ± 0.11
Oxotremorine	85 ± 2	7.01 ± 0.10	7.00 ± 0.06	0.86 ± 0.03	25 ± 4	n/a	n/a	n/a
Pilocarpine	78 ± 8	6.00 ± 0.17	n/a	0.92 ± 0.05	n/a ^a	n/a	n/a	n/a

Agonist intrinsic activities and potencies determined by [³⁵S]-GTPγS binding to Gα_{q/11} at membrane preparations from CHO-m1 and CHO-m3 cell membranes. Data shown are means ± S.E.M of a least three independent experiments performed in duplicate

⁺ Agonist intrinsic activity expressed relative to that of MCh (10μM) , ⁺⁺ Agonist intrinsic activity expressed relative to that of MCh (300μM)

⁺⁺⁺ Data taken from tables 3.2. and 3.3. for comparative purposes – * indicates pEC₅₀ significantly different from those obtained from immunoprecipitation with G_{q/11} (column 3)

^a no significant increase above basal

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carbachol = methacholine = oxotremorine-M > oxotremorine* > pilocarpine*

(*significantly different maximal stimulation ($E_{\max}$) to that obtained for 1 mM methacholine: $p < 0.05$, Student's *t*-test)

3.4.4.2.2. G α_{i1-3} -specific immunoprecipitation

To address whether M₁ mACh receptor agonists exhibit a uniform or agonist-specific activation of different G α subunits in CHO-m1 membranes, we examined the ability of ligands to mediate GDP/GTP exchange on G α_{i1-3} subunits. Unfortunately, the highly variable basal/background G α_i activity precluded the accurate determination of concentration-response curves. Therefore, only intrinsic activity measurements were possible. Maximal stimulations for agonist were: 7404 ± 828 ; 8058 ± 661 ; 8795 ± 1068 ; 2566 ± 393 and 625 ± 153 c.p.m.-over-basal for methacholine, carbachol, oxotremorine-M, oxotremorine and pilocarpine, respectively (**Fig. 3.21A**). Intrinsic activity, assessed as the percentage stimulation compared to that obtained for 1 mM methacholine yielded $E_{\max}$ values of 100; 98 ± 8 ; 118 ± 3 ; 36 ± 9 and 8 ± 6 for methacholine, carbachol, oxotremorine-M, oxotremorine and pilocarpine, respectively (**Fig. 3.21B**). $E_{\max}$ values (using supra-maximal agonist concentrations), can be used as an assessment of agonist relative efficacies (Kenakin, 2003). The rank order of relative efficacy with respect to G α_{i1-3} activation was:

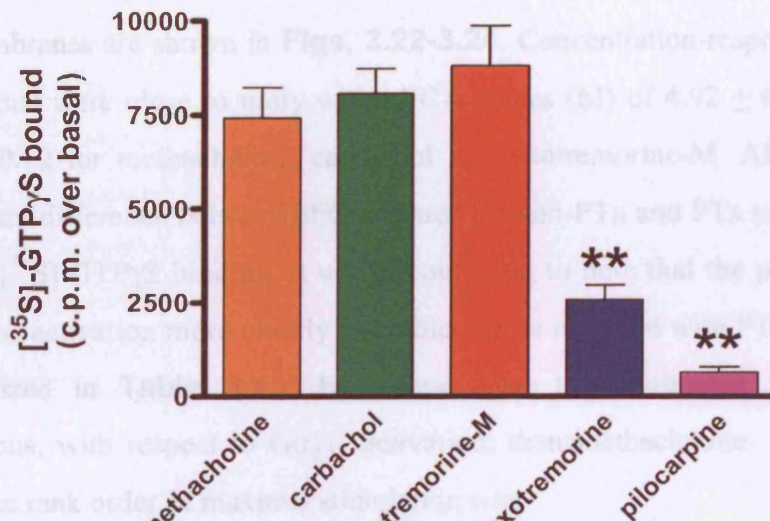
oxotremorine-M = carbachol = methacholine > oxotremorine * > pilocarpine*

(*significantly different maximal stimulation ($E_{\max}$) to that obtained for 1mM methacholine $p < 0.01$, Student's *t*-test)

3.4.4.3. Agonist-mediated [³⁵S]-GTP γ S binding and subsequent G α -specific immunoprecipitation at CHO-cell membrane preparations expressing M₃ mACh receptors

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A.



B.

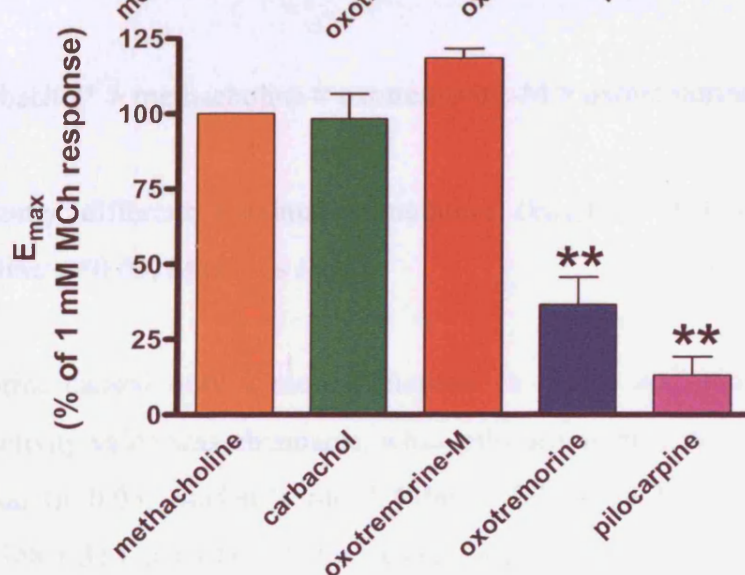


Fig. 3.21. Intrinsic activities of agonist-stimulated [³⁵S]-GTPγS binding to Gα_{i1-3} in CHO-m1 cell membranes.

Maximal agonist stimulation of [³⁵S]-GTPγS binding to G_{i1-3} in CHO-m1 membrane preparations. Panel A shows [³⁵S]-GTPγS binding to Gα_{i1-3} as c.p.m. (with the basal and non-specific binding subtracted). **Panel B** shows transformed data from **panel A** as a percentage of [³⁵S]-GTPγS binding elicited with 1 mM MCh. Data are shown as means ± S.E.M. for 4 experiments carried out in triplicate. Agonists with E_{max} values statistically different from the reference MCh value are indicated as ***p*<0.01.

3.4.4.3.1. G $\alpha_{q/11}$ -specific Immunoprecipitation

Concentration-response curves for activation of G $\alpha_{q/11}$ by the different agonists in CHO-m3 membranes are shown in **Figs. 3.22-3.24**. Concentration-response curves had slope factors that were close to unity with pEC₅₀ values (M) of 4.92 ± 0.12 , 4.98 ± 0.17 and 5.48 ± 0.12 for methacholine, carbachol and oxotremorine-M. Although there was no significant difference between pEC₅₀ values for non-PTx and PTx pre-treated membranes in total [³⁵S]-GTP γ S binding, it was encouraging to note that the pEC₅₀ values obtained for G $\alpha_{q/11}$ activation more closely resembled those obtained with PTx pre-treatment (Data summarized in **Table 3.4**). E_{max} data show that carbachol appears to be more efficacious, with respect to G $\alpha_{q/11}$ activation, than methacholine ($p < 0.05$, Student's *t*-test). The rank order of maximal stimulation was:

carbachol* > methacholine = oxotremorine-M > oxotremorine* >> pilocarpine*

(*significantly different maximal stimulation (E_{max}) to that obtained for 1 mM methacholine: $p < 0.05$, Student's *t*-test)

Oxotremorine caused only a modest increase in G $\alpha_{q/11}$ activation, such that only an intrinsic activity value was obtainable, while pilocarpine mediated no significant binding above basal ($p > 0.05$, Student's *t*-test) (Max [³⁵S]-GTP γ S binding values above basal (c.p.m.) 1348 ± 314 and 403 ± 92 for oxotremorine and pilocarpine, respectively; mean $\pm$ S.E.M, n=3).

3.4.4.3.2. G α_{i1-3} -specific immunoprecipitation

As with CHO-m1 membrane preparations, full concentration curves for M₃ mACh receptor-mediated [³⁵S]-GTP γ S binding to G α_{i1-3} could not be obtained due to high background noise. Full agonists, which elicited robust [³⁵S]-GTP γ S binding to G $\alpha_{q/11}$ at CHO-m3 membranes, also caused significant [³⁵S]-GTP γ S binding to G α_{i1-3} with increases above basal of 7133 ± 1667 ; 10505 ± 719 and 9590 ± 936 for methacholine, carbachol and oxotremorine-M, respectively (mean (c.p.m.) $\pm$ S.E.M., n $\geq$ 3, **Fig. 3.25A**).

Chapter 3: Evaluation of the G-protein coupling profile of CHO-cells stably expressing the M₁ and M₃ human muscarinic acetylcholine receptors

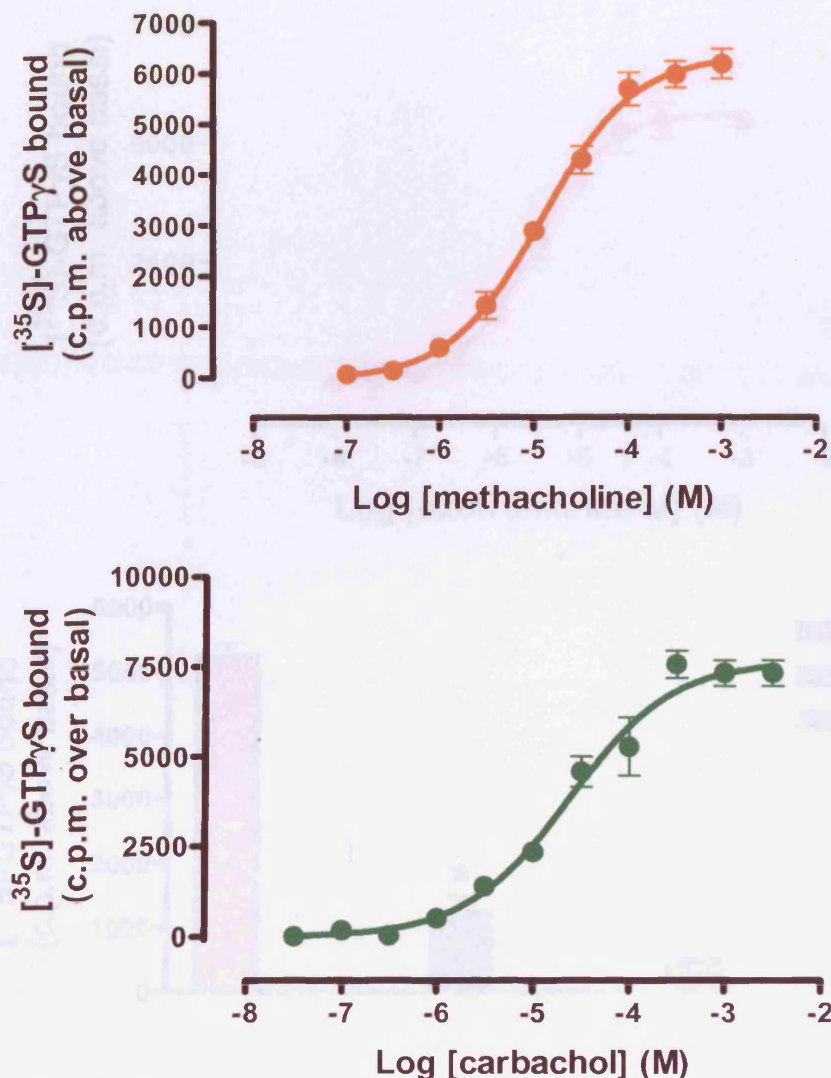


Fig. 3.22. Concentration-dependencies of agonist-stimulated [³⁵S]-GTPγS binding to Gα_{q/11} in CHO-m3 cell membranes.

Concentration-response curves for methacholine (upper panel) or carbachol (lower panel) - stimulated [³⁵S]-GTPγS binding to Gα_{q/11} in CHO-m1 cell membranes. Basal values (determined in the absence of agonist) and non-specific binding (determined in the presence of 10 μM GTPγS) have been subtracted. Data are shown as means ± S.E.M. for 3-6 experiments carried out in duplicate. E_{max}, pEC₅₀, and slope factors are summarized in **Table 3.4**.

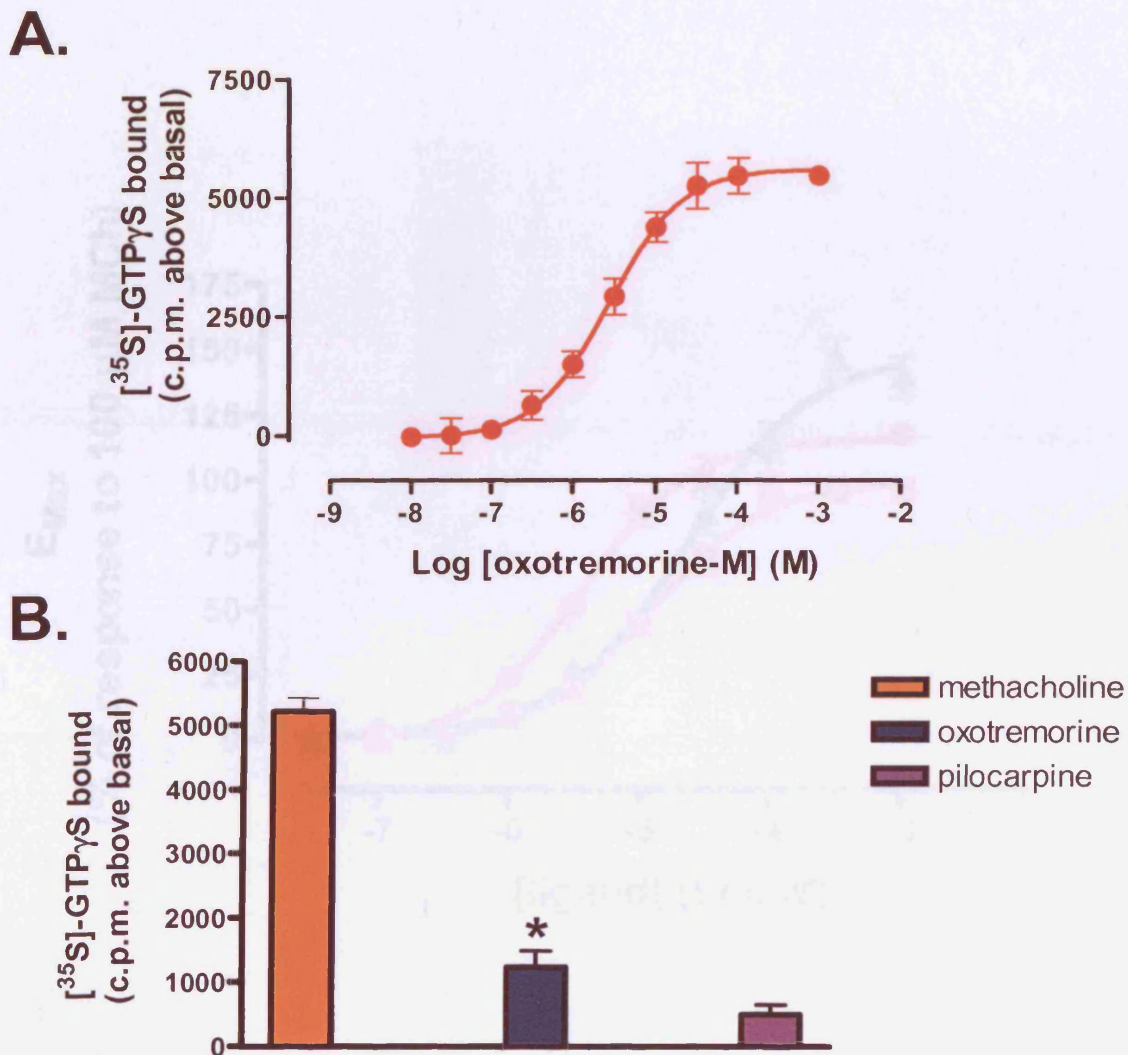


Fig. 3.23. Concentration-dependencies of agonist-stimulated [³⁵S]-GTPγS binding to Gα_{q/11} in CHO-m3 cell membranes.

A. Concentration-response curve for oxotremorine-M-stimulated [³⁵S]-GTPγS binding to Gα_{q/11} in CHO-m1 cell membranes. **B.** Maximal stimulations by oxotremorine (100 μM) and pilocarpine (1 mM) of [³⁵S]-GTPγS binding to Gα_{q/11} in the same membrane preparation (*) significant increase above basal. For both panels **A & B**, basal values (determined in the absence of agonist) and non-specific binding (determined in the presence of 10 μM GTPγS) have been subtracted. Data are shown as means ± S.E.M. for 3-6 experiments carried out in duplicate. E_{max}, pEC₅₀, and slope factors are summarized in **Table 3.4**.

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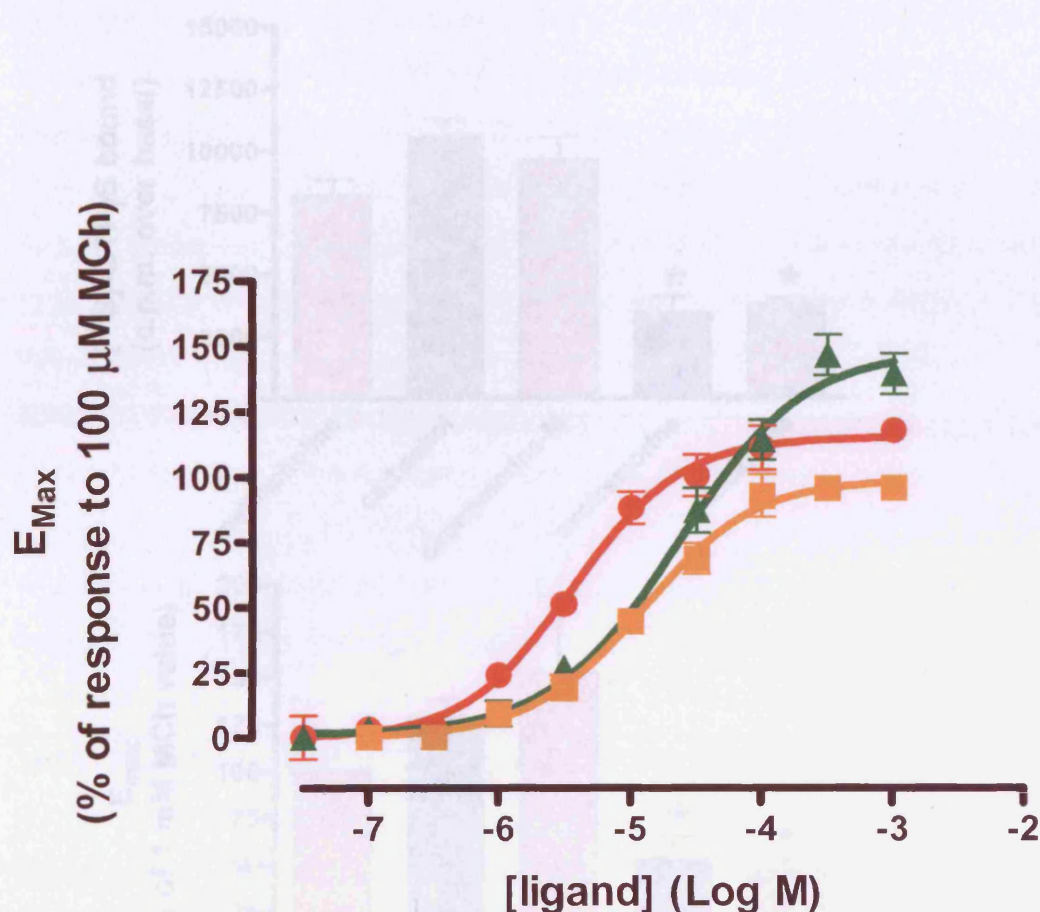


Fig. 3.24. Intrinsic activities of agonist-stimulated [³⁵S]-GTPγS binding to Gα_{q/11} in CHO-m3 cell membranes.

Intrinsic activities of carbachol (▲), oxotremorine-M (●), as a percentage of 100 μM methacholine (■) stimulated [³⁵S]-GTPγS binding to Gα_{q/11} in CHO-m3 cell membranes. Basal values (determined in the absence of agonist) and non-specific binding (determined in the presence of 10 μM GTPγS) have been subtracted. Data are shown as means ± S.E.M. for 3-6 experiments carried out in duplicate. E_{max}, pEC₅₀, and slope factors are summarized in Table 3.4.

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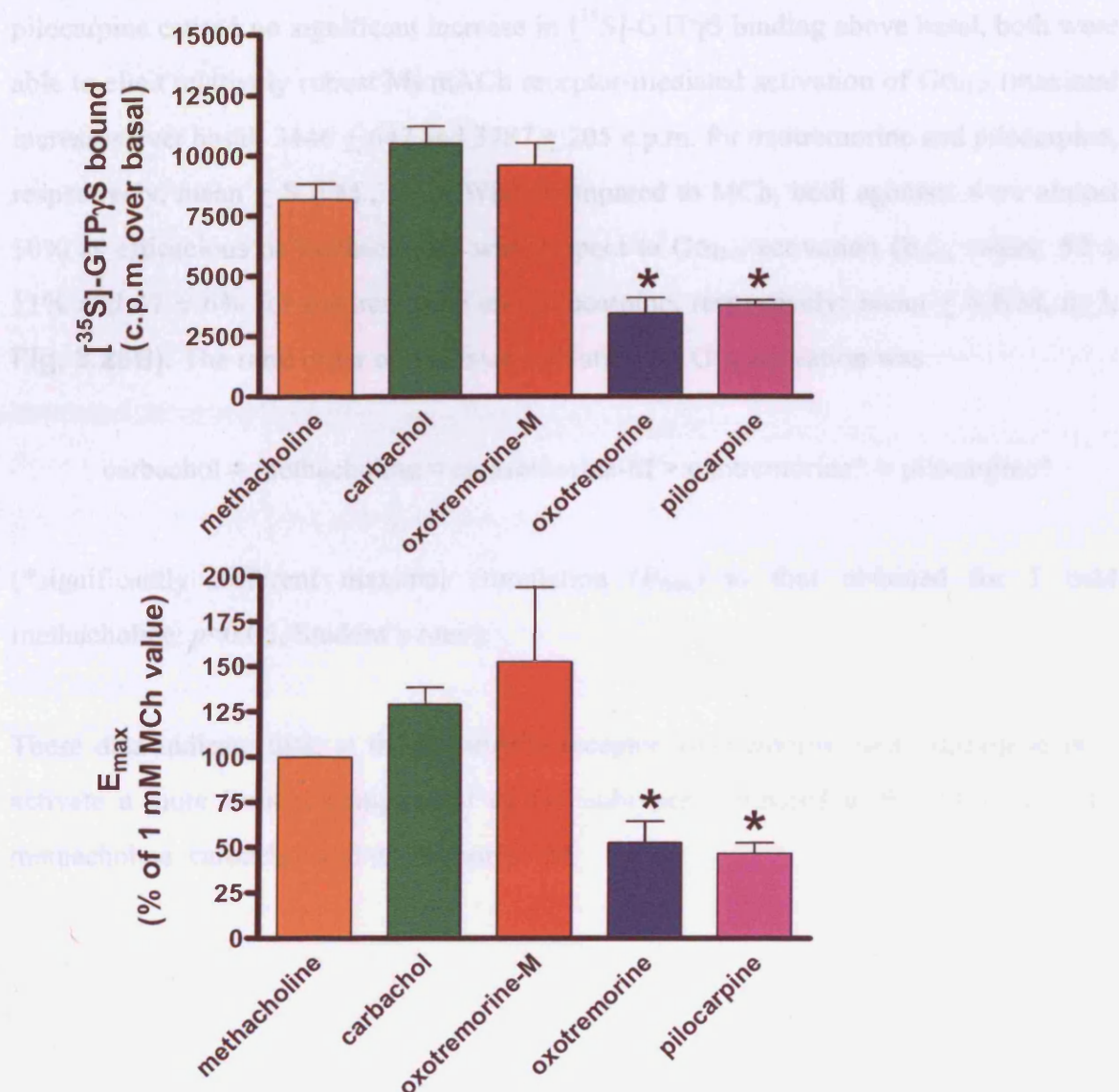


Fig. 3.25 Intrinsic activities of agonist-stimulated [³⁵S]-GTPγS binding to Gα_{i1-3} in CHO-m3 cell membranes.

Maximal agonist stimulation of [³⁵S]-GTPγS binding to G_{i1-3} at CHO-m1 membrane preparations. Panel **A** shows [³⁵S]-GTPγS binding to Gα_{i1-3} as c.p.m. (with basal and non-specific binding subtracted). Panel **B** shows transformed data from panel A as a percentage of [³⁵S]-GTPγS binding elicited by 1 mM methacholine. Data are shown as means ± S.E.M. for 4 experiments carried out in triplicate. Agonists with E_{max} values statistically different from the reference value (1 mM MCh) are indicated **p* < 0.05.

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Interestingly, although oxotremorine was relatively poor at G $\alpha_{q/11}$ activation and pilocarpine caused no significant increase in [³⁵S]-GTP γ S binding above basal, both were able to elicit relatively robust M₃ mACh receptor-mediated activation of G α_{i1-3} (maximal increases over basal: 3446 ± 642 and 3787 ± 205 c.p.m. for oxotremorine and pilocarpine, respectively; mean $\pm$ S.E.M., $n \geq 3$). When compared to MCh, both agonists were almost 50% as efficacious as methacholine with respect to G α_{i1-3} activation ($E_{\max}$ values, $52 \pm 11\%$ and $47 \pm 6\%$ for oxotremorine and pilocarpine, respectively; mean $\pm$ S.E.M., $n \geq 3$, **Fig. 3.25B**). The rank order of maximal activation for G α_i activation was:

$$\text{carbachol} = \text{methacholine} = \text{oxotremorine-M} > \text{oxotremorine}^* = \text{pilocarpine}^*$$

(*significantly different maximal stimulation ($E_{\max}$) to that obtained for 1 mM methacholine: $p < 0.05$, Student's t -test)

These data indicate that, at the M₃ mACh receptor, oxotremorine and pilocarpine may activate a more limited complement of G α subtypes compared to the other agonists, methacholine, carbachol and oxotremorine-M.

3.5 Discussion

3.5.1. Total [³⁵S]-GTPγS binding

Assessment of total [³⁵S]-GTPγS binding has been used extensively as an indicator of the global population of Gα-subunits activated in response to ligand binding at G-protein coupled receptors (Akam *et al.*, 1997; Zhu *et al.*, 1997; Zaworski *et al.* 1999; McLoughlin and Strange, 2000; Feng *et al.*, 2002). Other studies have also employed the technique to highlight differences in receptor-mediated activation of PTx-sensitive and -insensitive G-proteins (Offermanns *et al.*, 1991; Negishi *et al.*, 1993; Alberts *et al.*, 1999; Wenzel-Seifert and Seifert, 2000). Using this approach it has previously been demonstrated that the M₁ and M₃ mACh receptors can couple to both Gα_{i/o} and Gα_{q/11} proteins (Offermanns *et al.*, 1994; Burford *et al.*, 1995b), whilst the M₂ and M₄ mACh receptors appear predominantly, if not exclusively, Gα_{i/o} coupled (Lazareno *et al.*, 1993). Here we have also employed a complementary approach with a range of agonists to try to dissect the individual sub-populations of Gα proteins activated, at and beyond the level of PTx sensitivity.

It has frequently been stated that the quantification of total [³⁵S]-GTPγS binding stimulated by receptors predominantly coupled to Gα_{q/11} is technically more demanding than for those receptors coupled to Gα_i-type G-proteins. This is due in part to poor signal over background and the comparatively low G_{q/11}α expression level in many cell-types (Porter *et al.*, 2002; Milligan, 2003). Here, we have demonstrated that concentration-response curves can be generated for M₁ and M₃ mACh receptors under optimal assay conditions. More importantly, total agonist-stimulated [³⁵S]-GTPγS binding in CHO-m1 cell membrane preparations can be dissected into high and low potency components if sufficient agonist concentration data-points are accumulated. A comparison of the magnitude of response and potency for agonists at M₁ mACh receptors, comparing data obtained in control and PTx pre-treated membranes, yields several interesting observations. Firstly, following PTx pre-treatment, concentration-response curves in CHO-m1 membranes are reduced in magnitude, but exhibit an increased agonist potency, compared to untreated membranes. This supports previous observations (Akam *et al.*,

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2001) that total [³⁵S]-GTPγS binding curves (+/-PTx) reveals agonist-mediated receptor activation of Gα_{q/11} and Gα_{i/o}-like G-proteins. Secondly, the low slope factor (<0.55) is suggestive of differential G-protein coupling with strikingly different potencies, as any other possible (artefactual) cause of low slope factors (*e.g.* G-protein/receptor depletion) is likely to produce slope factors with minimal slope factors of 0.7 (Lazareno *et al.*, 1993). Lastly, the significant correlation between the pEC₅₀ values obtained from PTx pre-treated and untreated membranes leads to the proposal that at low agonist concentrations, M₁ mACh receptors preferentially facilitate GDP/GTPγS exchange on Gα_{q/11} subunits.

Total [³⁵S]-GTPγS binding curves obtained from CHO-m3 cell membranes are reduced in potency and smaller in magnitude than those obtained for respective agonists at CHO-m1 cell membranes. This supports other experimental evidence, which suggests that M₃ mACh receptors, when expressed in CHO cells are less well coupled to their respective G-proteins than M₁ mACh receptors (Lazareno *et al.*, 1993; Burford *et al.*, 1995a,b). Despite the smaller signal-to-noise ratio, total [³⁵S]-GTPγS binding curves for PTx pre-treated and untreated membranes could still be assessed for 'full' agonists and indicated coupling to both Gα_{q/11} and Gα_{i/o} subclasses. However, despite low slope factors, these curves could not be resolved into high and low potency components. This could reflect the difficulty in obtaining consistent data because of the poorer signal-to-noise ratio. However if we accept that the low slope factor can be indicative of coupling to different G-proteins (Costa *et al.*, 1992; Ehlert, 1985), rather than to different receptor activation states, an alternative conclusion should be considered - that M₃ mACh receptors expressed in CHO-cells couple to Gα_{q/11} and Gα_{i/o}, but with potencies too similar to allow accurate dissection into high and low potency components.

3.5.2 Gα-specific immunoprecipitation

As with any assay relying on specific antibodies, it is important to appreciate the potential differences in antibody immunoprecipitation efficiency (Milligan, 2003b). This makes it unrealistic *quantitatively* to compare the proportions of agonist-mediated Gα_{q/11} and Gα_{i/o} activation by either receptor subtype. However, it does not preclude the qualitative

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relative assessment of the G α complement activated by the agonists at the M₁ and M₃ mACh receptor.

3.5.2.1 G α -specific immunoprecipitation at the M₁ mACh receptor

Where possible, assay conditions, including the GDP concentration, have been comparable across the two [³⁵S]-GTP γ S assays, to facilitate direct comparisons of the pharmacological data. Considering the strong correlation between agonist potency at specific G $\alpha_{q/11}$ activation, and the pEC₅₀ obtained for total [³⁵S]-GTP γ S binding after PTx pre-treatment, it seems reasonable to predict that at low agonist concentrations the M₁ mACh receptor (when expressed in CHO-cells) preferentially couples to G $\alpha_{q/11}$. This preferential coupling to G $\alpha_{q/11}$ does not appear to be related to the efficacy of the agonist, as a similar situation pertains for G $\alpha_{q/11}$ activation for the partial agonists pilocarpine and oxotremorine. The apparent full agonism of oxotremorine and pilocarpine when isolating the G $\alpha_{q/11}$ component is likely to represent a receptor reserve for this particular pathway (Bymaster *et al.*, 2001). Using a similar methodology, pathway-specific receptor reserve has been demonstrated for recombinant (DeLapp *et al.*, 1999) and endogenous (Porter *et al.*, 2002) mACh receptors, as well as other GPCR subtypes e.g. 5-HT_{2C} receptor (Cussac *et al.*, 2002).

The high degree of variability, in both basal and agonist-mediated [³⁵S]-GTP γ S binding to G α_{i1-3} subunits, precluded the construction of meaningful concentration-response curves. Despite being unable to determine accurate pEC₅₀ values, the intrinsic efficacy (E_{max}) data demonstrate that only full agonists (methacholine, carbachol and oxotremorine-M) are able to elicit robust [³⁵S]-GTP γ S binding to G α_{i1-3} subunits. The partial agonist, oxotremorine, is only 25% as efficacious as methacholine with respect to G α_{i1-3} activation, whilst the addition of pilocarpine does not cause any significant [³⁵S]-GTP γ S above that of basal. Therefore, in contrast to the activation of G $\alpha_{q/11}$, the activation of G α_{i1-3} is directly related to the efficacy of the agonist.

The biphasic curves obtained in total [³⁵S]-GTP γ S binding assays correlate well with the data obtained by the immunoprecipitation of specific G α subunits. The M₁ mACh

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receptor when expressed in CHO-cells shows a preferential coupling to G $\alpha_{q/11}$ subunits. The efficiency of the coupling is likely to result in a high degree of receptor reserve for this pathway, which results in partial agonists having comparable efficacies to that of full agonists when the activation of the G $\alpha_{q/11}$ pathway is isolated. The apparent PTx-sensitivity of the low potency (pEC₅₀ K_L) component is suggestive of G α_i coupling only at high agonist concentrations, when G $\alpha_{q/11}$ coupling is likely to be saturated. Only agonists of high efficacy are able to initiate coupling to the more poorly coupled G α_i subunits. The exact molecular basis of partial and full agonism is currently poorly understood, although it has been suggested that differences in GDP/GTP exchange rates (Selley *et al.*, 1998; Seifert *et al.*, 2000), amounts of ternary complex stabilized (Pauwels *et al.*, 1997a,b) receptor-G-protein contact sites (Jackson *et al.*, 1998), differences in affinities for **R** and **R*** (Leff *et al.*, 1997) or differences in affinity for **R*** and **R*G** (ability to stabilise the high-affinity ternary complex) (De Lean *et al.*, 1980) may all underlie the magnitude of observed efficacy. Despite this, the M₁ mACh receptor expressed in the CHO cell background exhibits ‘agonist-strength-of-signal’ G-protein coupling, whereby agonists of high efficacy are able to activate both well-coupled and poorly-coupled G α subtypes, whereas agonist of low efficacy elicit receptor-mediated activation of only the most efficiently coupled G-protein(s) (**Fig. 3.26A**).

3.5.2.2 G α -specific immunoprecipitation at the M₃ mACh receptor

G-protein coupling elicited by agonist activation of the M₃ mACh receptor stably expressed in CHO-cells is smaller in magnitude than for the M₁ receptor when total [³⁵S]-GTP γ S binding is measured. Only full agonists (methacholine, oxotremorine-M and carbachol) are able to promote significant [³⁵S]-GTP γ S binding above basal, such that concentration-response curves could be generated. Although we were unable to resolve total [³⁵S]-GTP γ S binding curves into high and low potency components several lines of evidence support the notion that only the **full** agonists stabilize a conformation of the M₃ mACh receptor that is more strongly coupled to G $\alpha_{q/11}$: **(1.)** Only the full agonists gave sufficient [³⁵S]-GTP γ S binding, when the isolation of G $\alpha_{q/11}$ was performed, to allow the construction of robust concentration-response curves. **(2.)** The EC₅₀ values for G $\alpha_{q/11}$ activation were similar to those obtained for the total [³⁵S]-GTP γ S binding concentration

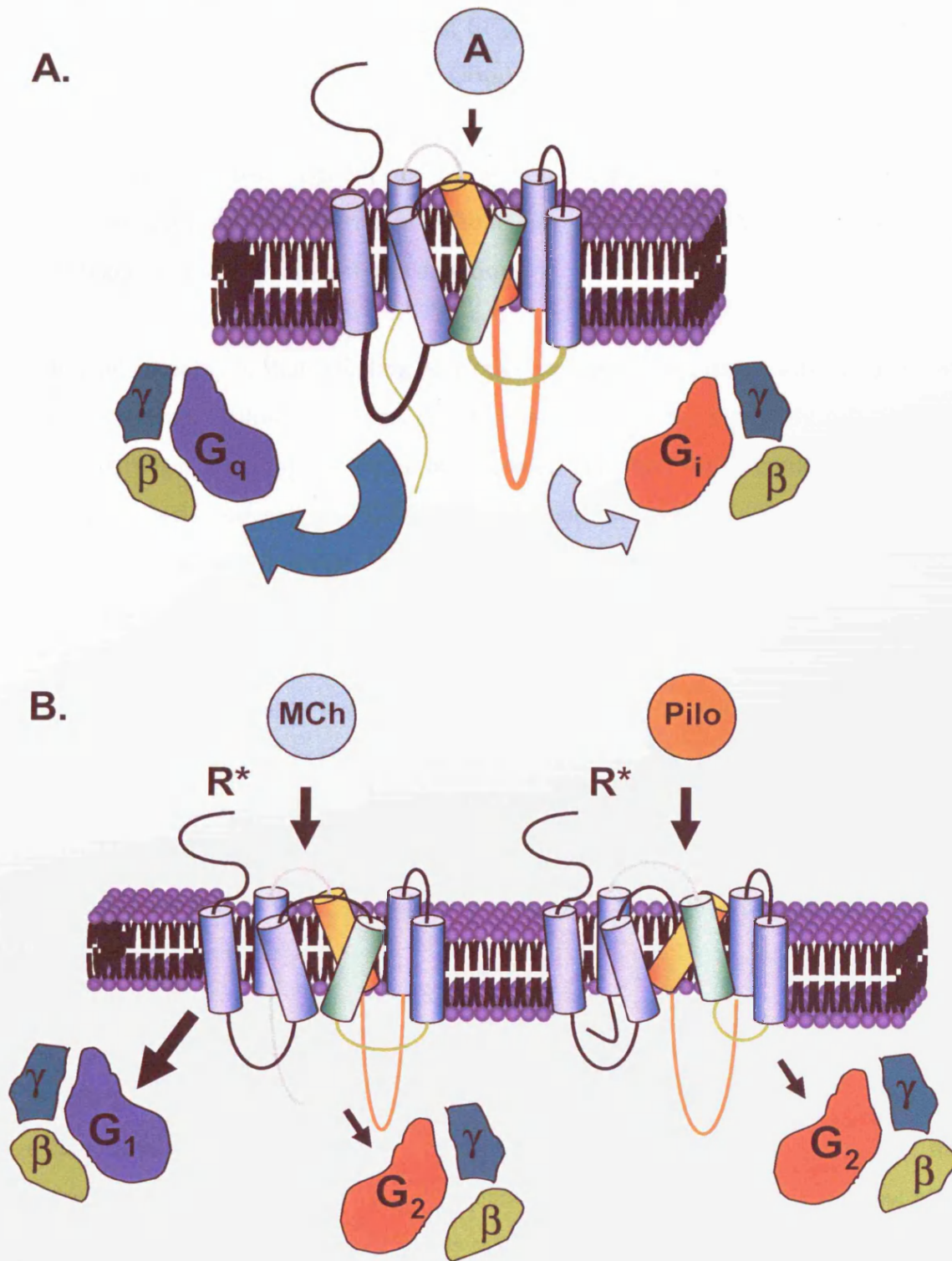


Fig. 3.26 Proposed signalling for M_1 (A) and M_3 (B) mACh receptors stably expressed in CHO-cells.

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curves constructed in membranes prepared from PTx-treated cells (although there were no significant differences between agonist EC₅₀ values in +/- PTx treated membranes). (3.). Significantly, [³⁵S]-GTPγS binding to Gα_{i1-3} subunits could only be detected at relatively high concentrations of the full agonists. In support of this notion, Offermanns *et al.* (1994) suggested that both M₁ and M₃ mACh receptors, stably expressed in HEK293 cells, mediated agonist activation of both Gα_q and Gα_i, but that Gα_i activation was detectable only at high agonist concentrations.

Although it appears that coupling to Gα_{q/11} via agonist-mediated activation of the M₃ mACh receptor follows the 'strength-of-signal' hypothesis, a slightly different pattern emerges when coupling to Gα_{i1-3} is assessed. The three full agonists (methacholine, carbachol and oxotremorine-M) stimulate comparable levels of [³⁵S]-GTPγS binding to Gα_{i1-3} subunits, and therefore the M₃ mACh receptor appears promiscuously coupled to both Gα_{q/11} and Gα_i, as also demonstrated by the low slope factors reported for total [³⁵S]-GTPγS binding curves (although due to possible differences in immunoprecipitating efficiency we are unable to comment on the relative proportions of Gα_{q/11} and Gα_{i1-3} activated). However, in contrast pilocarpine, despite eliciting no significant [³⁵S]-GTPγS binding to Gα_{q/11} subunits, produces [³⁵S]-GTPγS binding to Gα_{i1-3}, which is 50% as efficacious (in terms of E_{max} values) as methacholine. Similarly, oxotremorine, which was comparatively poor at Gα_{q/11} activation, is equally as effective as pilocarpine with respect to Gα_{i1-3} activation.

As evidence has already been presented to suggest that, at least for full agonists, the M₃ mACh receptor expressed in CHO cells is more productively coupled to Gα_{q/11}, the results obtained for pilocarpine and oxotremorine seem paradoxical. If the M₃ mACh receptor signals in 'strength-of-signal' manner, we might expect agonists of low efficacy to couple only to the most productively coupled G-protein subtype. Here we report that partial agonists elicit poor (an in the case of pilocarpine no significant) coupling to Gα_{q/11}, but are approx. 50% as efficacious as full agonists with respect to Gα_{i1-3} activation. This is suggestive of separate agonist-specific states, within which the full agonists appear to stabilize an active conformation of the receptor that is better coupled to Gα_{q/11} and less

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efficiently to G α_i . However, the partial agonists, and in particular pilocarpine, may stabilize a different conformation of the M₃ mACh receptor that is more robustly coupled to G α_i , a concept termed ‘agonist-specific stimulus trafficking’ (**Fig 3.26B**) (Kenakin, 1995b).

The possibility of distinct agonist-specific mACh receptor conformations was suggested by early studies on a pharmacologically unclassified mACh receptor from rat cerebral cortex, which provided some evidence for distinct oxotremorine and acetylcholine signalling conformations (Gurwitz and Sokolovsky, 1985). Akam *et al.* (2001) have presented evidence that pilocarpine appears to stabilize a conformation of the M₃ mACh receptor which favours coupling to G α_{i1-3} in preference to G $\alpha_{q/11}$. In this study pilocarpine elicited greater incorporation of [³⁵S]-GTP γ S to G $\alpha_{i/o}$ than to G $\alpha_{q/11}$ subunits. Interestingly the magnitude of [³⁵S]-GTP γ S binding into G $\alpha_{i/o}$, after activation of the M₃ receptor, was approximately equal for both methacholine and pilocarpine. In the present study we have used a wider range of structurally diverse compounds to show that the M₁ and M₃ mACh receptors appear to signal via separate mechanisms. Agonist-specific receptor conformations have been predicated to exist for a wide-range of GPCRs, including the D_{2S} dopamine receptor (Wiens *et al.*, 1998), A₁ adenosine receptor (Cordeaux *et al.*, 2000), 5-HT₄ receptor (Claeysen *et al.*, 2001), β_2 adrenoceptor (Azzi *et al.*, 2003) and NTS1 neurotensin receptor (Skrydelski *et al.*, 2003). For a recent review, please see Kenakin, 2003.

Whilst many studies have presented convincing data that seem irreconcilable with a single active receptor conformation, the exact reason why different agonists stabilize different conformations and the physiological relevance of this is poorly understood. **Fig. 3.8.** shows the chemical structures of the five agonists used in this study, the three ‘full’ agonists (methacholine, carbachol and oxotremorine-M) possess a tetramethylammonium group that is also present in the natural ligand, acetylcholine. The exact binding sites of ligands at most GPCRs is poorly characterized, however, it has been proposed that the process of receptor activation with respect to the M₁ mACh receptor involves the closure of an aromatic cage (residues found in several transmembrane domains) around the

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tetramethylammonium headgroup (for a recent review please see Hulme *et al.*, 2001, 2003). The ‘tightening’ of residues in the M₁ mACh receptor is predicted to cause local movements in transmembrane domains 6 and 7 relative to TM3, which may lead to receptor activation and exposure of G-protein binding sites on the intracellular receptor surface.

Interestingly, both pilocarpine and oxotremorine do not possess a tetramethylammonium substituent. Although most research into agonist activation of the mACh receptors has attempted to delineate the acetylcholine binding site on the M₁ mACh receptor, it seems reasonable to suggest that ligands that do not possess the tetramethylammonium group may bind to distinct residues to those that do. The structural consequences of this could be to stabilize the active receptor in a distinct conformation, which is favourable for a different G-protein coupling profile to that of the tetramethylammonium-containing full agonists.

Subtle changes in the conformations of the third intracellular loop, and proximal regions of the transmembrane domains 5 and 6, have been demonstrated to selectively impair the efficiency of one G-protein coupled pathway for a dual-coupled GPCR (Wade *et al.*, 1999). Progressive substitution of three arginine residues for alanines in the carboxyl terminal portion of the α_{2A} -adrenoceptor has been shown to lead to a progressive decrease in receptor potency with respect to $G\alpha_s$ -mediated cyclic AMP accumulation, whilst $G\alpha_i$ coupling appeared unaffected. Similar experiments on other basic amino acid residues located closer to the intracellular end of TM6 resulted in reciprocal effects, with normal $G\alpha_s$ coupling but a 50-fold reduction in potency with respect to $G\alpha_i$ coupling (Wade *et al.*, 1999). A single point mutation in the carboxyl-terminal end of the thyrotropin receptor (⁶²³Ala) resulted in a selective loss of the inositol phosphate response, but did not affect cyclic AMP production (Kosugi *et al.*, 1992, 1993). Mutagenesis experiments on the α_{1A} and α_{1B} adrenoceptor (McWhinney *et al.*, 2000) and D₂ dopamine receptor (Senogles *et al.*, 2004) have also implicated subtle changes in the third intracellular loop that result in modulation of one of (at least) two pathways predicted to be affected by the receptor via activation of distinct $G\alpha$ sub-populations. Although it is possible that a

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particular amino acid change directly compromises a crucial receptor-G-protein binding site these results suggest that different conformations of receptor:G-protein contact sites, in particular of the intracellular loops (and by extrapolation the receptor) may lead to the activation of different G α complements (Christopoulos and Kenakin, 2002).

We have therefore presented evidence that the M₁ and M₃ mACh receptor, expressed in CHO cells, may couple to different G α subunits by distinct mechanisms related to the conformations stabilised by the agonists. The agonist-specific-receptor trafficking exhibited by the M₃ mACh receptor may have some basis in the inherent structure of the agonists employed. The challenge is to understand the molecular basis for this differential signalling and its physiological relevance. Differences in the sequence and tertiary structure of the third intracellular loop between the M₁ and M₃ mACh are possible candidates to explain the divergent signalling pathway(s). In order to gain an insight into the processes that regulate the receptor activation and G-protein coupling properties of these two receptors we present data over the next two Chapters that investigate the effect of homologous discrete point mutations in TM6 of both receptors. These mutations are predicted to 'mimic' receptor activation and we have therefore examined the effect of structurally diverse antagonists and agonists on the subsequent increases in levels of downstream effector activity.

Chapter Four.

Creation and characterisation of a constitutively active, mutant M_3 mACh receptor

4.1 Introduction

Mutations that increase the constitutive activity of the muscarinic acetylcholine receptors have been described for amino acids in many regions of the proposed three dimensional structure for most of the mACh subtypes (for a recent review please see Spalding and Burstein, 2001). However, a cluster of residues, of which mutation confers varying degrees of constitutive activity, has been described whose locations are predicted to be located within, or proximal to transmembrane domains 3 and 6.

Spalding *et al.*, (1995) isolated a double mutation (S465Y and T466P) from random mutagenesis of the human M₅ receptor that gave robust constitutive activity in the absence of agonist. Further studies on the single residue, S465, showed that its substitution by 11 structural diverse amino acids conferred a range of constitutive activities (Spalding *et al.*, 1997). The authors concluded that these different residues were unlikely to make similar interactions within the receptor, and therefore the mutation of S465 disrupted local interactions, which were normally responsible for holding the receptor in an inactive conformation. A stabilising role for transmembrane domain 6 was also suggested by a random mutagenesis study on residues within TM 6 of the M₅ receptor, substitutions on one face of the helix was shown to confer agonist-independent activity. Similarly, it was suggested that unrelated, multiple substitutions were unlikely to strengthen specific interactions within the active conformation but more likely to disrupt the receptors ability to maintain its inactive conformation (Spalding *et al.*, 1998). Bluml *et al.*, (1994d) described a point mutation at Asn-507 in the rat M₃ receptor, which is predicted to lie within transmembrane 6, which gave a 2-fold increase in basal inositol phosphate levels.

These observations align with those from other receptors, which suggest that the receptor activation process involves a specific rotation of receptor regions particularly affecting the relative positions of the 3 and 6 transmembrane domains (Farrens *et al.*, 1996; Gether *et al.*, 1997; Javitch *et al.*, 1997). Although the receptor-G-protein contact sites are as yet poorly defined, it has been suggested that the α -helical cytoplasmic extensions of TM5 and TM6 may provide the surface for G-protein interaction (Kristiansen, 2004) and that

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one function of the inactive receptor must be to prevent these activating domains from interacting with the G-protein (Kristiansen, 2004). Therefore, movement of TM3 and TM6 away from each other, during the receptor activation process, may expose previously hidden residues/conformations some of which allow the insertion of the G α -subunit C-terminal into contact regions within the seven-helix bundle (Iiri *et al.*, 1994). In support of this model mutagenesis of the C-terminal end of the third intracellular loop in the of the human M₁ (Hogger *et al.*, 1995), the rat M₃ (Schmidt *et al.*, 2003), and the human M₂ (Liu *et al.*, 1996) mACh receptors have all been shown to cause constitutive activity.

On the basis of these previous studies we decided to create a point mutation in the M₃ mACh receptor at position Asn-514, which is predicted to lie at the junction of transmembrane domain 6 and the third extracellular loop for three reasons:

(1). Convincing data for the M₅ receptor suggest that this residue, although not conserved amongst all the mACh subtypes, may serve as a general switch to disrupt interactions which constrain the inactive state (Spalding *et al.*, 1995)

(2). The extracellular end of TM6 was chosen because although mutagenesis of residues at the intracellular end have been shown to elicit constitutive activity, residues in this region are also important for the direct contact and specificity of G-proteins (Burstein *et al.*, 1998). The interaction between the G α -subunit and the C-terminal end of the third intracellular loop has been documented for the M₂ mACh receptor (Liu *et al.*, 1995b). Therefore, mutations proximal to the intracellular end of TM6 may directly modify G-protein contact points (or even the physiological specificity), whilst revealing little of the actual receptor activation process.

(3). The choice of tyrosine was chosen, as the replacement of Ser-465 (homologous to 514 in the M₃ mACh) in the M₅ mACh receptor by bulky residues elicited the greatest levels of constitutive activity (Spalding *et al.*, 1997).

Experimental objectives

By creating a N514Y mutation of the M₃ mACh receptor (predicted position shown to **Figs. 4.1, 4.2**) we hoped to mimic (at least) in part the receptor activation process, and avoid the potential problems of creating point mutations within regions proposed to be important for G-protein specificity and activation. A range of structurally diverse antagonists was tested to assess their ability to decrease any agonist-independent activity. A wide range of both binding and functional assays have been employed, using antagonists and agonists, to gain insight into the possible conformations of the M₃ mACh receptor involved in different aspects of cell signalling.

4.2 Methods

For details of the methodologies used in this Chapter please refer to **Chapter 2 – Materials and Methods**:

2.1.2.2. Cell adhesion

2.2. Transfection procedures

2.3. Cell membrane preparation

2.4.1.1. [³H]-NMS saturation binding to membrane preparations

2.4.1.2. [³H]-NMS saturation binding to plated whole cells

2.4.1.3. [³H]-NMS saturation binding to plated whole cells pre-treated with antagonists

2.4.2.1. [³H]-NMS displacement binding

2.4.2.2. Effects of GTP on [³H]-NMS displacement binding

2.7. [³H]-inositol phosphate accumulation

2.8. Assessment of receptor phosphorylation

4.3 Data analysis

All data are shown as means $\pm$ S.E.M. for at least three separate experiments performed in duplicate except where indicated.

[³H]-NMS binding for each individual experiment was analysed by non-linear regression using a commercially available programme (GraphPad Prism version 3.0/4.0; GraphPad

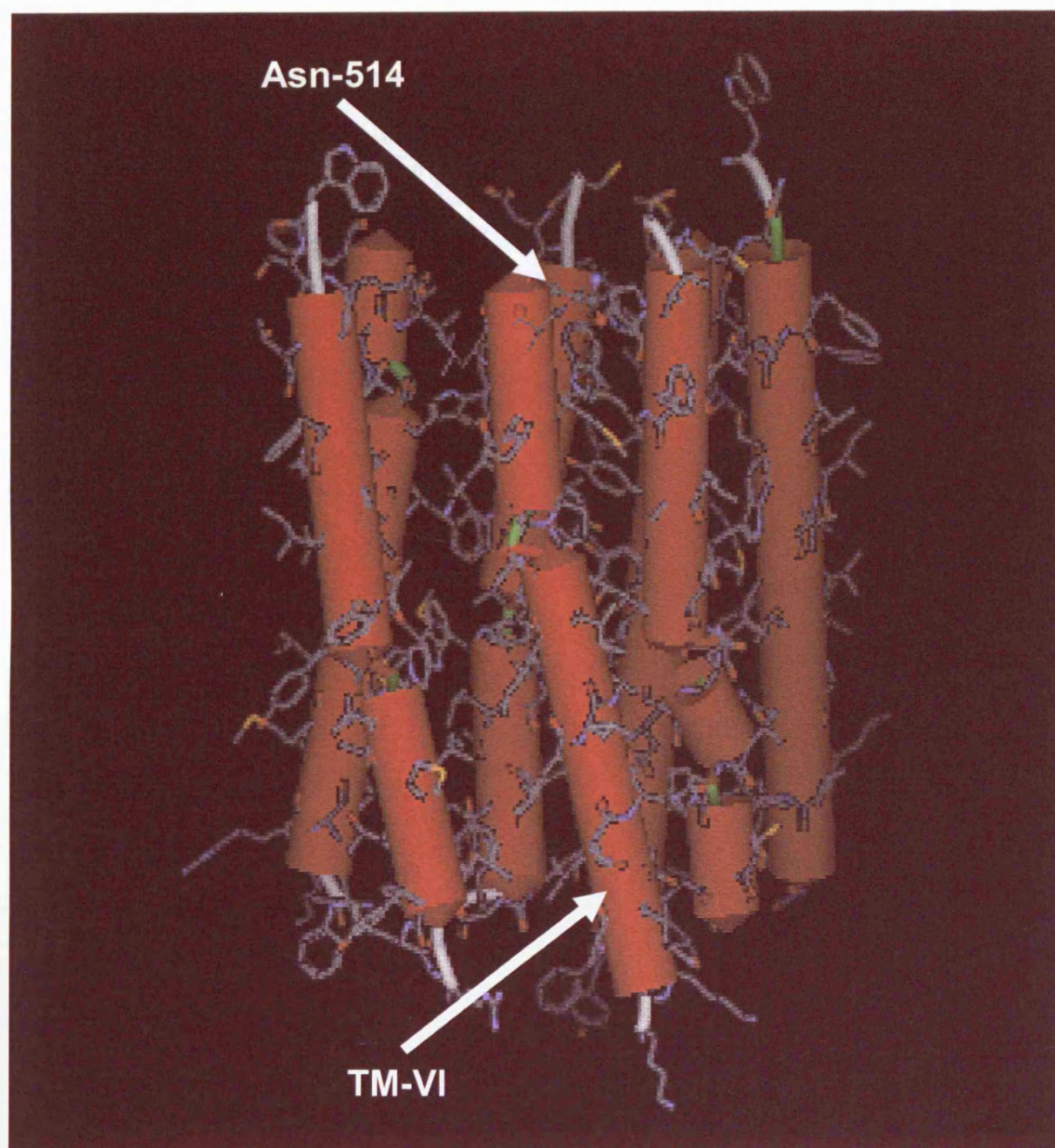


Fig. 4.1. Lateral view of the M_3 mACh receptor showing the proposed location of amino acid residue asparagine-514.

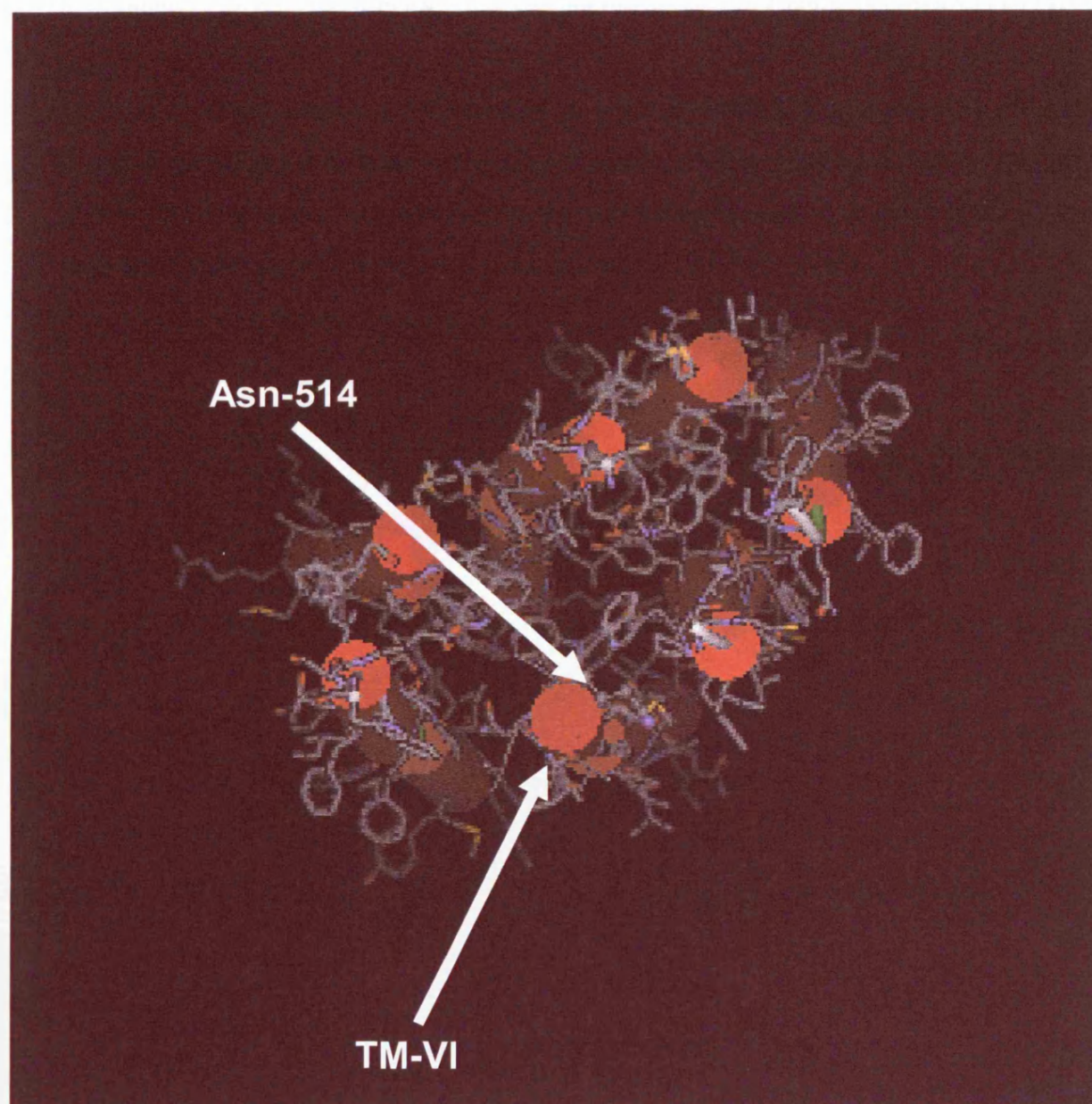


Fig. 4.2. Aerial view of the M_3 mACh receptor showing the proposed location of amino acid residue asparagine-514.

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Software, San Diego, CA, USA) to give B_{max} and K_D estimates. Displacement binding data were fitted to sigmoidal (variable slope) curves using the same analysis package, IC₅₀ values were converted to pK_i values using the method of Cheng and Prusoff (1973).

All concentration response curves data, from either [³H]-InsP_x accumulation or receptor upregulation assays, were fitted using the sigmoidal (variable slope) curve-fitting function of the same programme. All data were compared for a one- and two-site fit and only the most statistically significant value is quoted (*F*-test, GraphPad Prism).

4.4. Results

4.4.1. Part I - Creation of the M₃ mACh mutant receptor

4.4.1.1. Primer design

pcDNA3 encoding the full-length human M₃ mACh receptor encoded within the *Bam* HI and *Eco* RI restriction sites, in the multiple cloning region (MCR) was a kind gift from Dr A.B. Tobin. The mutations were carried out using the QuikChange[®] site-directed mutagenesis kit (Stratagene), using the following designed primers:

Sense (Primer 1)

5'-CCA TAC AAC ATC ATG GTT CTG GT**AC** ACC TTT TGT GAC AGC TGC-3'

Antisense (Primer 2)

5'-GCA GCT GTC ACA AAA GGT GT**AT** CAG AAC CAT GAT GTT GTA TGG-3'

The bases highlighted in **RED** encode the amino acid residue substitution asparagine to tyrosine at position 514 (N514Y). The mutation in **BLUE** encodes a silent mutation to incorporate a unique restriction site for *AccI*. This allows for initial confirmation of successful mutagenesis prior to conformation by dideoxynucleotide sequencing, see **Fig.**

4.3.

4.4.1.2. Mutant strand synthesis (thermal cycling)

Complementary oligonucleotide primers were diluted to a concentration of 125 µg ml⁻¹ in ddH₂O. The following reactions were prepared on ice in thin-walled PCR tubes:

Mutagenesis control reaction:	5 µl	10x reaction buffer
	2 µl	pWhitescript 4.5-kb control plasmid
	1.25 µl	Control primer #1
	1.25 µl	Control primer #2
	1 µl	dNTP mix

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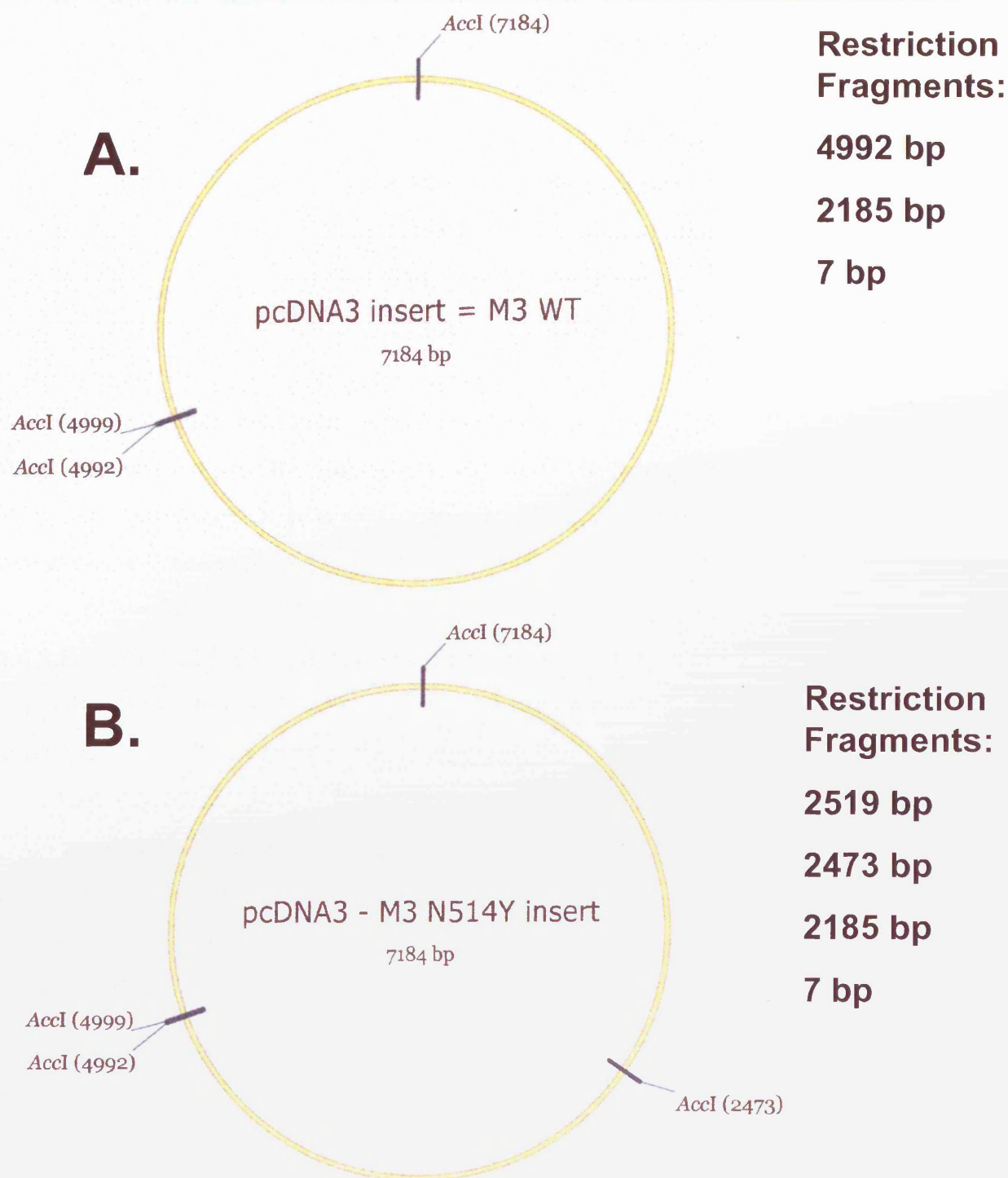


Fig. 4.3. *Acc* I restriction map and digestion fragments

The expected restriction digest maps of *Acc* I on M₃-WT and N^{514Y}M₃ cloned into pcDNA3. With the incorporation of a silent mutation in N^{514Y}M₃ the restriction fragments produced by the digest of *Acc* I on the two plasmids can clearly be differentiated.

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N ^{514Y} M ₃ sample reaction:	39.5 µl	ddH ₂ O
	5 µl	10x reaction buffer
	2 µl	pcDNA
		+ M ₃ - WT template (25 µmol ml ⁻¹)
	1.25 µl	Sense primer
	1.25 µl	Anti-sense primer
	1 µl	dNTP mix
	39.5 µl	ddH ₂ O

Each sample mixture was gently mixed and then 1 µl of pfu-Turbo DNA polymerase added. The samples were then subjected to the following thermal cycle: 1 cycle 30 sec at 95°C; and 16 cycles of 30 sec at 95°C, 1 min at 55°C, and 7.5 min at 68°C. Samples were then held at 4°C before proceeding.

4.4.1.3. *Dpn* I digestion of the PCR products

The pcDNA3 parental DNA plasmid was propagated from a strain of *E. coli*. Almost all strains of *E. coli* bacteria used for propagating DNA contain two site-specific DNA methylases (QuikChange® handbook). One of these, DAM methylase will add a methyl group to adenosine within the specific sequence **GATC**; this includes the foreign DNA transformed into the bacteria. The restriction enzyme *Dpn* I specifically recognises and digests DNA which is DAM-methylated and is therefore used to digest the parental (non-mutated) DNA (the mutated complementary strands will be DAM-methyl-deficient). Therefore, 1 µl of *Dpn* I restriction enzyme was added to the control and sample tubes, gently mixed and left to digest for 1 h at 37°C.

4.4.1.4. Transformation of XL1-Blue super-competent cells

One hundred µl of XL-Blue super-competent cells were thawed on ice and transferred into 1.5 ml Eppendorf tubes in 50 µl aliquots, to which 1 µl of the control or sample PCR reactions was added. Reaction mixtures were gently mixed and left on ice for 30 min before heat-shocking at 42°C for 2 min. Five hundred µl of pre-warmed (37°C) S.O.C media (1 L ddH₂O containing 20 g bacto-tryptone, 5 g bacto-yeast extract, 2 mL 5 M

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NaCl, 2.5 mL 1 M KCl sterilized by autoclaving) was added to each tube, which was subsequently shaken at 250 r.p.m. for 1 h at 37°C.

4.4.1.5. Plate preparation

Plates for screening the bacterial colonies were prepared by autoclaving 1 L of LB agar, and when cool to the touch filter-sterilized ampicillin was added to a final concentration of 100 µg ml⁻¹ (pcDNA3 contains an ampicillin-resistant marker and its inclusion should therefore aid selection for successful transformed bacteria). Approx. 25 ml of LB Agar was poured into 100-mm plates which, when cool were kept at 4°C until required.

4.4.1.6. Colony screening

The 4.5-kb control plasmid contains a stop codon at a position within a β-galactosidase gene, the control primers create a point mutation to revert this stop codon back to the wild-type sequence. Therefore, recombinant plasmids, which have incorporated the mutation, will contain the functional β-galactosidase gene and appear blue on plates containing IPTG and X-gal. This control serves as an indication as to how successful the mutagenesis control reactions were, if a high proportion of the colonies on the control plate are blue, unsuccessful mutagenesis in the samples may then be traced to some other cause than the experimental process itself (e.g. primer design).

For the control reaction, 100 µl of 10 mM IPTG and 100 µl of 2% X-gal was spread onto plates 30 min prior to bacterial plating. For the control 250 µl of the bacterial suspension was plated whilst 500 µl of the sample (entire volume) was spread. Plates were left for 30 min to absorb the fluid before inverting and incubating at 37°C for at least 16 h. **Fig. 4.4** shows the colony plates after 20 h incubation, indicating a mutagenesis efficiency (for the control reaction) of almost 100%.

4.4.1.7. Small-scale preparation of DNA

Twenty colonies were screened for assessment of successful mutagenesis, prior to sequencing, by small-scale DNA preparation and subsequent restriction enzyme fragment analysis. Twenty well-separated colonies were picked, using aseptic technique, and

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A.

A. Untransformed bacteria

B.

**B. pWhitescript 4.5kB
mutagenesis control**

Fig. 4.4. Colony growth after 16 h incubation

A. - untransformed bacteria (negative control) indicating all growth on subsequent plates was due to successful transformation with ampicillin-resistance plasmids. **B.** - mutagenesis plates, blue colonies indicate successful control mutagenesis.

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propagated in 3 ml of LB-Amp media and incubated at 37°C for 16 h. Miniprep DNA was isolated using SpinPrep™ plasmid kits as recommended by the manufacturer. Briefly, cells (from 1.5 ml culture) were collected by centrifugation and thoroughly re-suspended in 100 µl SpinPrep re-suspension buffer. Cell lysis was then completed with the addition of 200 µl lysis buffer and gently inverting the samples. To neutralize the solution, 400 µl of SpinPrep neutralization buffer was added and again gently inverted. Bacterial genomic DNA was separated by centrifugation and 600 µl of the supernatant was removed into SpinPrep columns. The columns were then centrifuged at 12,000 xg in a microcentrifuge for 30 sec and the flow-through discarded. The columns were washed with 650 µl SpinPrep wash buffer to remove salt and protein and centrifuged as before. The spin column was transferred to fresh collection tube and the DNA eluted with 50 µl pre-warmed elution buffer, followed by centrifugation for 1 min.

4.4.1.8. Restriction digest

The incorporation of the silent mutation into the ^{N514Y}M₃ mACh receptor gene produces a different restriction fragment pattern compared to the wild-type M₃-mACh receptor when digested with *Acc* I. Therefore, restriction digest followed by DNA gel electrophoresis allows for an initial assessment of successful mutagenesis clones before large scale-DNA preparation and sequencing. Restriction digest were carried out using the recipes below followed by incubation at 37°C for 2 h.

	M ₃ -WT	^{N514Y} M ₃
10 x supplied buffer	2 µl	2 µl
<i>Acc</i> I enzyme	1 µl	1 µl
DNA	1 µl	10 µl
ddH ₂ O	16 µl	7 µl

4.4.1.9. Gel electrophoresis

DNA gels were cast from 1.5% (w/v) agarose in 1xTAE buffer containing 0.5 µg ml⁻¹ ethidium bromide to detect the DNA fragments. To each sample 5x DNA loading buffer

was added and the sample pipetted into the wells formed by an eight-tooth comb. Gels were run at constant voltage at 110 mV for 1-2 h and periodically inspected for mobility. DNA was visualized under UV, and photographs taken with an instant Polaroid film. Sizes of bands were estimated by comparison to markers of known molecular size.

Fig. 4.5 shows a selection of digested clones, the wild-type DNA has a characteristic fragment pattern of bands at approx. 5,000 bp and 2,000 bp. Clones indicating successful mutagenesis show fragmentation patterns which lose the 5,000 bp fragment with the gain of two bands at approx. 2,500 bp. This band appears bright due to the inability to resolve these into separate bands. On the basis of these digested clones 11, 13 and 19 were used for large scale DNA preparation. The lower band, still present in m3-WT DNA, at 2,000 bp is still visible in the ^{N514Y}M₃ mACh receptor digested DNA.

4.4.1.10. Large-scale preparation of DNA

Clones 9, 14 and 19, with the correct restriction digest fragments, were chosen for large scale DNA preparation. The residual bacterial culture, from the small-scale DNA preparation, was aseptically poured into a 500 ml conical flask containing 100 ml LB-Amp and shaken at 300 r.p.m. (37°C, 16 h). Prior to harvesting glycerol stocks were made to allow long-term storage (-80°C) of bacterial cultures by the addition of an equal volume of warmed glycerol to 0.75 ml bacterial culture. Cells were harvested by centrifugation at 5,000 xg for 10 min and plasmid DNA isolated with Mobius™ 1000 plasmid kit. Briefly, the bacterial pellet was re-suspended in 8 ml of bacterial re-suspension buffer with repeated pipetting until all visible clumps were dispersed. Eight ml of bacterial lysis buffer was added and cell lysis completed by gently inversion of tubes. After a 5 min incubation the solution was neutralized with 8 ml pre-chilled Mobius neutralization buffer and inverted gently. After a 5 min ice-cold incubation the flocculent material was separated by centrifugation at 10,000 xg for 2 min. The cleared supernatant was further clarified by centrifugation through a ClearSpin Filter at 2000 xg for 3 min. The clarified supernatant was transferred to a pre-equilibrated Mobius 1000 column to bind the DNA. After the supernatant had passed through by gravity the DNA was washed to remove impurities with 20 ml of Mobius wash buffer. The DNA was eluted in 5 ml of

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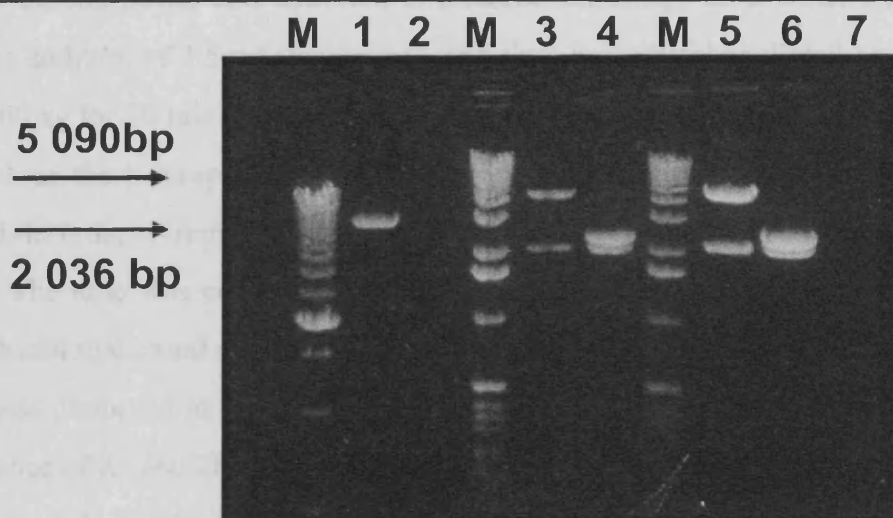


Fig. 4.5. Restriction enzyme analysis of DNA mini-prep clones

Restriction enzyme analysis, using *Acc* I, of $m3$ -N514Y receptor clones 9-14 (lanes 1-2, 3 and 5-7, respectively) and M_3 -WT receptor (lane 3). An Invitrogen 1 kb DNA ladder was used. Lanes 4 and 6 indicate $N^{514Y}M_3$ clones which have successfully incorporated the base-pair change due to the presence of a dense band at approx. 2500 bp (unresolved fragments at 2519 and 2473 bp) and another band immediately beneath (indicating the 2185 bp fragment). Digestion of the M_3 -WT produced the expected bands at approx. 5 and 2 kb).

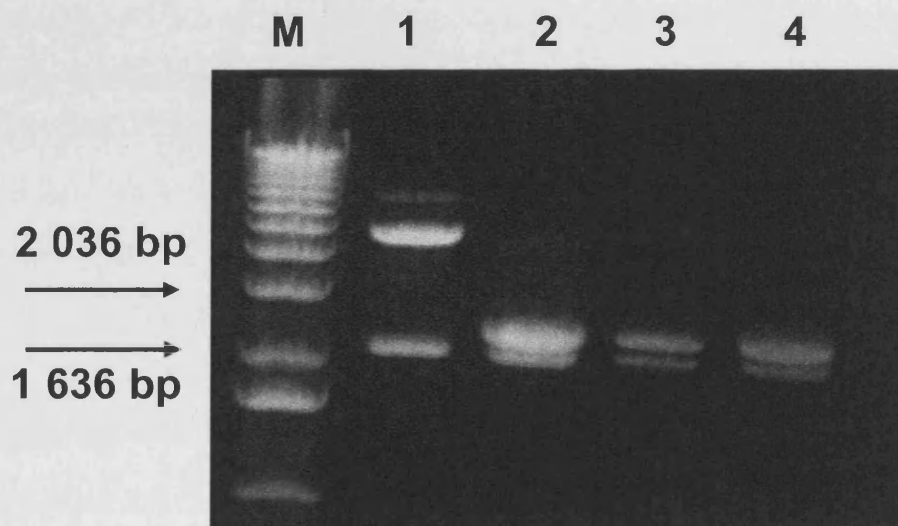


Fig. 4.6. Restriction enzyme analysis of DNA maxi-prep clones

Restriction enzyme analysis, using *Acc* I, of M_3 -WT (lane 1) and $N^{514Y}M_3$ mACh receptor clones 11, 13 and 19 (lanes 2-4, respectively). Bands of the expected sizes were produced by all the digested reactions as indicated on the mini-prep digestion above in **Fig. 4.6**.

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Mobius elution buffer and collected in a sterile centrifuge tube. DNA was precipitated with the addition of 3.5 ml isopropanol, and then immediately pelleted by centrifugation at 15,000 xg for 20 min. The position of the pellet was marked on the side of the tube and as much as the isopropanol was removed without disturbing the pellet. The pellet was washed, in order to remove remaining isopropanol with 3 ml of 70% ethanol and gently mixed. The tube was centrifuged at 15,000 xg for 30 min to collect the washed pellet. Any ethanol that could not safely be removed was allowed to evaporate by air. The DNA pellet was dissolved in 500 μl DNAase-free ddH_2O and its concentration determined by absorbance of $\lambda=260/280$ nm using a spectrophotometer. All DNA collected was diluted to 0.75 $mg\ ml^{-1}$ in ddH_2O .

Fig. 4.6. shows the *Acc* I digest fragments performed as above, except only using 2 μl of the clonal DNA. Prior to experimentation successful mutagenesis was confirmed by sequencing.

4.4.2. Part II - Pharmacological characterization of the constitutively active, mutant M₃ mACh receptor

4.4.2.1 Characterization of the M₃-WT and ^{N514Y}M₃ mACh receptors transiently transfected into HEK 293 cells: binding studies

4.4.2.1.1. Expression levels and [³H]-NMS affinity

HEK293 cells were selected as they express a small endogenous M₃ mACh receptor population and therefore may be more physiologically-relevant than other immortalized cell-lines. Additionally, they generally display high transfection efficiency levels. Initial experiments on whole cells revealed that the expression level of the ^{N514Y}M₃ mACh receptor was consistently 2.5 fold less than M₃-WT when equivalent amounts of cDNA were transfected per cell. When appropriate, this difference was corrected for in experiments allowing a relatively accurate matching of mutant/WT receptor densities.

The HEK-cell line is reported to express a small endogenous M₃ population. At saturating concentrations of [³H]-NMS (>1nM), untransfected HEK293 cells elicited a minor level of specific binding. However the signal was not sufficiently robust to produce full saturation curves and therefore the endogenous population is only estimated to be approximately 50 fmol mg⁻¹ protein. The concentrations of [³H]-NMS employed in displacement binding studies elicited no significant binding above non-specific in untransfected HEK-cells. However to ensure the endogenous population did not significantly change with passage, two controls were always utilized in binding experiments: untransfected and empty-vector-transfected cells.

Constitutively active receptors have been reported to display marked differences in the affinity of antagonists and agonists when compared to wild-type receptors (Samama *et al.*, 1993; Rossier *et al.*, 1999; Wade *et al.*, 2001). [³H]-NMS saturation binding experiments were used to assess the affinity of NMS in both the wild-type and mutated receptor. **Fig. 4.7A, B** shows representative saturation binding curves for the M₃-WT and

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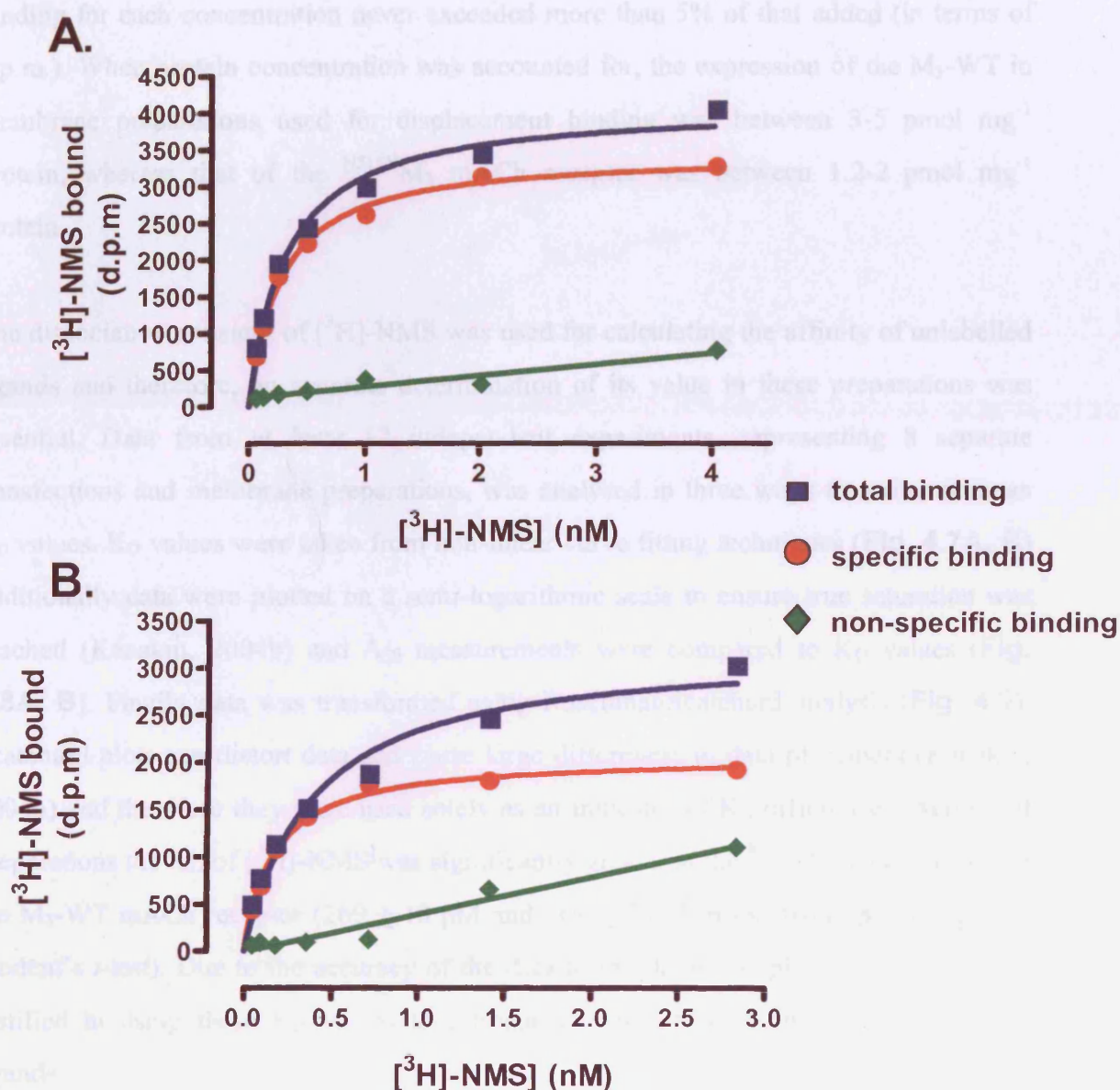


Fig. 4.7. [3 H]-NMS binding to wild-type and N514Y-mutant M_3 mACh receptors in HEK-cell membrane preparations

Membranes were prepared from HEK-cells transiently transfected with **A.** M_3 -WT and **B.** $N514Y$ - M_3 receptor cDNA, and incubated in the presence or absence of atropine for 60 min. All experiments were performed in duplicate and the graphs are representative of 12-14 separate experiments. Receptor densities and dissociation constants for NMS are summarized in the main text (see 4.3.1).

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^{N514Y}M₃ mACh receptors. Membrane concentration was adjusted such that maximal binding for each concentration never exceeded more than 5% of that added (in terms of d.p.m.). When protein concentration was accounted for, the expression of the M₃-WT in membrane preparations used for displacement binding was between 3-5 pmol mg⁻¹ protein, whereas that of the ^{N514Y}M₃ mACh receptor was between 1.2-2 pmol mg⁻¹ protein.

The dissociation constant of [³H]-NMS was used for calculating the affinity of unlabelled ligands and therefore, an accurate determination of its value in these preparations was essential. Data from at least 12 independent experiments, representing 8 separate transfections and membrane preparations, was analysed in three ways to arrive at mean K_D values. K_D values were taken from non-linear curve fitting techniques (**Fig. 4.7A, B**) additionally data were plotted on a semi-logarithmic scale to ensure true saturation was reached (Kenakin, 2004b) and A₅₀ measurements were compared to K_D values (**Fig. 4.8A, B**). Finally data was transformed using Rosenthal/Scatchard analysis (**Fig. 4.9**). Scatchard plots can distort data and cause large differences in data placement (Kenakin, 2004b) and therefore they were used solely as an indicator of K_D differences. Across all preparations the K_D of [³H]-NMS was significantly greater at the ^{N514Y}M₃ receptor than at the M₃-WT mACh receptor (269 ± 10 pM and 169 ± 7 pM, respectively *p*<0.05; paired Student's *t*-test). Due to the accuracy of the data from a large sample population we felt justified in using these K_D values to estimate the affinities of other mACh receptor ligands.

4.4.2.1.2. Affinity of mACh receptor antagonists

Four antagonists were used throughout this study based on their diverse structures and M₃ mACh receptor affinities. **Fig. 4.10.** shows the structures and pK_B values for the antagonists, with respect to the M₃ mACh receptor, atropine is non-selective, whereas pirenzepine, methoctramine and 4-DAMP show some selectivity for the M₁, M₂ and M₃ mACh receptors, respectively. All of the values obtained in experiments were compared to atropine, which was employed here as a 'reference' antagonist.

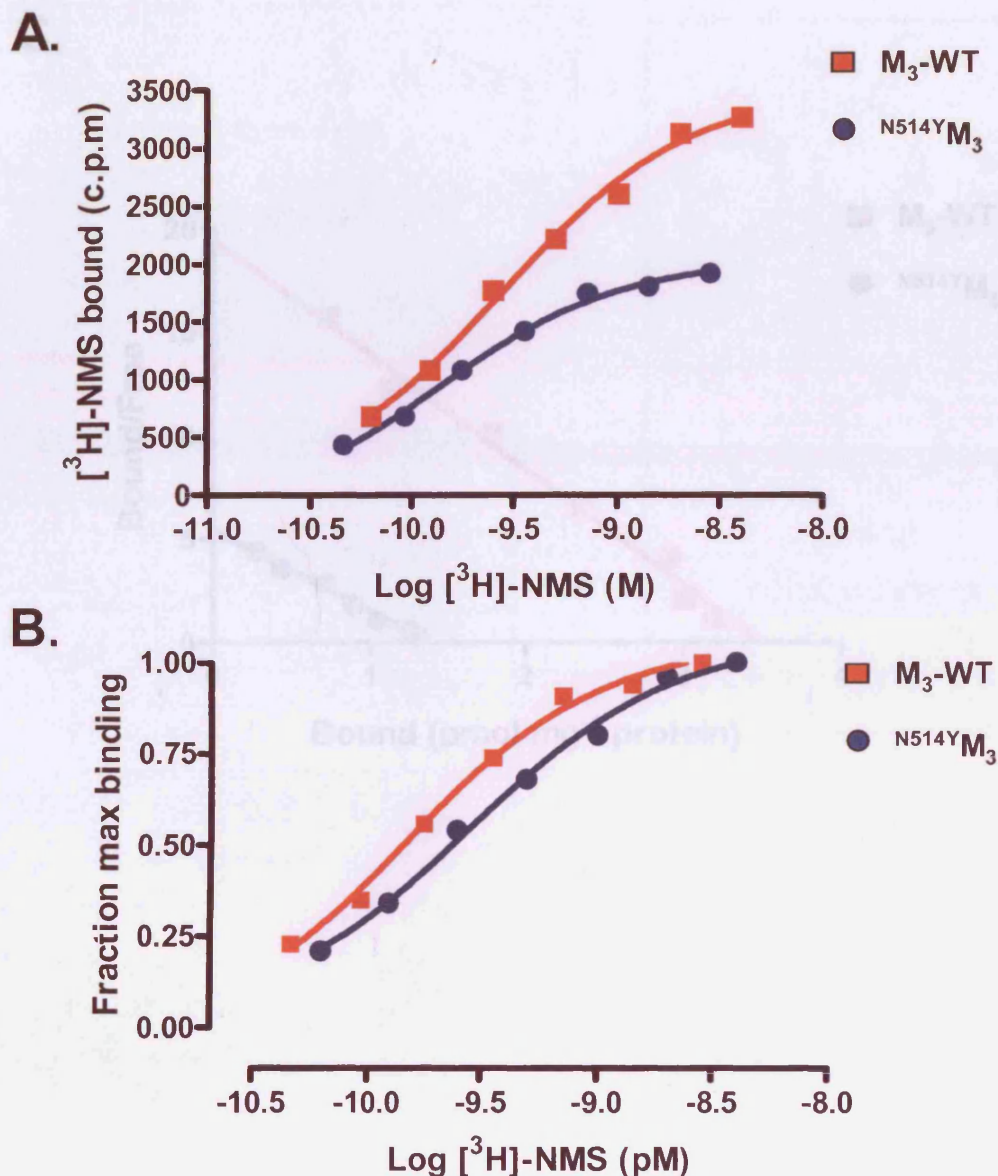


Fig. 4.8. $[^3\text{H}]\text{-NMS}$ binding to wild-type and N514Y-mutant M_3 mACh receptors in HEK-cell membrane preparations

Membranes were prepared from HEK-cells transiently transfected with **A.** $M_3\text{-WT}$ and **B.** $N514Y M_3$ receptor cDNA, and incubated in the presence or absence of atropine for 60 min. Data obtained have been transformed into **A.** A log-concentration plot, and **B.** a fraction of the maximal binding to display true saturation binding. Data shown are a representative of at least 12 separate experiments performed in duplicate.

C.

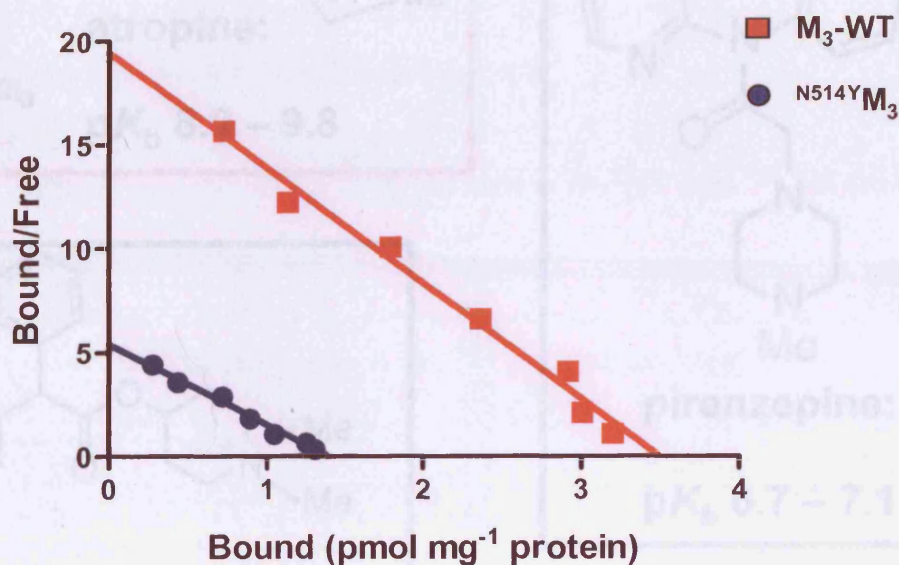


Fig. 4.9. [³H]-NMS binding to wild-type and N514Y-mutant M_3 mACh receptors in HEK-cell membrane preparations

Membranes were prepared from HEK-cells transiently transfected with **A.** M_3 -WT and **B.** $N514Y M_3$ receptor cDNA, and incubated in the presence or absence of atropine for 60 min. Data obtained have been transformed into a Scatchard plot to demonstrate the different affinity (K_D) of [³H]-NMS for the two receptor forms. Data shown are representative of at least 12 separate experiments performed in duplicate

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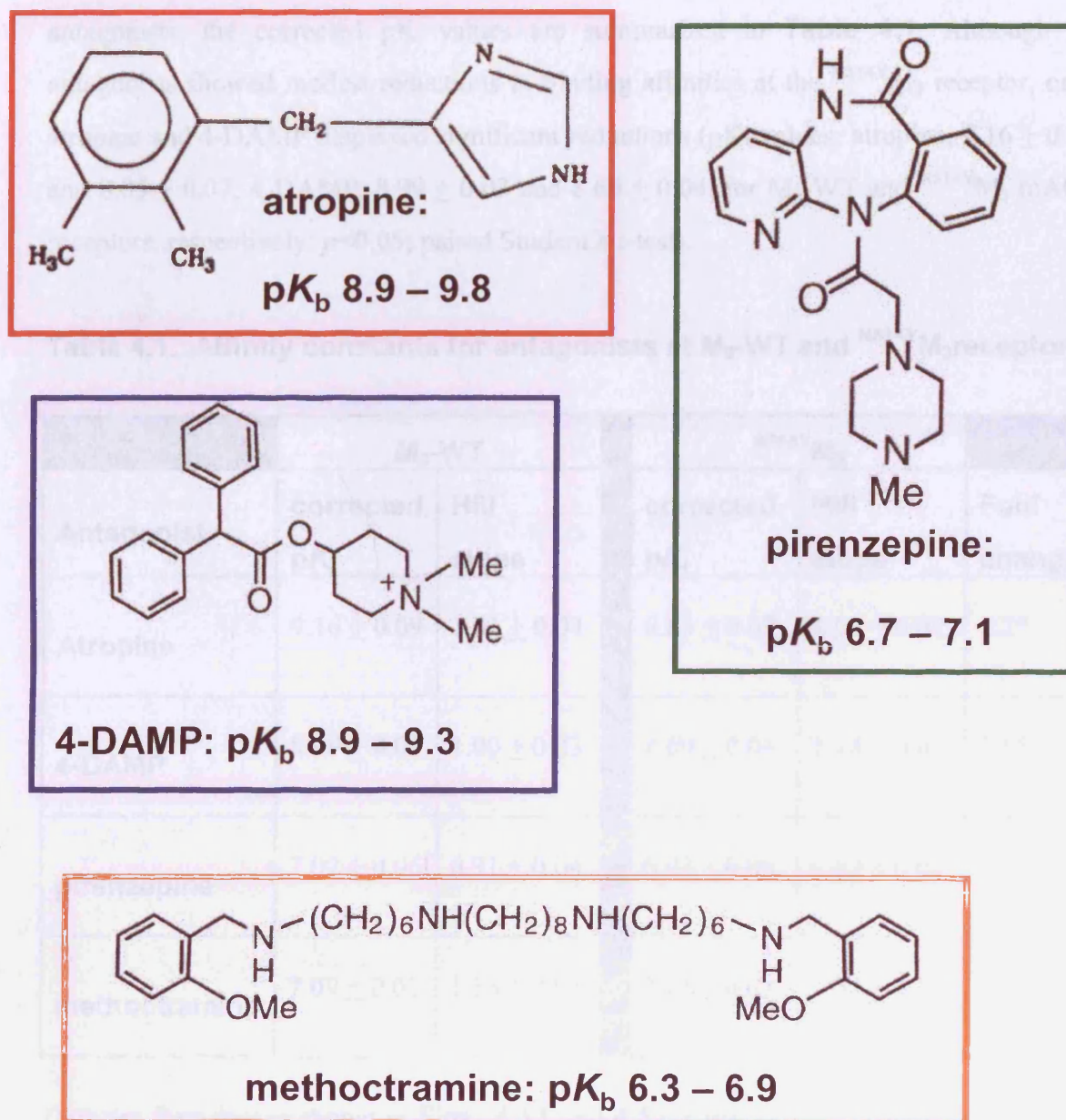


Fig. 4.10. Structure and pK_b values for the four antagonists used throughout the study

(pK_b values taken from Caulfield and Birdsall (1998) and represent estimates taken from experiments performed using a number of mammalian species.)

Figs. 4.11. and **4.12.** illustrate [³H]-NMS displacement curves for the different antagonists: the corrected pK_i values are summarized in **Table 4.1**. Although all antagonists showed modest reductions in binding affinities at the ^{N514Y}M₃ receptor, only atropine and 4-DAMP displayed significant reductions (pK_i values: atropine, 9.16 ± 0.09 and 8.85 ± 0.07; 4-DAMP, 8.99 ± 0.03 and 8.60 ± 0.04; for M₃-WT and ^{N514Y}M₃ mACh receptors, respectively: *p* < 0.05; paired Student's *t*-test).

Table 4.1. Affinity constants for antagonists at M₃-WT and ^{N514Y}M₃ receptors

	M ₃ -WT		^{N514Y} M ₃		
Antagonist	corrected pK _i	Hill slope	corrected pK _i	Hill slope	Fold change
Atropine	9.16 ± 0.09	1.01 ± 0.04	8.85 ± 0.07	1.02 ± 0.02	2.2*
4-DAMP	8.99 ± 0.03	1.00 ± 0.03	8.60 ± 0.04	1.14 ± 0.05	2.5*
pirenzepine	7.02 ± 0.06	0.91 ± 0.04	6.93 ± 0.06	0.89 ± 0.04	1.2
methoctramine	7.09 ± 0.01	1.56 ± 0.05	7.05 ± 0.03	1.62 ± 0.10	1.1

Data are from assays shown in **Figs. 4.11** and **4.12**. Corrected IC₅₀ values (pK_i values) were calculated according to the method of Cheng and Prusoff, (1973), using the appropriate [³H]-NMS K_D value. Fold changes represent the lower/higher affinity of the ^{N514Y}M₃ receptor (obtained by dividing the pK_i of mutated receptor by the pK_i of the wild-type receptor for antagonists). Data represent the mean ± S.E.M. for at least three experiments. *indicates ^{N514Y}M₃ corrected affinity is statistically different to that obtained for the M₃-WT receptor (*p* < 0.05; unpaired Student's *t*-test).

All pK_i values obtained for antagonists at the wild-type M₃ mACh receptor are in good agreement with those published in the literature (Caulfield and Birdsall, 1998). All slope

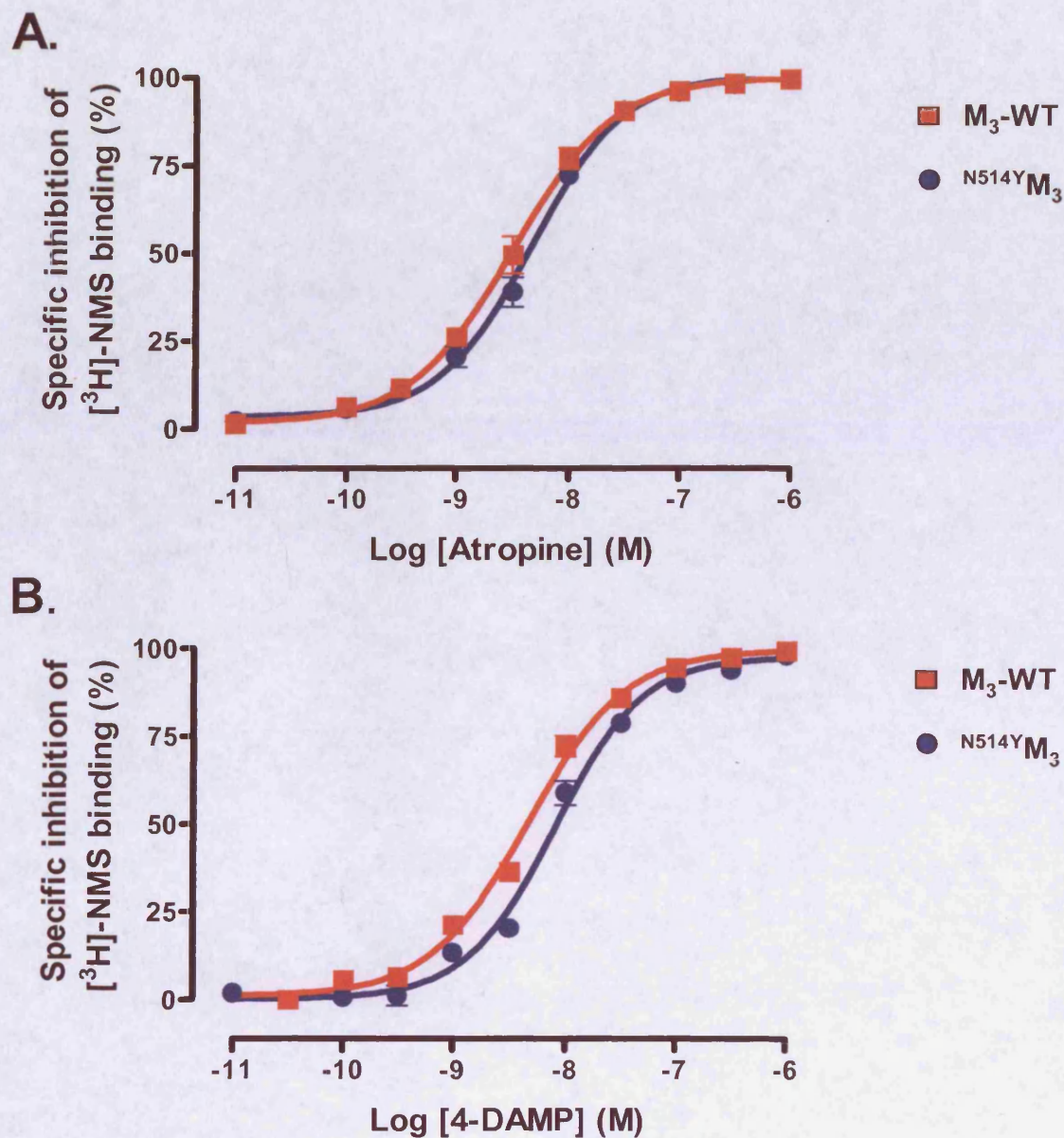


Fig. 4.11. Antagonist displacement of $[^3H]$ -NMS binding to HEK cell membranes from transiently transfected cells

Inhibition binding experiments on M_3 -WT (■) or $N514Y M_3$ (●) mACh receptors, performed on membrane preparations obtained from transiently transfected HEK-293 cells, in the presence of 300-500 pM $[^3H]$ -NMS. Data shown represent the means $\pm$ S.E.M from at least three experiments performed in duplicate. Summary data for the antagonists are shown in Table 4.1.

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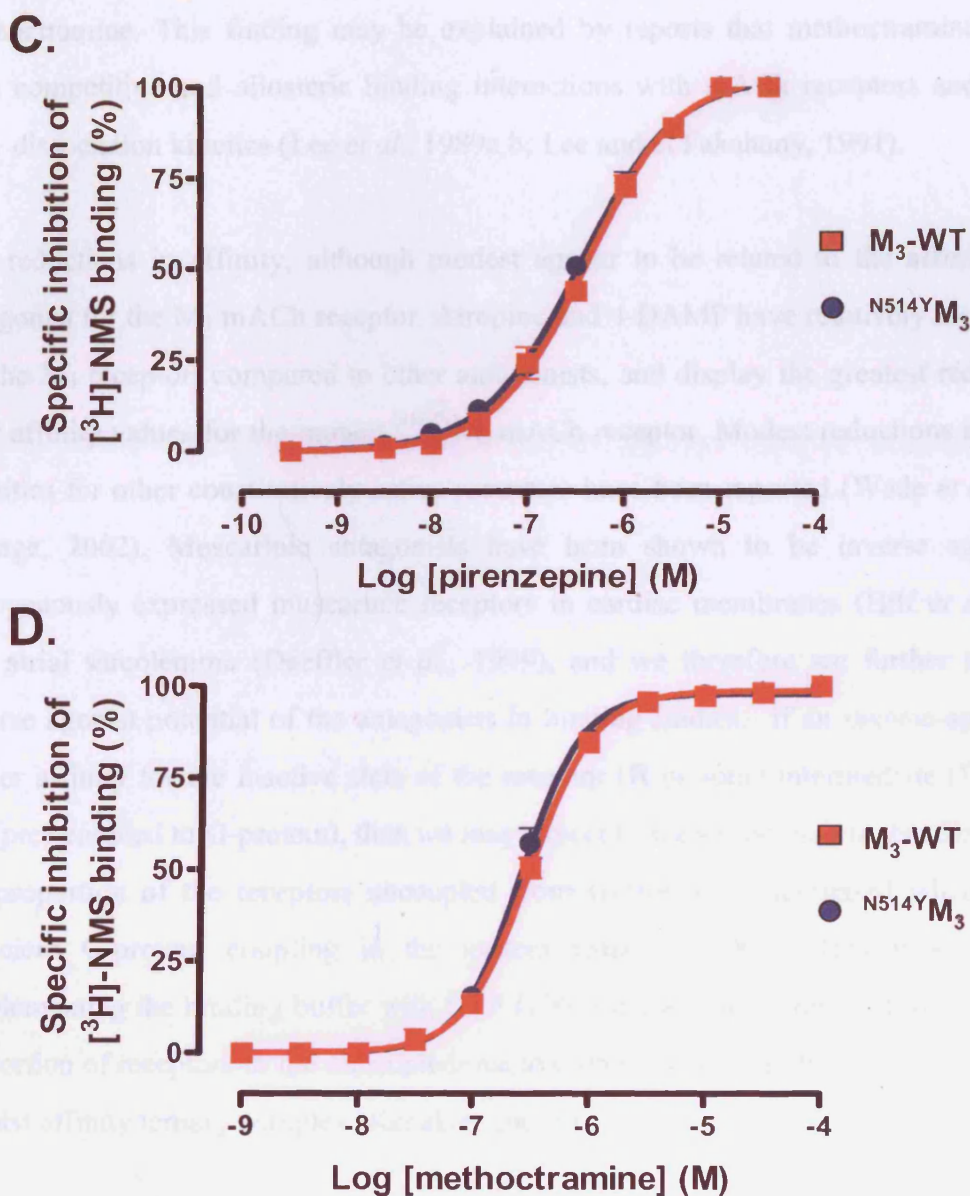


Fig. 4.12. Antagonist displacement of $[^3\text{H}]\text{-NMS}$ binding in HEK-cell membranes from transiently transfected cells

Inhibition binding experiments on $M_3\text{-WT}$ (■) or $N514Y M_3$ (●) receptors, performed on membrane preparations obtained from transiently transfected HEK-293 cells, in the presence of 300-500 pM $[^3\text{H}]\text{-NMS}$. Data shown represent the mean $\pm$ S.E.M from at least three experiments performed in duplicate. Summary data for the antagonists are shown in Table 4.1.

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factors were not significantly different from unity, apart from those obtained for methoctramine. This finding may be explained by reports that methoctramine exhibits both competitive and allosteric binding interactions with mACh receptors and exhibits slow dissociation kinetics (Lee *et al.*, 1989a,b; Lee and el Fakahany, 1991).

The reductions in affinity, although modest appear to be related to the affinity of the antagonist for the M₃ mACh receptor. Atropine and 4-DAMP have relatively high affinity for the M₃ receptor, compared to other antagonists, and display the greatest reduction in their affinity values for the mutant^{N514Y} M₃ mACh receptor. Modest reductions in binding affinities for other constitutively active receptors have been reported (Wade *et al.*, 2001; Strange, 2002). Muscarinic antagonists have been shown to be inverse agonists at endogenously expressed muscarinic receptors in cardiac membranes (Hilf *et al.*, 1992) and atrial sarcolemma (Daeffler *et al.*, 1999), and we therefore we further tested the inverse agonist potential of the antagonists in binding studies. If an inverse-agonist has higher affinity for the inactive state of the receptor (**R** or some intermediate (**R'**) that is not (pre)-coupled to G-protein), then we may expect to see an increase in its affinity when the proportion of the receptors uncoupled from G-proteins is increased when there is sufficient G-protein coupling in the system (Strange, 2002). This was tested by supplementing the binding buffer with GTP (100 μ M), which is proposed to increase the proportion of receptors in the uncoupled/inactive state by causing dissolution of the high-agonist affinity ternary complex (Kenakin, 2004b).

Fig. 4.13. shows the effect of the inclusion of GTP (100 μ M) on the displacement of [³H]-NMS by atropine. Under the binding conditions employed GTP had no significant effect on the pIC₅₀ values derived from the atropine displacements curves (M₃-WT, 8.72 $\pm$ 0.02 and 8.67 $\pm$ 0.02; ^{N514Y}M₃, 8.57 $\pm$ 0.03 and 8.59 $\pm$ 0.04 in the absence and presence of GTP, respectively, $p > 0.05$; unpaired Student's *t*-test). Similarly, no significant differences were seen for the other three antagonists tested ($p > 0.05$; unpaired Student's *t*-test) (data not shown). Given the small reduction in binding affinity with the ^{N514Y}M₃ mACh receptor for some antagonists, the absence of a GTP-shift is disappointing, though not wholly surprising. Alternatively, the conformation of the ^{N514Y}M₃ receptor stabilised

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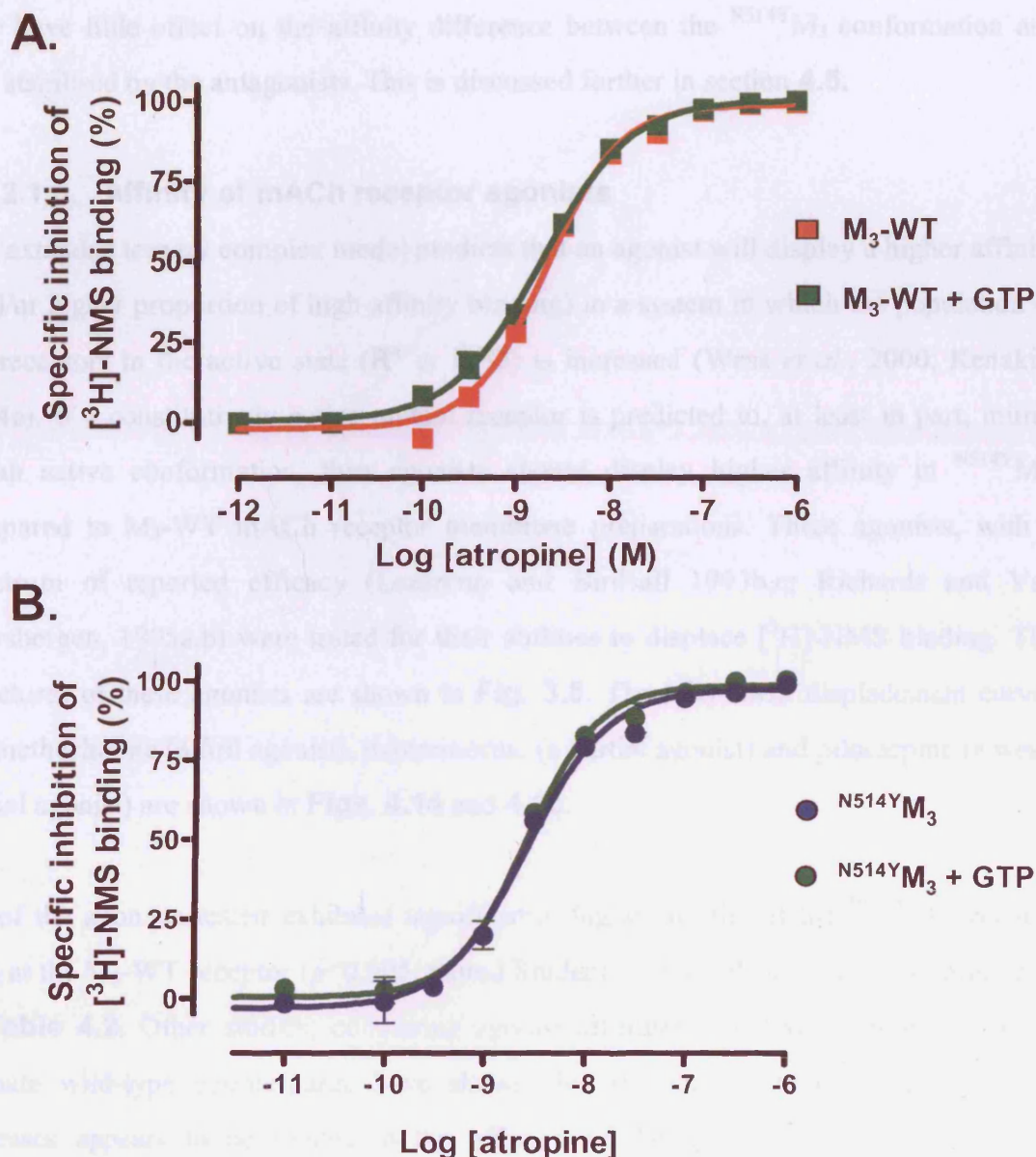


Fig. 4.13. Effect of GTP on antagonist displacement of $[^3H]$ -NMS binding in transiently transfected HEK-cell membranes

Inhibition binding experiments on M_3 -WT (■) or $N514Y M_3$ (●) mACh receptors, performed in the absence or presence (■ / ●) of 100 μM GTP, on membrane preparations obtained from transiently transfected HEK-293 cells, in the presence of 300-500pM $[^3H]$ -NMS. Data shown represent the mean $\pm$ S.E.M from at least three experiments performed in duplicate.

Summary data for the antagonists are shown in **Table 4.1.**

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by the inverse agonists need not equate to the ground state of the M₃-WT receptor. GTP may have little effect on the affinity difference between the ^{N514Y}M₃ conformation and that stabilised by the antagonists. This is discussed further in section 4.5.

4.4.2.1.3. Affinity of mACh receptor agonists

The extended ternary complex model predicts that an agonist will display a higher affinity (and/or higher proportion of high affinity binding) in a system in which the population of the receptors in the active state (**R*** or **R*G**) is increased (Wess *et al.*, 2000; Kenakin, 2004c). If a constitutively active mutant receptor is predicted to, at least in part, mimic the/an active conformation, then agonists should display higher affinity in ^{N514Y}M₃, compared to M₃-WT mACh receptor membrane preparations. Three agonists, with a spectrum of reported efficacy (Lazareno and Birdsall 1993b,c; Richards and Van Giersbergen, 1995a,b) were tested for their abilities to displace [³H]-NMS binding. The structures of these agonists are shown in **Fig. 3.8**. The [³H]-NMS displacement curves for methacholine (a full agonist), oxotremorine (a partial agonist) and pilocarpine (a weak partial agonist) are shown in **Figs. 4.14** and **4.15**.

All of the agonists tested exhibited significantly higher affinity at the ^{N514Y}M₃ receptor than at the M₃-WT receptor ($p < 0.005$, paired Student's *t*-test); these data are summarized in **Table 4.2**. Other studies, comparing agonist affinities at CAM receptors and their cognate wild-type counterparts, have shown that the magnitude of agonist affinity increases appears to be related to the efficacy of the agonist in functional studies (Samama *et al.*, 1993). Affinity increases for methacholine and pilocarpine appear to exhibit this relationship as methacholine exhibited a greater increase (13 fold) compared to pilocarpine (5 fold). On the basis of this it would be predicted that oxotremorine would exhibit an intermediate affinity increase (having a reported intermediate efficacy). However, despite having a significantly higher affinity at the ^{N514Y}M₃ receptor the increase was only 2.4 fold, significantly less than that seen for pilocarpine. Interestingly, oxotremorine was the only agonist to show a reduced slope factor at the ^{N514Y}M₃ mACh receptor (nH, 0.76 ± 0.08 versus 0.57 ± 0.02 for M₃-WT and ^{N514Y}M₃ mACh receptors, respectively; $p < 0.05$, paired Student's *t*-test). It appears that, unlike other constitutively

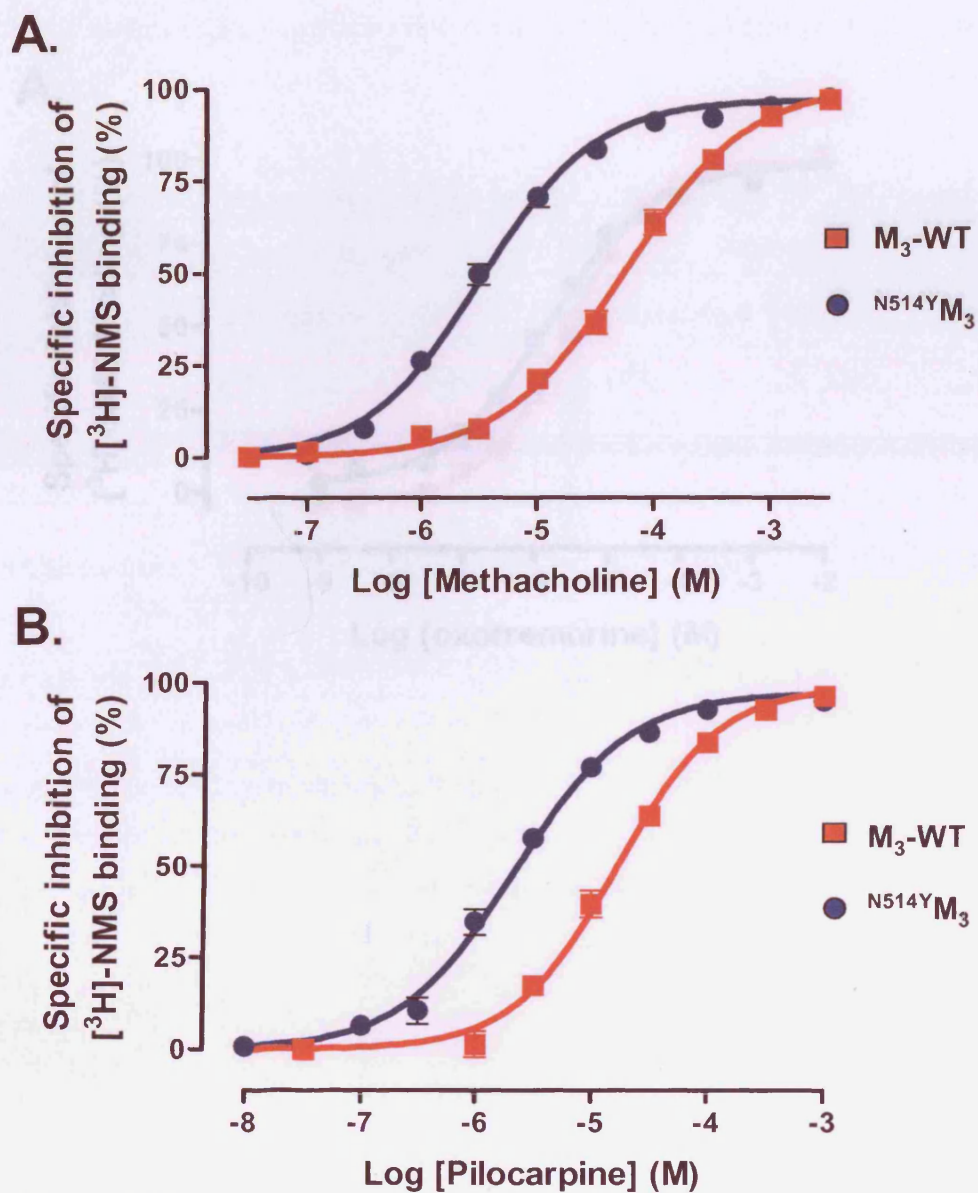


Fig. 4.14. Agonist displacement of $[^3\text{H}]\text{-NMS}$ binding in HEK-cell membranes from transiently transfected cells

Inhibition binding experiments on $M_3\text{-WT}$ (■) or $N514Y M_3$ (●) mACh receptors, performed on membrane preparations obtained from transiently transfected HEK-293 cells, in the presence of 300-500 pM $[^3\text{H}]\text{-NMS}$. Data shown represent the mean $\pm$ S.E.M from at least three experiments performed in duplicate. Summary data for the antagonists are shown in Table 4.2.

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A.

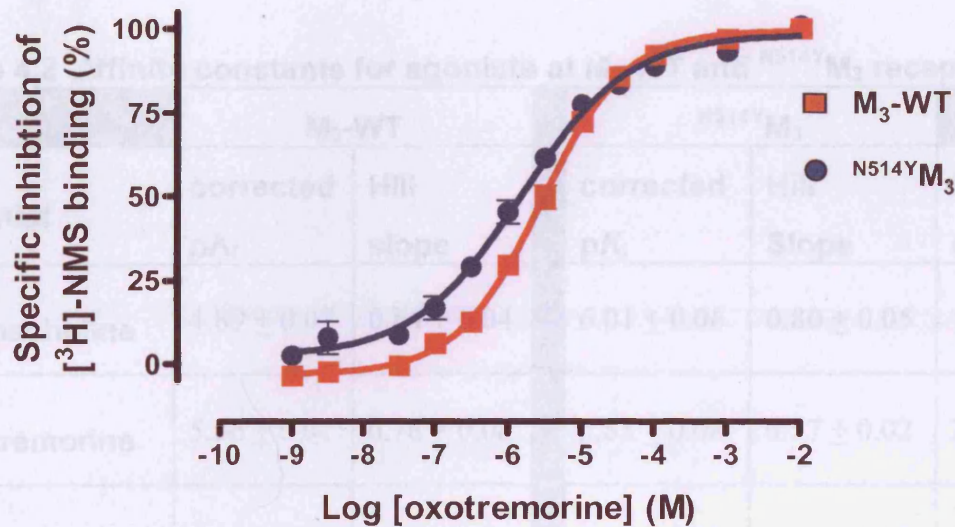


Fig. 4.15. Agonist displacement of $[^3H]$ -NMS binding in HEK-cell membranes from transiently transfected cells

Inhibition binding experiments on M_3 -WT (■) or $N514Y M_3$ (●) mACh receptors, performed on membrane preparations obtained from transiently transfected HEK-293 cells, in the presence of 300-500 pM $[^3H]$ -NMS. Data shown represent the mean $\pm$ S.E.M from at least three experiments performed in duplicate. Summary data for the antagonists are shown in

Table 4.2.

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active mutant receptors, there is no direct correlation between affinity increase and efficacy, this may again suggest a more complex interaction between the ^{N514Y}M₃ receptor and agonists.

Table 4.2 Affinity constants for agonists at M₃-WT and ^{N514Y}M₃ receptors

Agonist	M ₃ -WT		^{N514Y} M ₃		fold change
	corrected pK _i	Hill slope	corrected pK _i	Hill Slope	
methacholine	4.89 ± 0.07	0.84 ± 0.04	6.01 ± 0.06	0.80 ± 0.05	13***
oxotremorine	5.56 ± 0.01	0.76 ± 0.08	5.85 ± 0.08	0.57 ± 0.02	2.4***
Pilocarpine	5.47 ± 0.04	0.93 ± 0.06	6.16 ± 0.06	0.84 ± 0.02	5***

Data are taken from data shown in **Figs. 4.14** and **4.15**. Corrected IC₅₀ values (pK_i values) were calculated according to the method of Cheng and Prusoff, (1973), using the appropriate [³H]-NMS affinities. Fold change represents the lower/higher affinity of the ^{N514Y}M₃ receptor (obtained by dividing the pK_i for the mutated receptor by the pK_i for the wild-type receptor). Data represent the mean ± S.E.M for at least three experiments. *indicates ^{N514Y}M₃ pK_i is statistically different to that obtained for the M₃-WT receptor (*p* < 0.005, unpaired Student's *t*-test)

In contrast to inverse-agonists, agonists would be predicted to show a decreased affinity in the presence of GTP (Samama *et al.*, 1993), and it is predicted that these effects should be more pronounced at the ^{N514Y}M₃ mACh receptor. **Fig. 4.16.** shows the effects of 100 μM GTP on methacholine displacement binding at M₃-WT and ^{N514Y}M₃ mACh receptors. No significant effects of GTP were detected in M₃-WT receptor preparations, with similar IC₅₀ values reported for ± GTP conditions. Similarly there was no significant difference in potency or slope factors obtained at the ^{N514Y}M₃ receptor (for MCh: IC₅₀ values, 5.49 ± 0.04 and 5.32 ± 0.05; slope factors, 0.78 ± 0.05 and 0.82 ± 0.07; in the absence and presence of GTP, respectively) and a similar trend was observed for both oxotremorine

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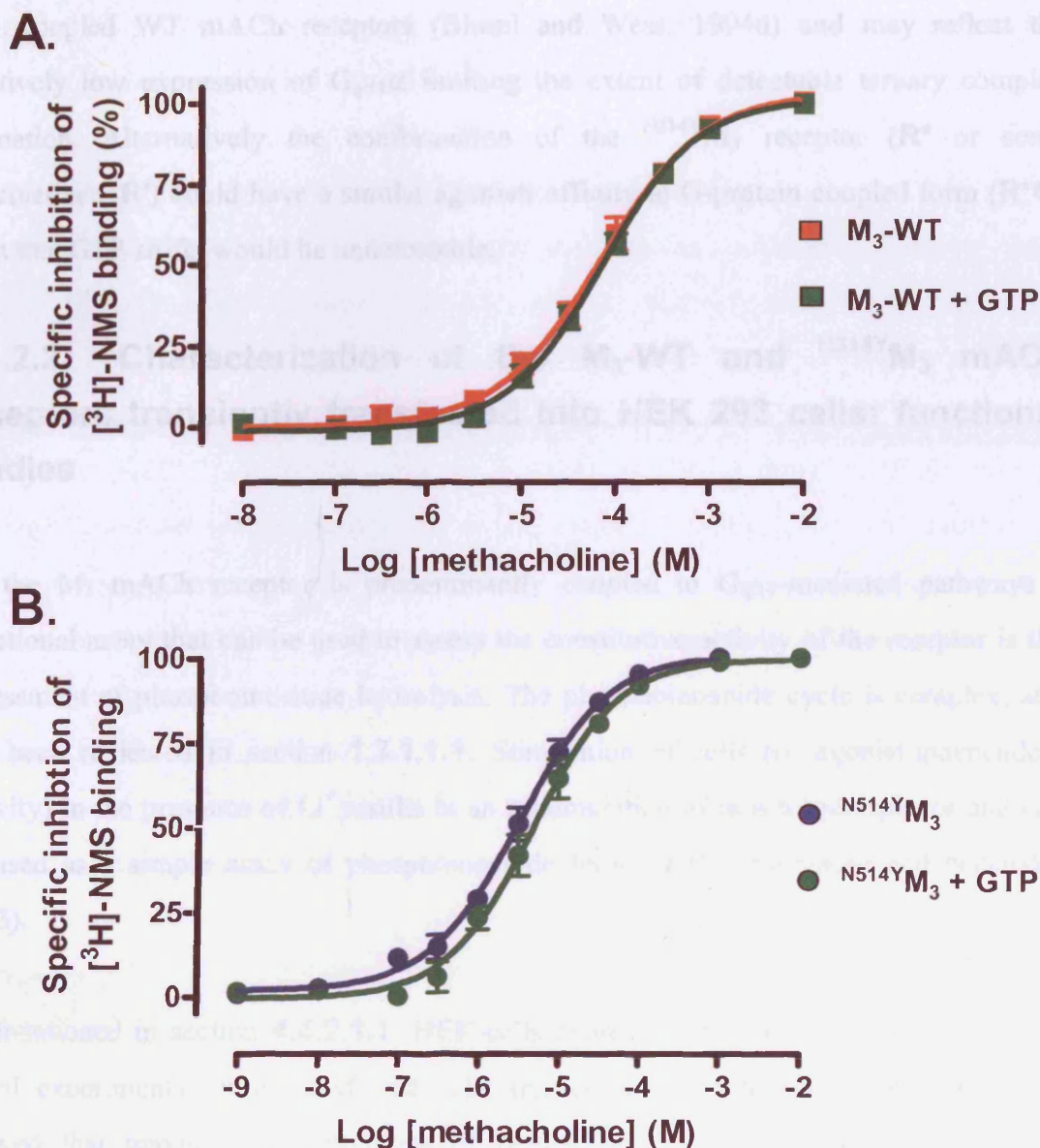


Fig. 4.16. Effect of GTP on antagonist displacement of $[^3\text{H}]\text{-NMS}$ binding in HEK-cell membranes from transiently transfected cells

Inhibition binding experiments on $M_3\text{-WT}$ (■) or $N514Y M_3$ (●) mACh receptors, performed in the absence or presence of 100 μM GTP, on membrane preparations obtained from transiently transfected HEK-293 cells, in the presence of 300-500 pM $[^3\text{H}]\text{-NMS}$. Data shown represent the mean $\pm$ S.E.M from at least three experiments performed in duplicate. Summary data for the antagonists are shown in **Table 4.2**.

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and pilocarpine (data not shown). The lack of a GTP-shift has been reported for other G_{q/11}-coupled WT mACh receptors (Bluml and Wess, 1994d) and may reflect the relatively low expression of G_{q/11}α limiting the extent of detectable ternary complex formation. Alternatively the conformation of the ^{N514Y}M₃ receptor (**R*** or some intermediate, **R'**) could have a similar agonists affinity to G-protein coupled form (**R*G**) such that GTP-shifts would be undetectable.

4.4.2.2 Characterization of the M₃-WT and ^{N514Y}M₃ mACh receptors transiently transfected into HEK 293 cells: functional studies

As the M₃ mACh receptor is predominantly coupled to G_{q/11}-mediated pathways a functional assay that can be used to assess the constitutive activity of the receptor is the assessment of phosphoinositide hydrolysis. The phosphoinositide cycle is complex, and has been reviewed in section 1.3.1.1.1. Stimulation of cells (or agonist-independent activity) in the presence of Li⁺ results in an accumulation of inositol phosphates and can be used as a simple assay of phosphoinositide turnover (Wojcikiewicz and Nahorski, 1993).

As mentioned in section 4.4.2.1.1. HEK-cells express a small endogenous population, initial experiments on untransfected cells (representative data shown in **Fig. 4.20**) showed that maximal concentrations of methacholine (~1 mM) elicited [³H]-InsP_x accumulation that was minor compared to transfected cells. At methacholine concentrations ≤10 μM no significant [³H]-InsP_x accumulation could be detected in untransfected cells (unlike transfected M₃-WT). Therefore any concentration-response for methacholine at the endogenous M₃ mACh receptor was significantly right-shifted, with a weak potency compared to transfected receptors. This low-potency, small [³H]-InsP_x accumulation was therefore a relatively insignificant component of the levels obtained with transfected cells. HEK293 cells have been used in many other studies, to assess agonist-mediated Ca²⁺ mobilization of transfected muscarinic receptors, with no significant reports of endogenous M₃ contributions (Yang *et al.*, 1995; Van Koppen *et al.*,

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2001; Stope *et al.*, 2003). However, to ensure the endogenous population response did not change with passage, two controls were always utilized in binding experiments: untransfected and empty-vector-transfected cells.

4.4.2.2.1. Expression levels used in functional studies

In order to produce a robust, agonist-independent [³H]-InsP_x accumulation assay, either the N^{514Y}M₃ or M₃-WT receptors were expressed at relatively high levels. Fig. 4.17. shows the effect of increasing plasmid load (in terms of µg DNA per well on a 24 well plate). Maximal expression of the receptors could be achieved at 0.50 and 0.40 µg well⁻¹ for M₃-WT and N^{514Y}M₃ mACh receptors, respectively. Under these conditions average receptor expression for inverse agonist functional studies were 3210 ± 159 and 1140 ± 80 fmol mg⁻¹ protein for M₃-WT and N^{514Y}M₃ mACh receptors, respectively. The discrepancies between the maximal expression levels of the M₃-WT and N^{514Y}M₃ mACh receptors suggests that the conformation of the receptor responsible for constitutive activity is either structurally instable, constitutively down-regulated or poorly trafficked to the cell membrane (Zeng *et al.*, 2003). Although both receptors have different maximal receptor expression levels, the concentration of DNA transfected which yields maximal expression is similar (between 0.40-0.50 µg well⁻¹), this suggests that above this concentration the cellular capacity or the toxicity of the transfection reagents becomes a limiting factor.

As receptor expression is known to influence both efficacy and potency (Kenakin 1997b) efforts were made to ensure similar levels of expression in agonist studies. On an experiment-to-experiment basis titrations of mutant versus WT receptor cDNA proved difficult to produce constant expression levels, yielding expression levels between 1000-1500 fmol mg⁻¹ protein for both receptor variants. However, the differences in expression levels between the receptor variants were minimized.

4.4.2.2.2. Time-course of agonist-independent [³H]-InsP_x accumulation

In order to ensure that agonist-independent [³H]-InsP_x accumulation, under Li⁺ block, was linear at the time-point measured, an initial time-course was established (Fig. 4.18).

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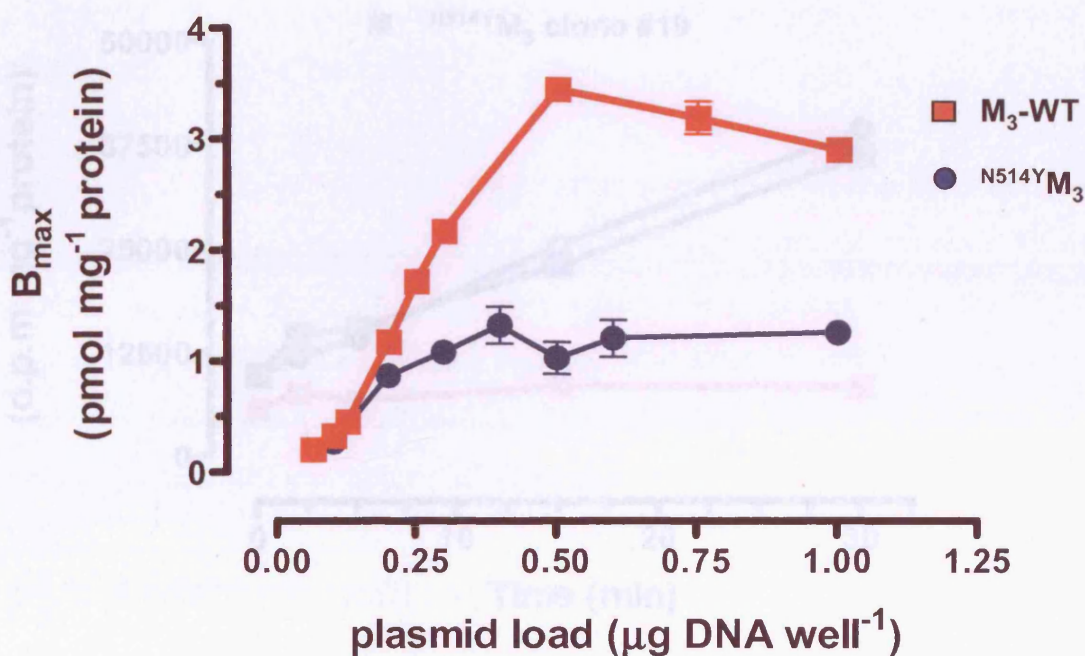


Fig. 4.17. Effect of increasing plasmid load on the maximal expression of wild-type and mutant receptors in HEK 293 cells

M₃-WT (■) or N514Y M₃ (●) mACh receptor cDNA was titrated in ddH₂O and the various concentrations transiently transfected into monolayers of HEK-293 cells as detailed in section 2.2. After a 48-hour incubation period the medium was removed and receptor expression quantified by [³H]-NMS binding as detailed in section 2.4.1.2. The data shown is mean ± S.E.M for at least twelve separate experiments performed in triplicate.

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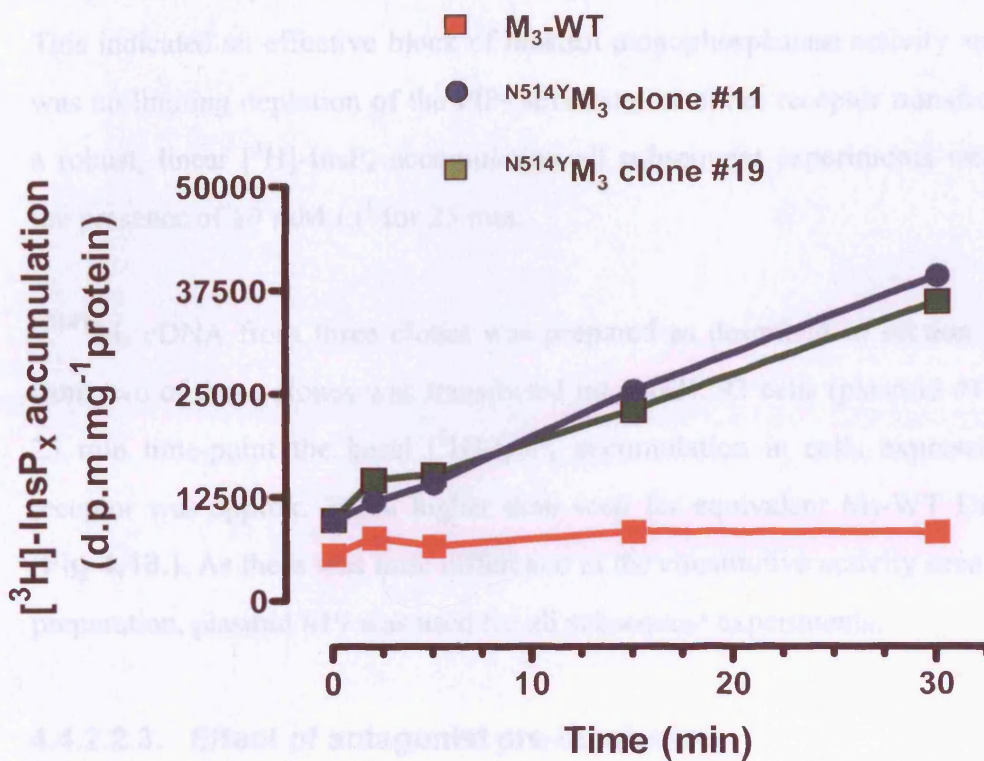


Fig. 4.18. Effect of Li⁺ on agonist-independent [3H]-InsP_x accumulation in transiently transfected HEK-293 cells

M_3 -WT (■) or $N^{514Y}M_3$ (●/■) mACh receptor cDNA was diluted to give approx. equal receptor expression levels and transiently transfected into monolayers of HEK-293 cells as detailed in section 2.7. After a 48-hour incubation period the medium was replaced by KHB, and Li⁺ was added at a final concentration of 10 mM. [3H]-IP_x were extracted as per section 2.7. Data shown represent the mean $\pm$ S.E.M from at least three experiments performed in duplicate.

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After an initial rapid accumulation there was a slower, but sustained accumulation over the 30 min time-period in cells expressing either the M₃-WT or ^{N514Y}M₃ mACh receptor. This indicated an effective block of inositol monophosphatase activity and ensured there was no limiting depletion of the PIP₂ substrate for either receptor transfection. To ensure a robust, linear [³H]-InsP_x accumulation all subsequent experiments were conducted in the presence of 10 mM Li⁺ for 25 min.

^{N514Y}M₃ cDNA from three clones was prepared as described in section 4.4.1.10, DNA from two of these clones was transfected into HEK293 cells (plasmid #11 and #19). At a 25 min time-point the basal [³H]-InsP_x accumulation in cells expressing the ^{N514Y}M₃ receptor was approx. 300% higher than seen for equivalent M₃-WT DNA transfection (Fig 4.18.). As there was little difference in the constitutive activity seen for either DNA preparation, plasmid #19 was used for all subsequent experiments.

4.4.2.2.3. Effect of antagonist pre-incubation

As any inverse-agonist would be competing against the Li⁺-induced [³H]-InsP_x accumulation it was important that any differences were not due to non-equilibration. Additionally, we wanted to establish whether allowing antagonists time to equilibrate with receptor would diminish any agonist-independent [³H]-InsP_x accumulation. Initial experiments were conducted on transfected HEK293 monolayers to establish the effects of pre-incubation with antagonists on agonist-independent [³H]-InsP_x accumulation (Fig. 4.19). With a 15 min pre-incubation the absolute magnitude of basal [³H]-InsP_x accumulation was smaller, when compared to adding the antagonist with Li⁺, but was robust enough to allow an accurate concentration-inhibition curve to be obtained. However the different assay conditions had no significant effect on the EC₅₀ of the antagonists ability to decrease basal [³H]-InsP_x accumulation (pEC₅₀ (no incubation) -8.19 ± 0.29 and -6.05 ± 0.25; pEC₅₀ (with incubation) 8.52 ± 0.07 and -6.13 ± 0.10 for 4-DAMP and methoctramine respectively, *p*>0.05, paired Student's *t*-test), but the improvement in the quality of the data-points was such that all further assays were run as follows:

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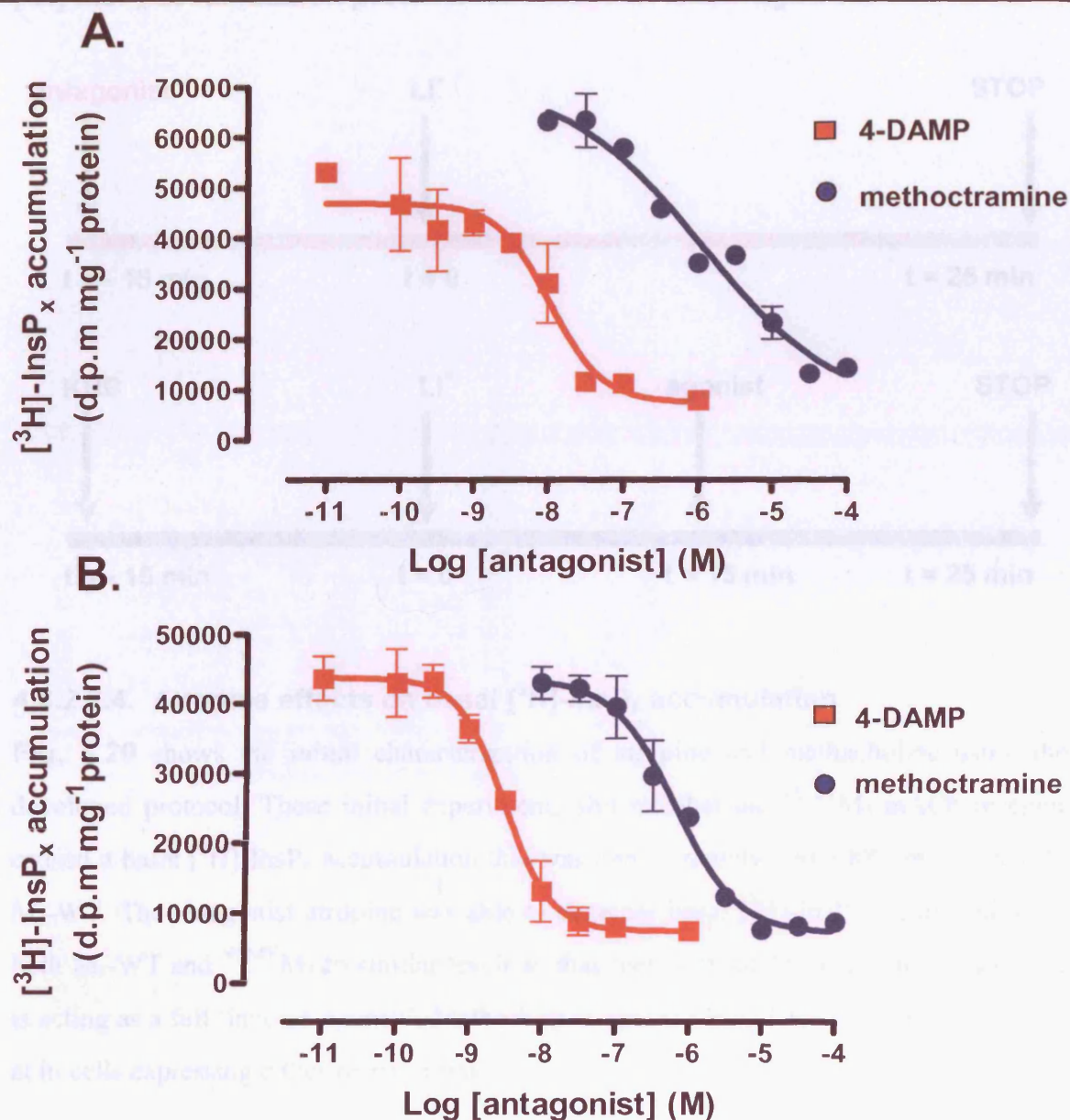
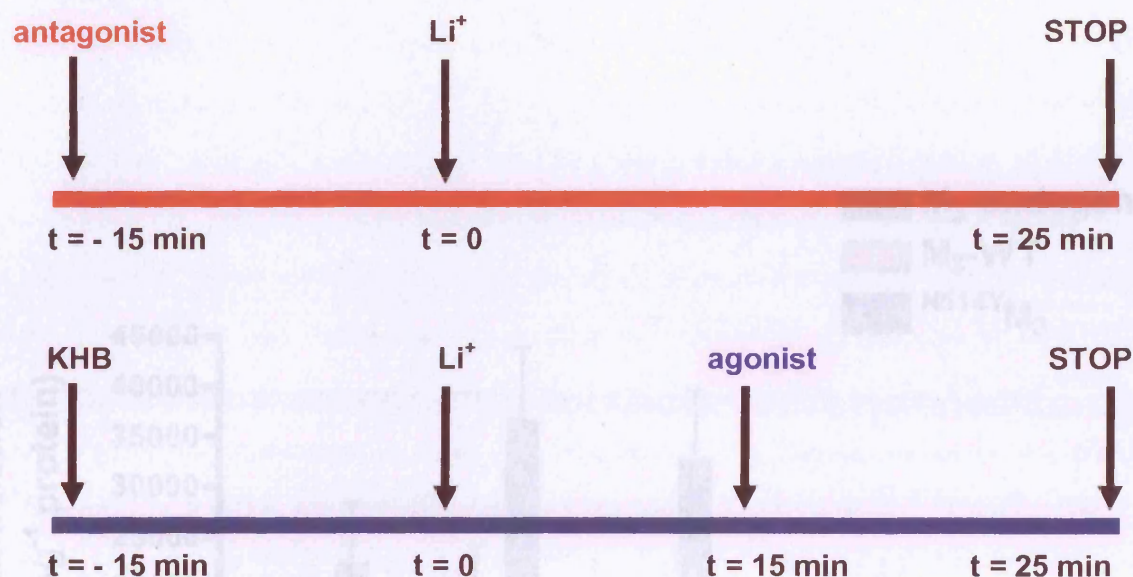


Fig. 4.19. Effect of pre-incubation with antagonists on basal $[^3\text{H}]\text{-InsP}_x$ accumulation in $N^{514Y}M_3$ -transfected HEK-293 cells

HEK-cell monolayers, transiently expressing the M_3 - N^{514Y} receptor were assessed for their ability to inhibit basal $[^3\text{H}]\text{-InsP}_x$ formation in the presence of 10 mM Li^+ . Antagonists with either added simultaneously with Li^+ (**A**) or 15 min prior to Li^+ addition (**B**). In either case incubations were continued for a further 25 min prior to $[^3\text{H}]\text{-InsP}_x$ extraction as per section 2.7. All data are means $\pm$ S.E.M for three experiments performed in duplicate.

$[^3H]$ -InsP_x accumulation protocol for **antagonists** and **agonists**



4.4.2.2.4. Atropine effects on basal $[^3H]$ -InsP_x accumulation

Fig. 4.20 shows the initial characterization of atropine and methacholine using the developed protocol. These initial experiments showed that the $N^{S14Y}M_3$ mACh receptor caused a basal $[^3H]$ -InsP_x accumulation that was approximately 300-400% that seen with M_3 -WT. The antagonist atropine was able to decrease basal $[^3H]$ -InsP_x accumulation, in both M_3 -WT and $N^{S14Y}M_3$ to similar levels to that seen with no Li^+ addition, i.e. atropine is acting as a full 'inverse-agonist'. Methacholine caused robust $[^3H]$ -InsP_x accumulations at in cells expressing either receptor type.

Based on the above observations the four antagonists and three agonists were tested for their effects on $[^3H]$ -InsP_x accumulation, following the developed protocol. For each experiment parallel $[^3H]$ -NMS binding experiments were carried out in order to check receptor density levels. For antagonist studies the basal level was determined in the absence of Li^+ and antagonists were assessed for their ability to inhibit $[^3H]$ -InsP_x accumulation down from the $+Li^+$ level to the basal level. For either agonist or antagonist experiments the functionality of receptors was always confirmed by running separate control wells incubated with 1 μ M atropine or 1 mM methacholine.

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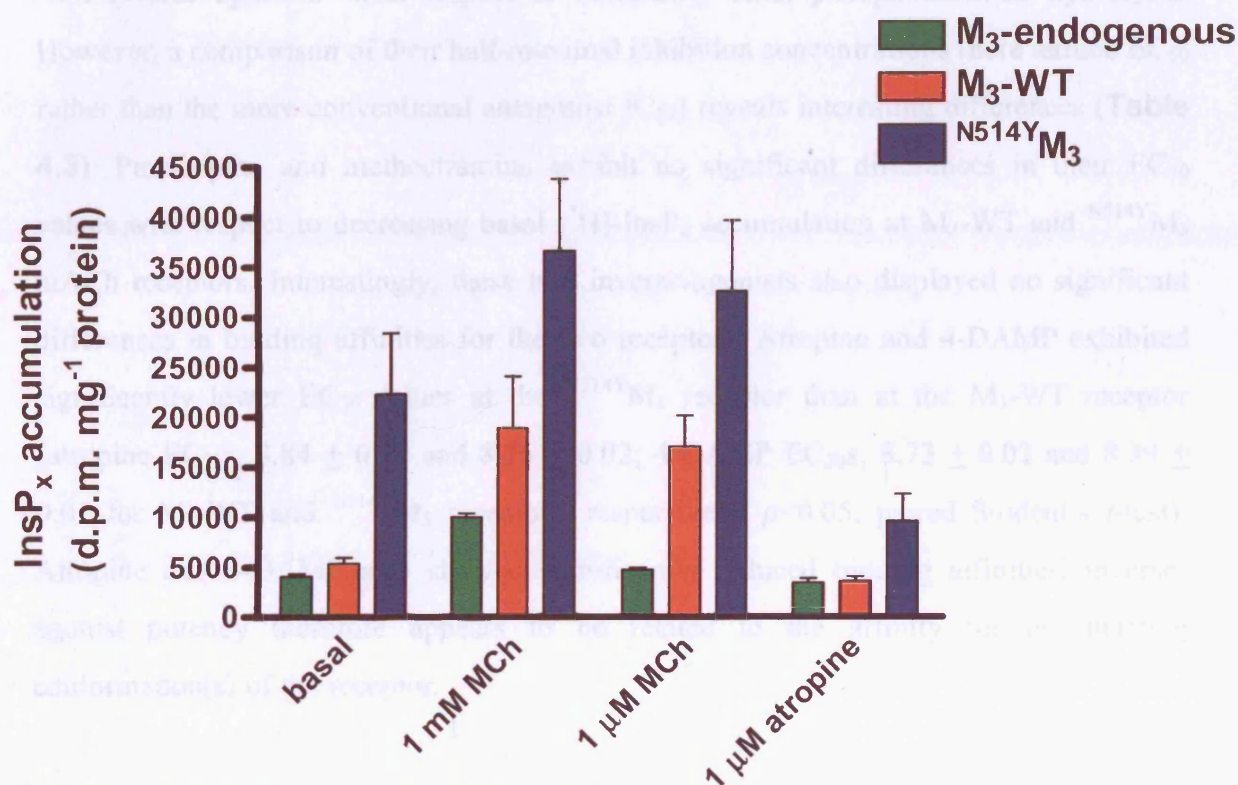


Fig. 4.20. Effect of ligands on $[^3H]$ -InsP_x accumulation in HEK-293 cells expression either wild-type or mutant M_3 receptor

HEK-cell monolayers, transiently expressing the M_3 -WT or $N514Y M_3$ mACh receptors were incubated with the indicated concentrations of antagonists/agonists as per section 4.3.2. All data are means $\pm$ S.E.M for three experiments performed in duplicate.

4.4.2.2.5. Comparative antagonist effects on basal [³H]-InsP_x accumulation

All four antagonists tested were able to concentration-dependently decrease basal [³H]-InsP_x accumulation to levels seen with no Li⁺ addition (**Figs. 4.21 - 4.23**) in cells expressing either the M₃-WT or ^{N514Y}M₃ receptor. Thus, all four antagonists were termed ‘full inverse agonists’ with respect to decreasing basal phosphoinositide hydrolysis. However, a comparison of their half-maximal inhibition concentrations (here termed EC₅₀ rather than the more conventional antagonist IC₅₀) reveals interesting differences (**Table 4.3**). Pirenzepine and methoctramine exhibit no significant differences in their EC₅₀ values with respect to decreasing basal [³H]-InsP_x accumulation at M₃-WT and ^{N514Y}M₃ mACh receptors. Interestingly, these two inverse-agonists also displayed no significant differences in binding affinities for the two receptors. Atropine and 4-DAMP exhibited significantly lower EC₅₀ values at the ^{N514Y}M₃ receptor than at the M₃-WT receptor (atropine EC₅₀s, 8.84 ± 0.08 and 8.26 ± 0.02; 4-DAMP EC₅₀s, 8.73 ± 0.02 and 8.39 ± 0.01 for M₃-WT and ^{N514Y}M₃ receptors, respectively, *p*<0.05, paired Student’s *t*-test). Atropine and 4-DAMP also showed significantly reduced binding affinities, inverse-agonist potency therefore appears to be related to the affinity for the inactive conformation(s) of the receptor.

All slope factors were not significantly different from unity except that for pirenzepine (0.67 ± 0.22 and 0.63 ± 0.18 for M₃-WT and ^{N514Y}M₃ receptor, respectively), although the relatively large errors on this measurement make the relevance of this finding questionable.

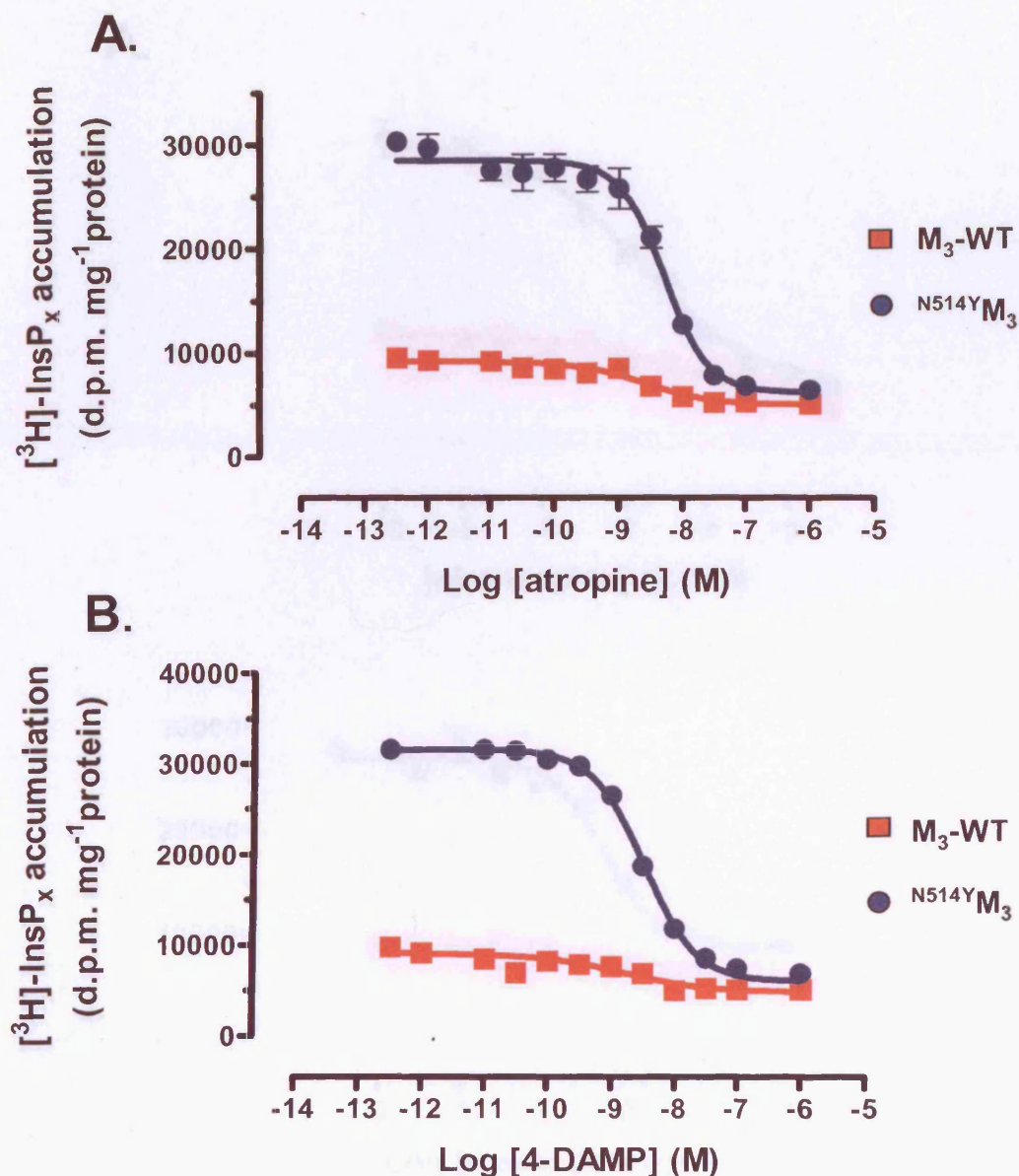


Fig. 4.21. Concentration-dependence of antagonist inhibition on agonist-independent Li^+ -mediated $[^3\text{H}]\text{-InsP}_x$ accumulation

Antagonist-mediated decreases in phosphoinositide hydrolysis on M_3 -WT or $N514Y M_3$ mACh receptors transiently transfected into HEK-293 cells. Monolayers were incubated as per the protocol in section 4.2.2, labelled inositol-phosphates ($[^3\text{H}]\text{-InsP}_x$) were extracted as described in section 2.7. Data represent the means $\pm$ S.E.M from at least three experiments performed in duplicate. Data are summarized in Table 4.3.

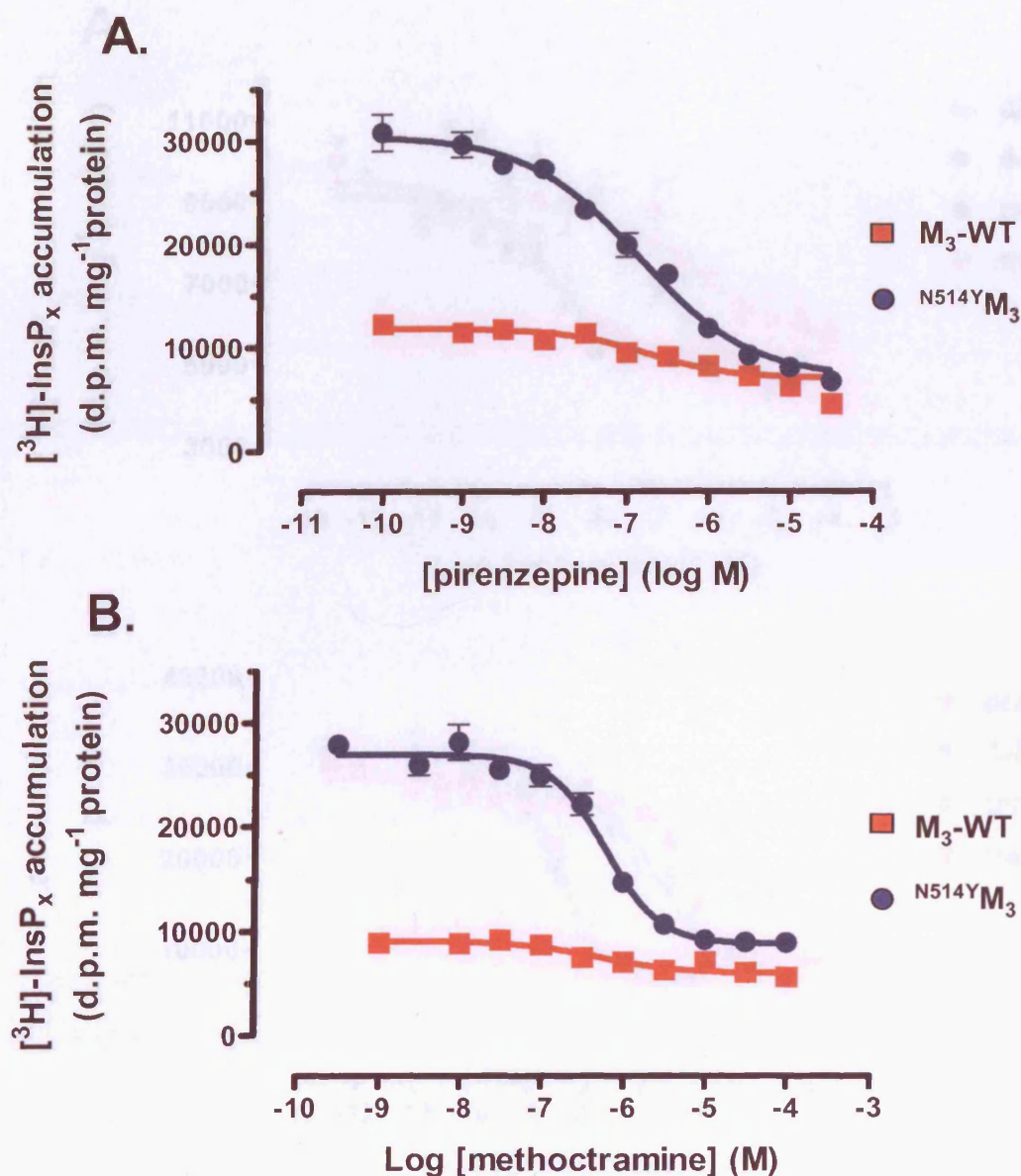


Fig. 4.22. Concentration-dependence of antagonist inhibition on agonist-independent Li⁺-mediated [³H]-InsP_x accumulation

Antagonist-mediated decreases in phosphoinositide hydrolysis on M₃-WT or N514YM₃ mACh receptors transiently transfected into HEK-293 cells. Monolayers were incubated as per the protocol in section 4.2.2, [³H]-InsP_x were extracted as described in section 2.7. Data represent the means $\pm$ S.E.M from at least three experiments performed in duplicate. Data are summarized in Table 4.3.

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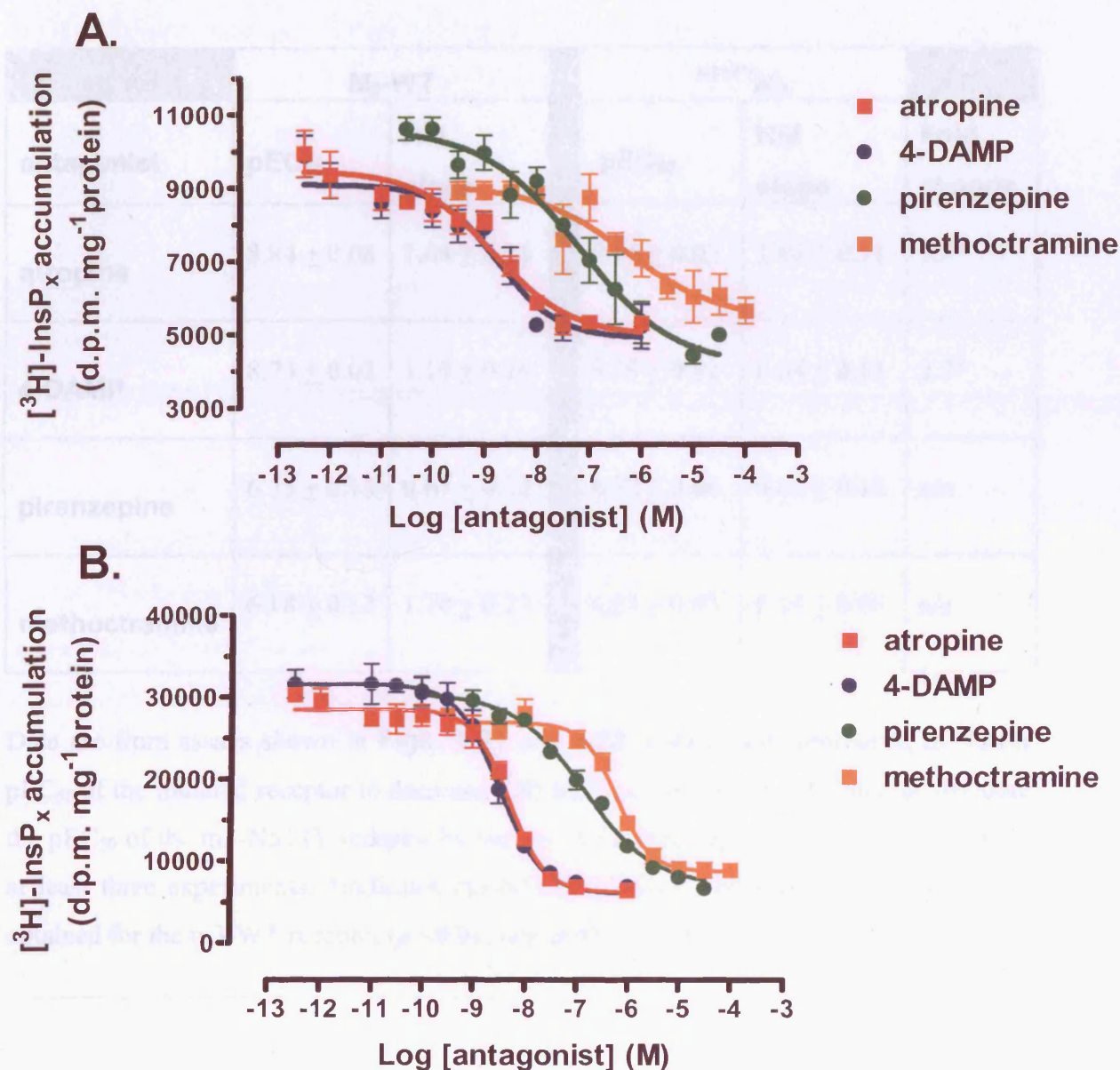


Fig. 4.23. Concentration-dependence of antagonist inhibition on agonist-independent Li^+ -mediated $[^3\text{H}]\text{-InsP}_x$ accumulation

Antagonist-mediated decreases in phosphoinositide hydrolysis in (A) M_3 -WT or (B) $\text{NS14Y } M_3$ mACh receptors transiently transfected into HEK-293 cells. Monolayers were incubated as per the protocol in section 4.2.2., $[^3\text{H}]\text{-InsP}_x$ were extracted as described in section 2.7. Data represent the means $\pm$ S.E.M from at least three experiments performed in duplicate. Data are summarized in Table 4.3.

Table 4.3 Comparative antagonist effects on basal [³H]-InsP_x accumulation

	M ₃ -WT		N514Y M ₃		
antagonist	pEC ₅₀	Hill slope	pEC ₅₀	Hill slope	Fold change
atropine	8.84 ± 0.08	1.08 ± 0.11	8.26 ± 0.02	1.07 ± 0.11	3.8*
4-DAMP	8.73 ± 0.02	1.10 ± 0.18	8.39 ± 0.01	0.94 ± 0.13	2.2*
pirenzepine	6.75 ± 0.13	0.67 ± 0.22	6.77 ± 0.06	0.63 ± 0.18	n/a
methoctramine	6.18 ± 0.12	1.10 ± 0.23	6.28 ± 0.03	1.14 ± 0.06	n/a

Data are from assays shown in **Figs. 4.21** and **4.22**. Fold change represents the lower pEC₅₀ of the mutated receptor to decrease [³H]-InsP_x accumulation (obtained by dividing the pEC₅₀ of the m3-N514Y receptor by the m3-WT). Data represent means ± S.E.M for at least three experiments. *indicates m3-N514Y pEC₅₀ is statistically different to that obtained for the m3-WT receptor (*p* < 0.01, unpaired Student's *t*-test).

4.4.2.2.6. Effects of increasing receptor expression on atropine effects on basal [³H]-InsP_x accumulation

The effects of increasing receptor density on the EC₅₀ value for atropine's effect to decrease basal [³H]-InsP_x accumulation was assessed by transfecting different concentrations of cDNA into HEK-monolayers. Interestingly, although M₃-WT DNA titrations produced graded receptor expression levels, in accordance with **Fig. 4.18.**, the maximal basal response did not change significantly. Transfecting M₃-WT in amounts below 0.2 µg DNA well⁻¹ produced no measurable basal [³H]-InsP_x accumulation (data not shown). Concentrations above 0.2 µg DNA well⁻¹ produced similar levels of basal

[³H]-InsP_x accumulation to those seen in **Figs. 4.21-4.22**. Increasing plasmid loads did not significantly increase the basal [³H]-InsP_x accumulation (data not shown). Therefore, although receptor density clearly varies with plasmid load, the M₃-WT dependence for constitutive activity and receptor expression showed a ‘threshold’ all-or-nothing relationship.

In contrast, increasing receptor expression of ^{N514Y}M₃ gave a respective increase in basal [³H]-InsP_x accumulation (**Fig. 4.24A**). Titrations of DNA produced receptor expression levels that were in accordance with the results obtained in **Fig. 4.17**. However, unlike M₃-WT DNA, **Fig. 4.24B** shows that increasing the concentration of ^{N514Y}M₃ DNA (in the range 0.1–0.3 µg DNA per well) gave basal [³H]-InsP_x accumulation levels that increased linearly with receptor density. However, at higher levels of DNA load (0.4 µg–0.5 µg DNA per well) both the receptor expression levels and the basal [³H]-InsP_x accumulation reached a plateau, where either the cellular capacity or the toxicity of the transfection reagent places a limitation on the upper value. The potency of atropine with respect to decreasing basal [³H]-InsP_x accumulation (**Fig 4.24A**) did not change significantly with receptor expression. ($p > 0.05$, Student’s *t*-test).

4.4.2.2.7. Agonist effects on [³H]-InsP_x accumulation

Agonist concentration-dependent increases in [³H]-InsP_x accumulation are shown in **Fig. 4.25**. At the ^{N514Y}M₃ receptor, agonists exhibit a spectrum of efficacy: compared to 1 mM methacholine, the partial agonists oxotremorine and pilocarpine had intrinsic activities of 73% and 51%, respectively (data are summarized in **Table 4.4**). pEC₅₀ values were in broad agreement with those reported in the literature (Richards and Van Giersbergen, 1995b). In contrast, all agonists exhibit potencies greater at the ^{N514Y}M₃ receptor (pEC₅₀ values: methacholine, 6.18 ± 0.06 and 7.40 ± 0.06 ; oxotremorine, 6.49 ± 0.08 and 7.67 ± 0.09 ; pilocarpine, 5.39 ± 0.04 and 6.53 ± 0.03 ; for M₃-WT and ^{N514Y}M₃ receptors, respectively). The extent of the potency increase also appeared to be related to the efficacy of the ligands being in the order: methacholine > oxotremorine > pilocarpine, although there was no statistically significant difference in fold-potency increases.

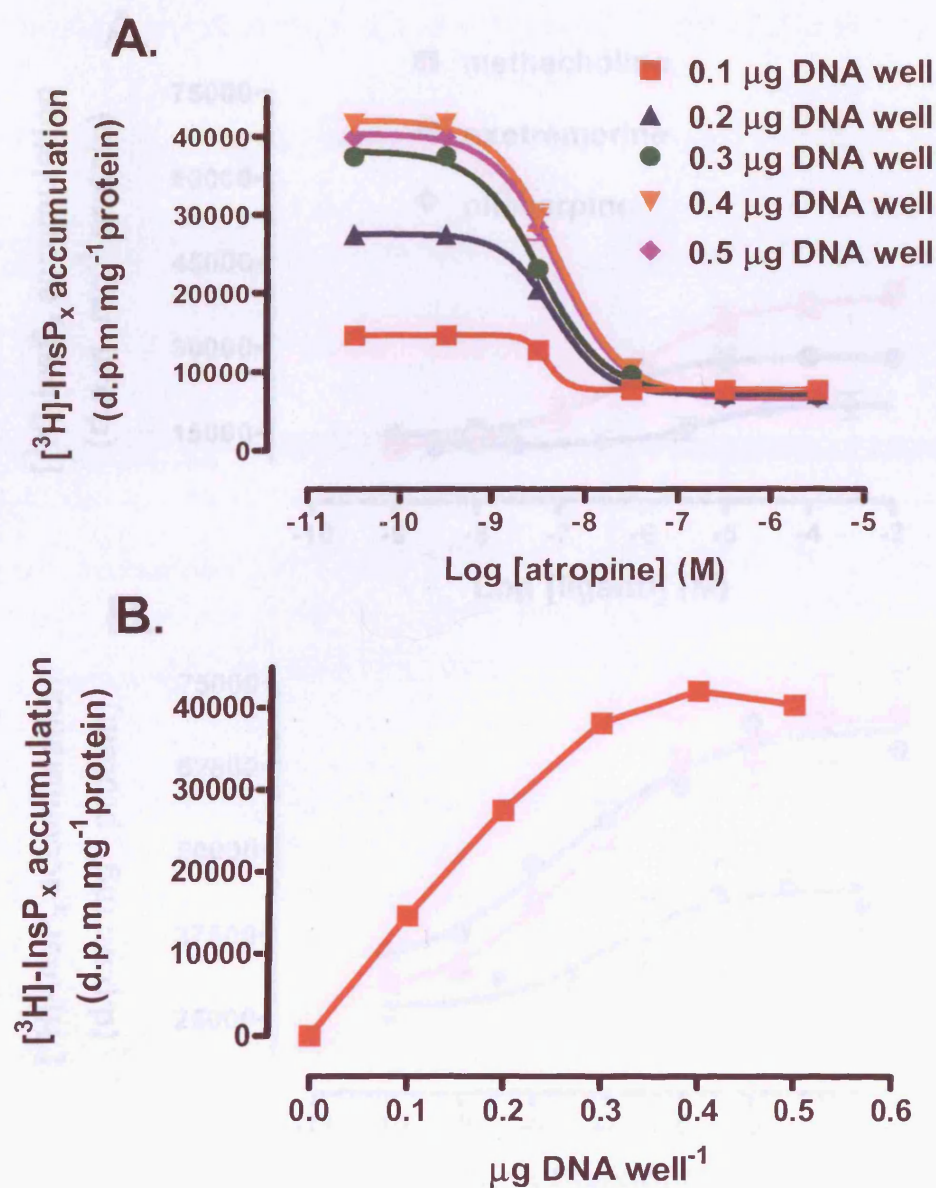


Fig. 4.24. Effects of atropine on agonist-independent Li⁺-mediated [³H]-InsP_x accumulation with increasing DNA load

N514Y M₃ mACh receptor cDNA was titrated in ddH₂O and the indicated concentrations transfected into HEK-monolayers on a 24 well-plate. Monolayers were incubated as per the protocol in section 4.2.2, [³H]-InsP_x were extracted as described in section 2.2. Data represent the means $\pm$ S.E.M from at least three experiments performed in duplicate.

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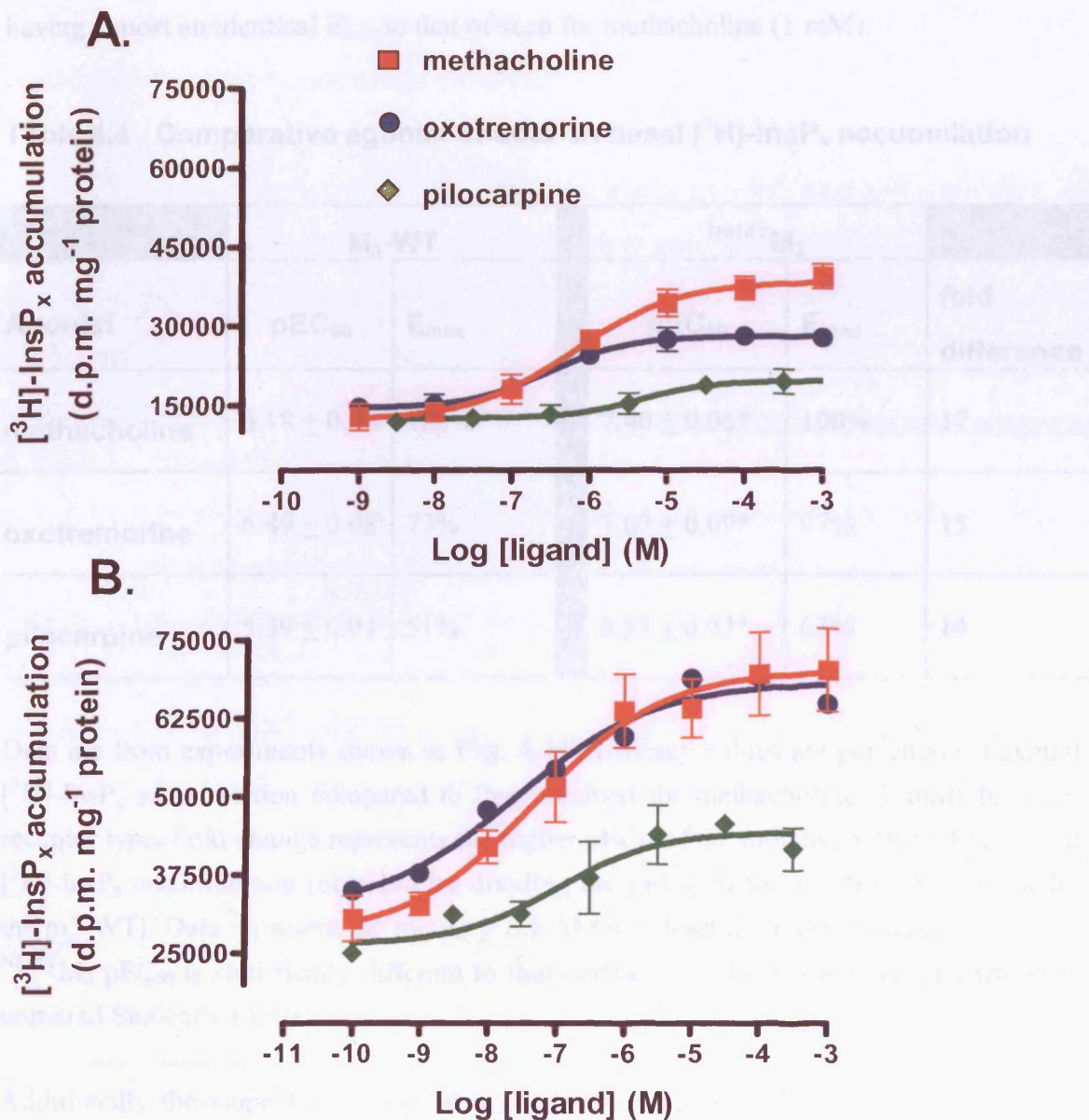


Fig. 4.25. Concentration-dependencies of agonist-stimulated increases in $[^3H]$ -InsP_(x) accumulation

Agonist-mediated increases in phosphoinositide hydrolysis in **(A)** M_3 -WT or **(B)** $N^{514Y}M_3$ mACh receptors transiently transfected into HEK-293 cells. Monolayers were incubated as per the protocol in section 4.2.2, $[^3H]$ -InsP_x were extracted as described in section 2.7. Data represent the means $\pm$ S.E.M from at least three experiments performed in duplicate. Data are summarized in **Table 4.4**.

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In addition, the intrinsic activity of the ligands was increased, with oxotremorine now having almost an identical E_{max} to that of seen for methacholine (1 mM).

Table 4.4 Comparative agonist effects on basal [³H]-InsP_x accumulation

Agonist	M ₃ -WT		N ^{514Y} M ₃		fold difference
	pEC ₅₀	E _{max}	pEC ₅₀	E _{max}	
methacholine	6.18 ± 0.06	100%	7.40 ± 0.06*	100%	17
oxotremorine	6.49 ± 0.08	73%	7.67 ± 0.09*	97%	15
pilocarpine	5.39 ± 0.04	51%	6.53 ± 0.03*	63%	14

Data are from experiments shown in **Fig. 4.25**. Efficacy values are percentage maximal [³H]-InsP_x accumulation compared to that obtained for methacholine (1 mM) for each receptor type. Fold change represents the higher pEC₅₀ of the mutated receptor to increase [³H]-InsP_x accumulation (obtained by dividing the pEC₅₀ of the m3-N514Y receptor by the m3-WT). Data represent the means ± S.E.M for at least three experiments. *indicates N^{514Y}M₃ pEC₅₀ is statistically different to that obtained for the M₃-WT receptor ($p < 0.01$, unpaired Student's *t*-test).

Additionally the slope factors for all agonists were significantly lower at the N^{514Y}M₃ receptor than the at the M₃-WT receptor (0.65 ± 0.10 and 0.46 ± 0.46 ± 0.09, 0.85 ± 0.05 and 0.43 ± 0.08, 0.95 ± 0.04 and 0.59 ± 0.09 for methacholine, oxotremorine-M and pilocarpine at M₃-WT and N^{514Y}M₃ respectively, $p < 0.01$, unpaired Student's *t*-test). This may indicate a more complex interaction with the N^{514Y}M₃ receptor with G-proteins and/or PLC-pools than the wild-type receptor.

4.4.2.3. Characterization of the M₃-WT and ^{N514Y}M₃ mACh receptors transiently transfected into HEK 293 cells: Additional indices of inverse-agonist activity

As all four antagonists decreased basal [³H]-InsP_x accumulation to similar levels this appears to support the hypothesis that they are acting by increasing the population of receptors in a common resting (G-protein uncoupled) state. We wanted to further test this notion by assessing inverse agonist activity of the four ligands in other assays, to experimental determine whether this is was a common property

4.4.2.3.1. Regulation of receptor number

[³H]-NMS binding experiments on transiently transfected HEK-cells showed that despite approximately equivalent plasmid loading, the ^{N514Y}M₃ receptor was expressed some 2.5 fold less than the M₃-WT mACh receptor (**Fig. 4.26**). The lower expression of constitutively active mutant receptors than their wild-type counterparts is frequently reported (Pauwels and Tardif, 2002), furthermore, this expression has been shown to be upregulated by pre-incubation with both inverse agonists (Smit *et al.*, 1996; Milligan *et al.*, 1997) and agonists (Milligan and Bond 1997). Transfected HEK-cell monolayers were therefore pre-incubated with inverse-agonists and the expression levels quantified by [³H]-NMS binding as detailed in section **2.4.1.3**.

The time-course of inverse-agonist specific receptor upregulation is shown in **Fig. 4.27A**. HEK-cells were transfected with either M₃-WT or ^{N514Y}M₃ DNA, after 24 h the medium was replaced with either fresh media or media containing maximal concentrations of inverse agonist (maximal concentrations were those that gave full inhibition of basal [³H]-InsP_x accumulation). In section **4.4.2.1.1**. we have shown that there is a system limitation on maximal receptor expression. In order to determine any ability of the inverse agonists to up-regulate the receptor expression, the HEK-cells were transfected with relatively low levels of DNA to yield modest receptor densities (0.2 µg DNA well⁻¹).

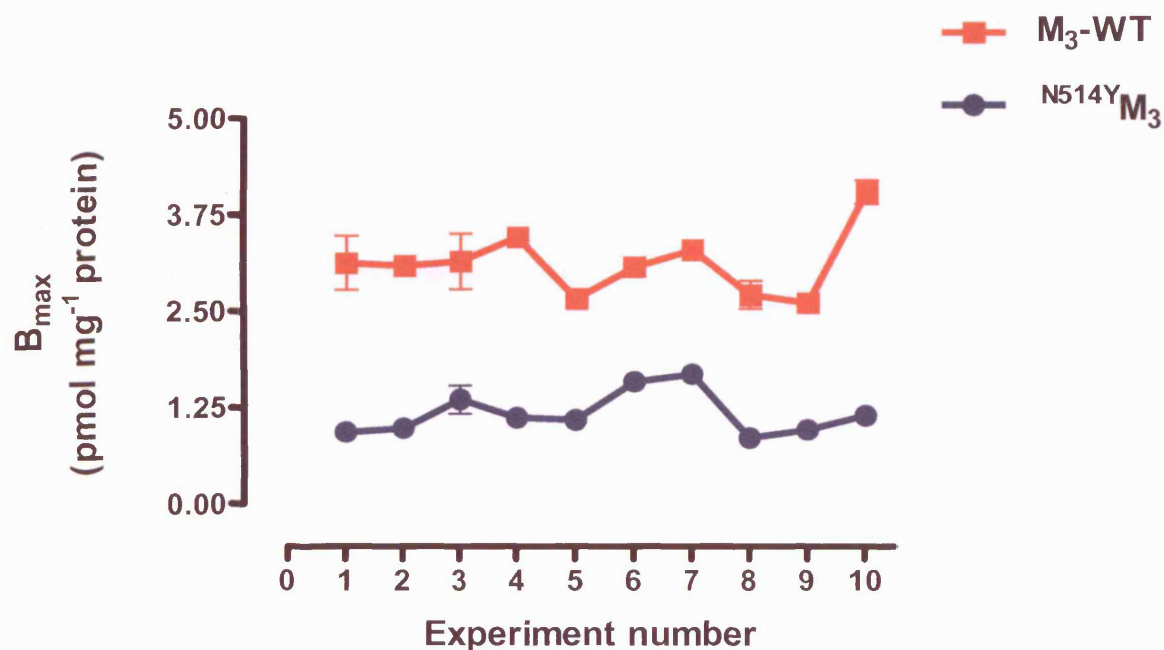


Fig. 4.26. [³H]-NMS binding to human mACh receptors transiently transfected HEK-cells

HEK-cell membranes, transiently transfected with M₃-WT (■) and N514Y M₃ (●) mACh receptor cDNA were prepared and incubated with [³H]-NMS in the presence or absence of atropine for 60 min to obtain an estimate of maximal saturable binding. HEK-monolayers were transfected with either 0.5 μg (M₃-WT) or 0.4 μg (N514Y M₃) DNA per well. Each data point shown represent the mean ± S.E.M from at independent receptor expression determination performed in quadruplicate.

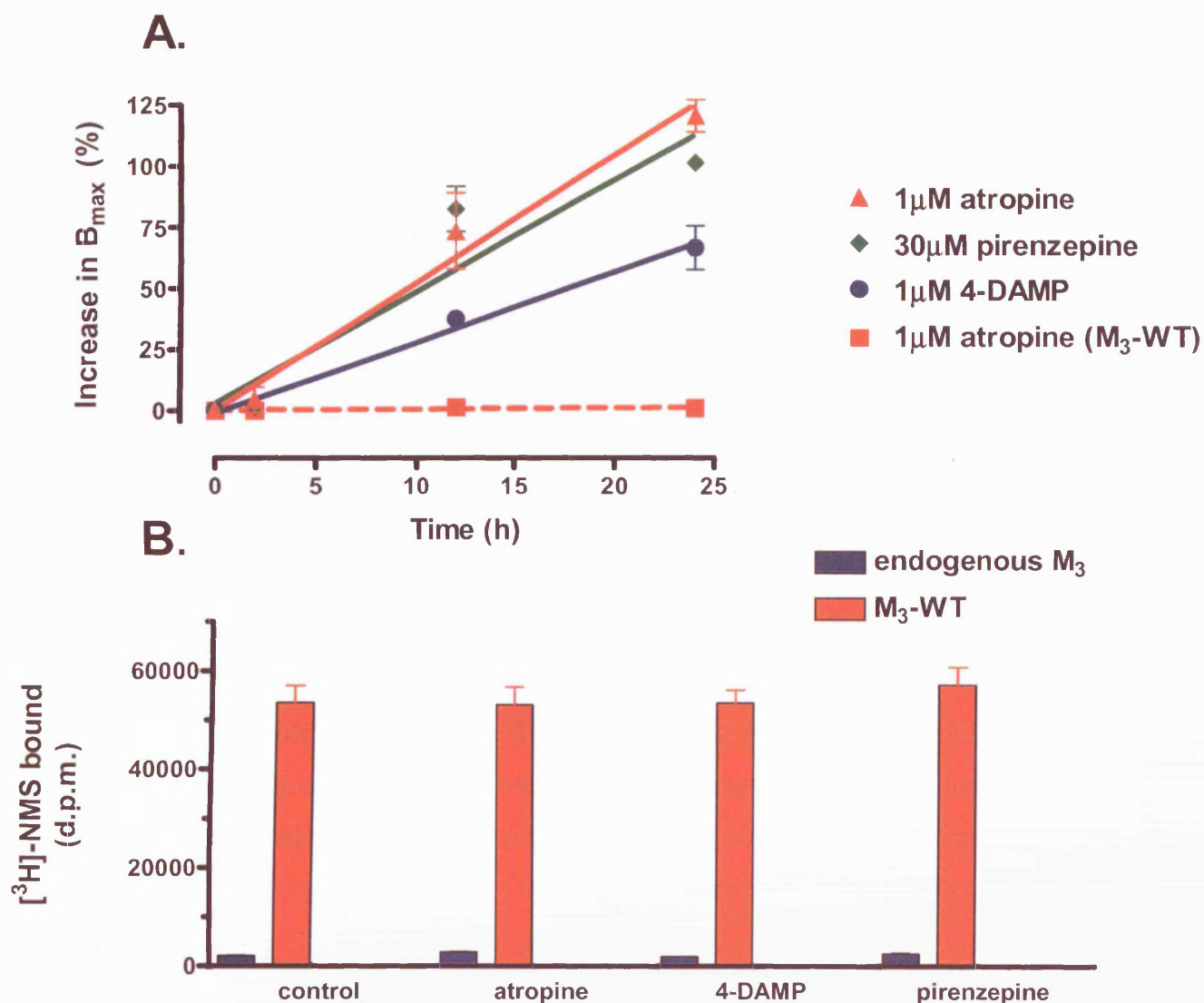


Fig. 4.27. $[^3H]$ -NMS binding to HEK-cells expressing endogenous M_3 , or transfected M_3 -WT/ $N^{514}Y$ M_3 mACh receptors

Membranes were prepared from HEK-cells untransfected or transiently transfected with either M_3 -WT or $N^{514}Y$ M_3 mACh receptor cDNA, and were incubated in the presence or absence of atropine for one hour to obtain an estimate of saturable binding. **A.** Time-course of receptor regulation on M_3 -WT or $N^{415}Y$ M_3 receptors **B.** Effects on endogenous and M_3 -WT expression levels, in the presence of maximal inverse agonist concentrations. Data shown represent the mean $\pm$ S.E.M from at least three experiments performed in duplicate.

Fig. 4.27A shows the inverse-agonist mediated receptor upregulation at the ^{N514Y}M₃ receptor was robust and still linear after a 24 h incubation period. All three antagonists tested (methoctramine has relatively slow dissociation kinetics and was therefore unsuitable for assessment by this assay due to wash-out difficulties) were able to significantly upregulate the ^{N514Y}M₃ receptor. Neither the M₃-WT (**Fig. 4.27A, B**), nor the endogenous HEK-m3 population (**Fig. 4.27B**) showed any significant receptor upregulation with maximal inverse agonist concentrations with 24 h incubation ($p > 0.05$, Student's *t*-test).

Fig. 4.28 shows the effects of increasing concentrations of inverse agonist after a 24 h incubation. All three antagonists tested were able to increase ^{N514Y}M₃ receptor expression levels in a concentration-dependent manner. Atropine and pirenzepine increased ^{N514Y}M₃ mACh receptor expression levels to a similar level ($93\% \pm 3$ and $84\% \pm 2$ increases respectively) albeit with different potencies (pEC_{50} 8.22 ± 0.11 and 6.33 ± 0.09 respectively) (Data summarized in **Table 4.5**). Interestingly although 4-DAMP was a 'full' inverse agonist with respect to decreasing basal [³H]-IP_x accumulation, when assessed for receptor regulation it was only 50% as efficacious as atropine (maximum upregulation $47\% \pm 4$, pEC_{50} 7.81 ± 0.20). It is also interesting to note that the pEC_{50} for upregulation are significantly lower than the pK_i values obtained from the radio-ligand binding experiments ($p < 0.05$, Student's *t*-test). This observation, along with the 4-DAMP partial inverse agonist activity, suggests that the mechanism of receptor up-regulation maybe more complex than the simple ligand stabilization of an inactive state of the receptor (Kenakin, 2002) and is discussed further in light of other experimental evidence (Petaja-Repo *et al.*, 2002).

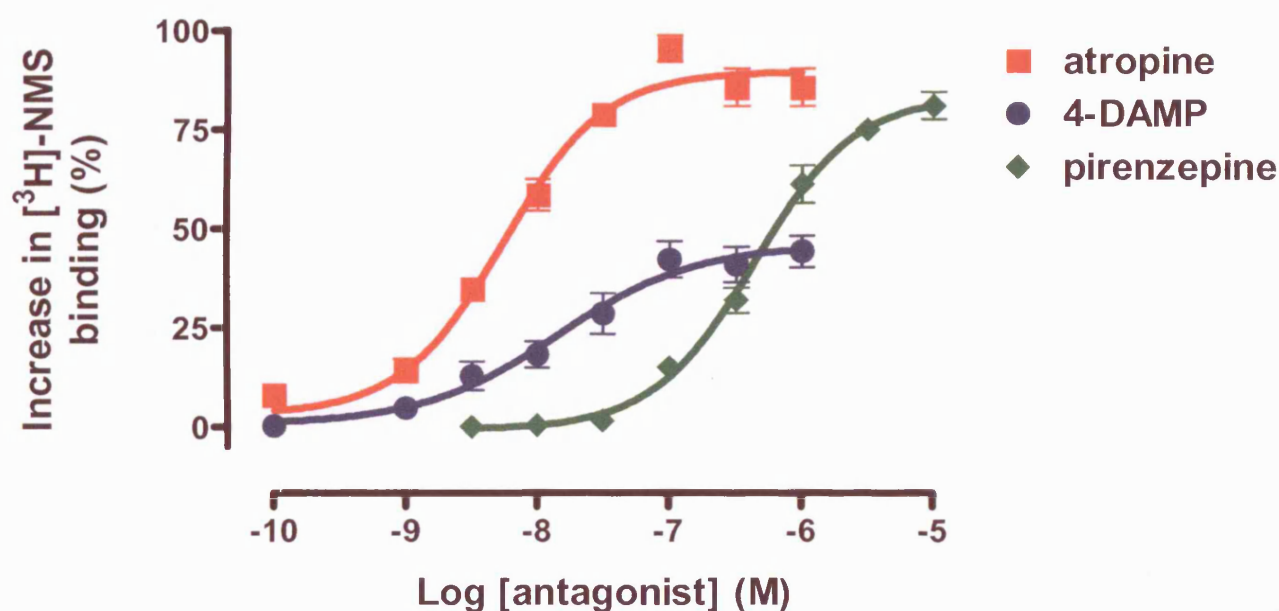


Fig. 4.28. Effect of inverse-agonist pre-incubation on [³H]-NMS binding to $N^{514Y}M_3$ receptors in HEK-293 cells

HEK-cell monolayers were incubated with the indicated concentrations of inverse agonist, 24 h post-transfection ($0.2 \mu\text{g DNA well}^{-1}$). After 24 h in the presence of the inverse agonist cells were thoroughly washed and [³H]-NMS binding completed as per section 2.4.1.3. Data shown represent the mean $\pm$ S.E.M from at least three experiments performed in triplicate. Data is summarised in Table 4.5.

Table 4.5 Potency and efficacy of inverse agonist with respect to the upregulation of the $N^{514Y}M_3$ receptor

Antagonist	$N^{514Y}M_3$		
	pEC ₅₀	Max. effect	Hill slope
Atropine	8.22 ± 0.11	93 ± 3%	1.00 ± 0.03
4-DAMP	7.81 ± 0.20	47 ± 4%	0.99 ± 0.08
pirenzepine	6.33 ± 0.09	84 ± 2	1.07 ± 0.07

Data are from experiments shown in **Fig. 4.28**. HEK293 cells, transfected with the $N^{514Y}M_3$ receptor, were incubated with appropriate concentrations of antagonists for twenty-four hours, and subsequent expression levels assessed using a saturating concentration of [³H]-NMS. Non-specific binding was determined in the presence of 1 μM atropine. Data shown represent the means ± S.E.M. from four experiments performed in triplicate. 'Max. effect' calculated as the percentage increase in $N^{514Y}M_3$ receptor expression levels incubated with buffer, compared to that with a maximal concentration of antagonist (atropine, 1 μM; 4-DAMP, 1 μM; and pirenzepine 10 μM).

4.4.2.3.2. M_3 -WT and $N^{514Y}M_3$ mACh receptor phosphorylation

The basal level of $N^{514Y}M_3$ receptor phosphorylation compared to that seen for the M_3 -WT was also assessed, as constitutively active receptors have been demonstrated to be subject to constitutive phosphorylation (Pei *et al.*, 1994). Furthermore, the possibility that inverse-agonists may reduce basal phosphorylation of the $N^{514Y}M_3$ receptor may provide another indication of inverse-agonist pharmacology.

$N^{514Y}M_3$ receptors demonstrated a robust phosphorylation in the absence of agonist, which, despite being matched for receptor numbers, was significantly greater than that seen for M_3 -WT receptors (**Fig. 4.29**) (12898 ± 496 versus M_3 -WT, 8304 ± 412

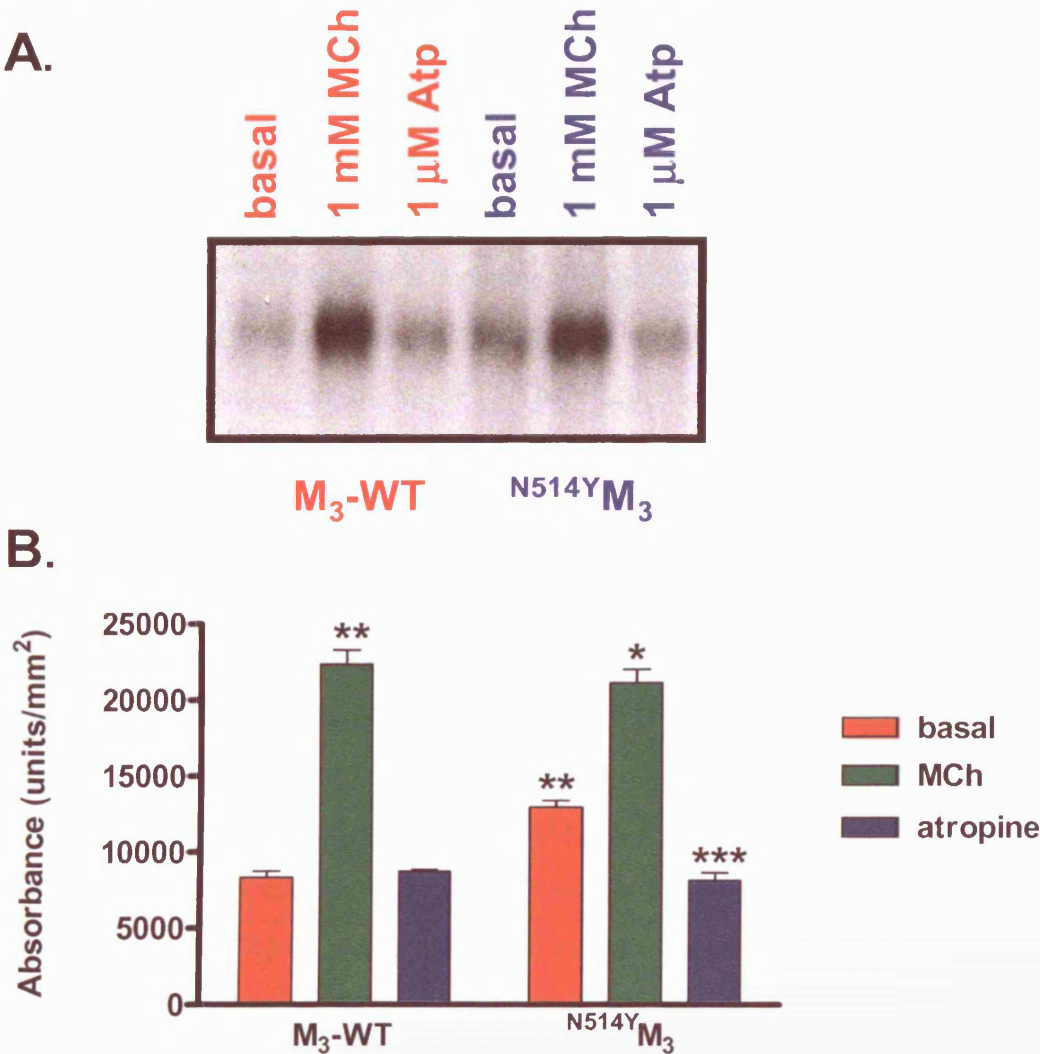


Fig. 4.29. Effects of ligands on the relative phosphorylation levels of M₃-WT and N514Y M₃ receptors expressed in HEK cells

HEK-293 cells were loaded with ³²P_i for 60 min in the presence or absence of a maximal concentration of each inverse-agonist at 37°C. Methacholine (1 mM) was added to some cells and incubated for a further 10 min. Cells were immediately lysed and phosphorylation levels assessed as detailed in section 2.9. **A.** shows a representative autoradiogram, and **B.** a histogram where the mean band intensity ($\pm$ S.E.M), has been quantified from four separate experiments performed in duplicate.

* indicates statistical significance c.f. N514Y M₃ basal, $p < 0.05$, Student's *t*-test
** indicates statistical significance c.f. M₃-WT basal, $p < 0.01$, Student's *t*-test
*** indicates statistical significance c.f. N514Y M₃ basal, $p < 0.005$, Student's *t*-test

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absorbance units quantified by densitometry; ($p < 0.01$, Student's t -test). Despite the increased basal receptor phosphorylation, ^{N514Y}M₃ mACh receptors still exhibited robust agonist-mediated increases in phosphorylation that were similar in band intensity to those seen for M₃-WT receptors (^{N514Y}M₃, 20158 ± 610 versus M₃-WT, 22324 ± 944 absorbance units, respectively). Inclusion of 1 μM atropine results in a significant attenuation of basal ^{N514Y}M₃ mACh receptor phosphorylation (reduction 56% ± 6, $n = 3$, ($p < 0.005$, Student's t -test).), whilst having no significant effect on the agonist-independent phosphorylation of M₃-WT. Therefore, the ^{N514Y}M₃ receptor exhibits an, inverse-agonist sensitive, agonist-independent phosphorylation.

We wanted to further characterize the efficacy of all the putative inverse-agonists to attenuate the agonist-independent level of phosphorylation at the ^{N514Y}M₃ receptor. Autoradiograms from at least 4 separate experiments were quantified, and the band intensities from the four antagonists were compared (**Fig. 4.30**). Atropine, 4-DAMP and pirenzepine caused significant reductions in band intensities (by 63 ± 7%, 76 ± 4% and 72 ± 8%, respectively, $n = 3$, $p < 0.05$, one-way ANOVA, Bonferroni's multiple comparison test). Although methoctramine was a 'full' inverse-agonist with respect to decreasing agonist-independent [³H]-IP_x accumulation, it failed to produce a significant reduction in basal band intensity (23 ± 10%, $p > 0.05$, Student's t -test). Therefore, as for receptor upregulation, the attenuation of ^{N514Y}M₃ mACh receptor agonist-independent phosphorylation provides evidence of differential inverse agonism that may be indicative of a more complex pharmacological action of inverse-agonists than stabilization of a single **R** conformation.

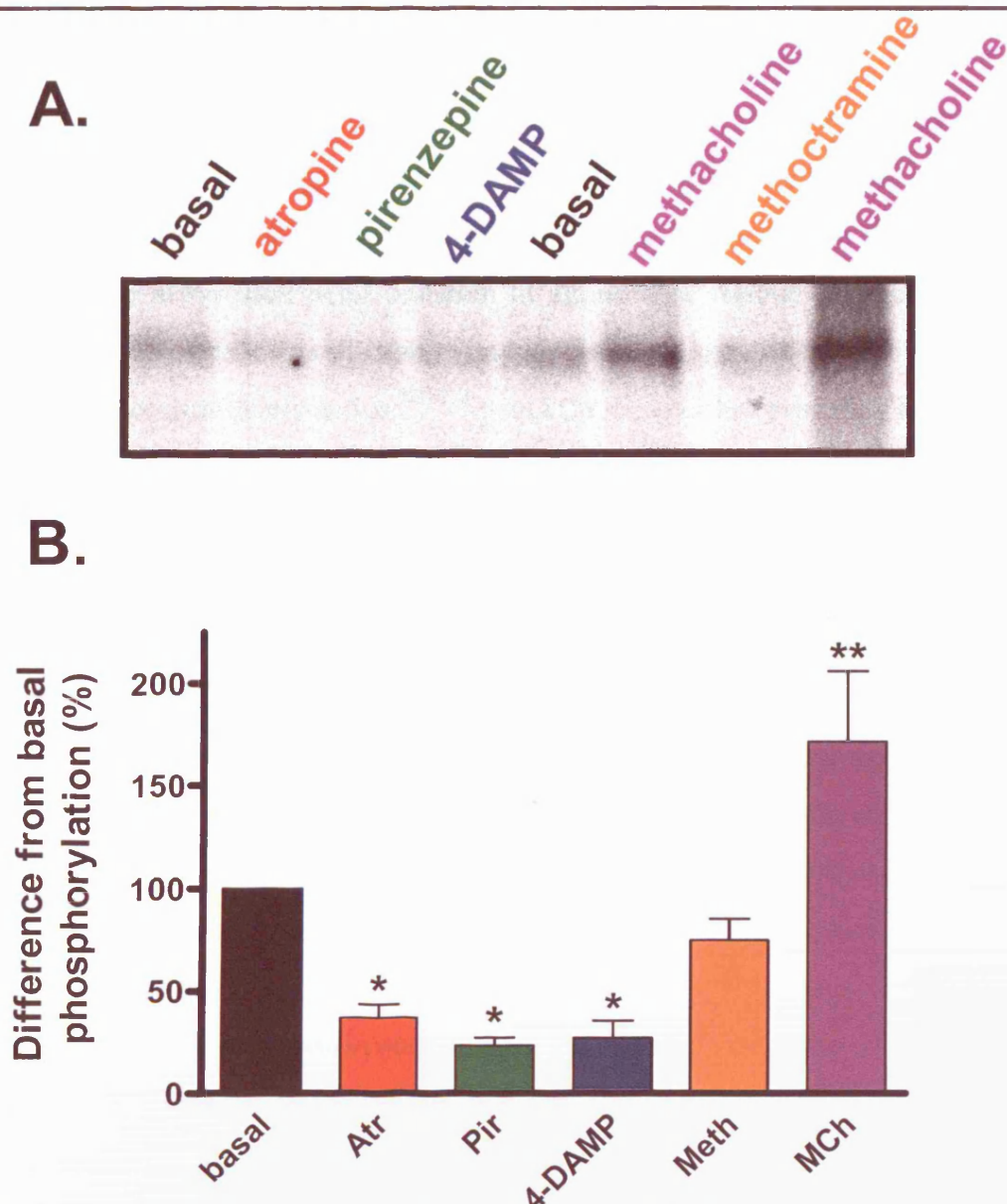


Fig. 4.30. Effects of ligands on the relative phosphorylation levels of the N^{514Y}M₃ mACh receptor expressed in HEK cells

HEK-293 cells were loaded with ³²P_i for 60 min, wither in the presence or absence of a maximal concentration of each inverse-agonist at 37°C. Methacholine (1mM) was added to some cells and incubated for a further 10 min. Cells were immediately lysed and phosphorylation levels assessed as detailed in section 2.9. **A.** shows a representative autoradiogram, and **B.** a histogram where the mean band intensity ($\pm$ S.E.M), has been quantified from four separate experiments performed in duplicate.

* indicates statistical significance c.f. N^{514Y}M₃ basal, $p < 0.05$, Student's *t*-test

4.5. Discussion

Constitutively active mutants of the M₁-M₅ (Ford *et al.*, 2002) mACh receptors have been previously reported by creating double point mutations of amino acids that reside at the junction of TM6 and the third extracellular loop. Here we show for the first time that a single asparagine to tyrosine point mutation at amino acid residue number 514 in the human M₃ mACh receptor is sufficient to induce a robust level of constitutive activity. Furthermore, the constitutively active ^{N514Y}M₃ mACh receptor has proved to be a valuable tool for pharmacological analysis, as it displays classical hallmarks of a constitutively active receptor: (i) increased agonist-independent PLC activity that is concentration-dependently inhibited by inverse agonists, (ii) decreased receptor stability/expression that can be rescued by inverse agonists, (iii) increased agonist efficacy and affinity; decreased antagonist affinity, and (iv) increased levels of ‘basal’ receptor phosphorylation. Despite the fact that we have classified all the antagonists tested as ‘full’ inverse-agonist based on their ability to decrease agonist-independent [³H]-InsP_x levels, there are some interesting differences in the pharmacology of these compounds. These are discussed further with reference to possible modes of inverse agonist action.

4.5.1 Mutational effects on (inverse)-agonist affinity

The extension of the ternary complex model was originally proposed, in part, to explain an increase in affinity of agonists that correlated with their efficacy at a constitutively active β₂-adrenoceptor (Samama *et al.*, 1993). Here we report an increase in affinity of methacholine and pilocarpine at the ^{N514Y}M₃ mACh receptor which correlates to with their relative efficacy at the M₃-WT receptor. Oxotremorine, an agonist with reported intermediate efficacy, demonstrated a minor increase in affinity at the ^{N514Y}M₃ mACh receptor. Interestingly, this was the only agonist tested gave a significantly decreased slope factor at the ^{N514Y}M₃ receptor, although a one-site fit was still preferred. In most reported examples of employing constitutively active mutants the increase in affinity (compared to wild-type) is directly related to the efficacy of the agonist (Rosenkilde *et al.*, 1999; Egan *et al.*, 2000; Ramsay *et al.*, 2001). Whether the surprisingly modest increase in the affinity of oxotremorine at the ^{N514Y}M₃ mACh receptor reflects a more

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complex interaction between different conformations of the active receptor remains to be investigated.

Antagonist binding profiles at the ^{N514Y}M₃-mACh receptor show that only those antagonists that have relatively high affinity/selectivity for the M₃-WT receptor exhibit a decreased binding affinity when ^{N514Y}M₃ values are compared to those obtained for the M₃-WT receptor. A decrease in inverse agonist affinity can be predicted, if we assume that the N514Y mutation induces a conformation which (at least partially) resembles the active state of the receptor and that the inverse agonist acts by preferentially binding to the inactive state of the receptor (**R**) (de Ligt *et al.*, 2000; Daeffler and Landry, 2000).

The absence of a reduced affinity for the antagonists pirenzepine and methoctramine, but a significant reduction for 4-DAMP and atropine at the ^{N514Y}M₃ receptor can be interpreted in two ways. 4-DAMP and atropine may bind preferentially to either **R** or **R'** (a partially activated form of the receptor) (Strange, 2002) over **R*G** (the conformation of the receptor that leads to productive G-protein coupling), or by having preferential affinity for **R** (**R'**) over **R***. However, it has been suggested that inverse agonists should show sensitivity to guanine nucleotides when substantial G-protein independent coupling is present (Strange, 2002). In contrast to the well characterized agonist effects, inclusion of GTP/GDP in the binding medium with a constitutively active mutant is predicted to *increase* the affinity of inverse agonists, if they favour binding to the inactive conformation (Kenakin, 2002a). No significant effect on the binding of either inverse agonists or agonists could be detected with the ^{N514Y}M₃ or the M₃-WT receptor in the presence of guanine nucleotides. This could reflect the fact that the increases in affinity in the presence of guanine nucleotides are likely to be quite modest (Seifert and Wenzel-Seifert, 2002) and are therefore difficult to demonstrate. However, as no significant GTP effects were seen for agonist binding, this might reflect the previous observation that guanine nucleotides frequently have no significant effect on ligand binding at G_{q/11}-coupled mACh receptors (Bluml *et al.*, 1994b,d). Additionally, the proportion of receptors (pre)-coupled to G-proteins, required to elicit a robust response, may be small

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(i.e. a substantial degree of receptor reserve exists for this pathway) and therefore the GTP-shift may be undetectable.

In comparison to the increases in agonist affinity seen for the mutant receptor, the reported reductions in antagonist affinity appear modest. However, it has recently been proposed for a constitutively active α_{2A} adrenoceptor, at which some inverse agonists exhibit a two-fold reduction in apparent affinity, that the fraction of receptors in the (partially) activated state was estimated to be 50% (Wade *et al.*, 2001). Therefore the modest reductions in antagonist affinity are in line with those reported in the literature. However if on the basis of a two-fold reduction in antagonist affinity, 50% of the ^{N514Y}M₃ receptors are coupled to G-proteins, the lack of GTP-shift for agonists seems surprising.

The lack of significant decreases in affinity for pirenzepine and methoctramine cannot be ignored, especially as they function as ‘full’ inverse agonists in PLC-dependent functional assays. One mode of inverse agonist action that allows an inverse agonist to suppress basal activity, but not exhibit a decreased affinity at constitutively active mutant receptors has recently been proposed (Strange, 2002). This does not involve a redistribution of receptor states, but one where the inverse agonist binds to all conformations with equivalent affinity. However the binding to the (partially)-active state switches the conformation of the receptor to an inactive state that still retains the ability to bind and sequester G-proteins, but is unable to activate them (Strange, 2002). Data consistent with this mode of inverse agonist action have been reported for several receptor systems (Bouboula *et al.* 1997; Gether and Kobilka, 1998; McLoughlin and Strange 2000; Costa *et al.*, 2003; Monczor *et al.*, 2003). including the M₂ mACh receptor (Vogel *et al.*, 1995).

4.5.2 Mutational effects on (inverse)-agonist efficacy

The potential of antagonists to exhibit inverse agonism by possessing preferential affinity for different receptor conformations may also explain the differences observed in functional studies. All antagonists tested were able to reduce basal levels of [³H]-InsP_x accumulation to a similar degree, however only 4-DAMP and atropine exhibited lower

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pEC₅₀s at the ^{N514Y}M₃ compared to the M₃-WT mACh receptor. Furthermore, only the concentration-response curves for 4-DAMP and atropine lay significantly to the right of the binding-curves for the ^{N514Y}M₃ receptor. If we assume that the conformation adopted by the ^{N514Y}M₃ receptor resembles (in part) that of the agonist-stabilized conformation of the M₃-WT receptor, then the following interpretations may be inferred. The lower EC₅₀s of 4-DAMP and atropine compared to their K_i values may be predicted to occur if they have preferential affinity for **R** (or **R'**) over **R*** (or **R/R*** over **R*G**) when the number of receptors in the **R*** (or **R*G**) conformation is increased. This is opposite to the effects seen with agonists, when the potency (and efficacy in the case of partial agonists) is increased through a higher receptor density in wild-type receptors, or through creation of constitutively active receptor mutants (**Fig. 4.26**). We attempted to demonstrate this effect by determining the potency of atropine, as the basal [³H]-InsP_x levels was elevated by increasing ^{N514Y}M₃ receptor levels (**Fig. 4.25**). Although there was a general trend for the potency of atropine to decrease with increasing receptor number, this did not reach significance. This probably reflects the difficulty in observing small affinity changes in the transient transfection system. Despite the decreases in potency, both 4-DAMP and atropine exhibited full efficacy with respect to decreasing the basal [³H]-InsP_x accumulation assessed against both receptors, this suggests that they are either 'full' inverse-agonists, or that a low degree of receptor reserve is present for this pathway. It would be interesting to test this hypothesis if/when partial-inverse agonists are found.

If we assume that pirenzepine and methoctramine bind to all receptor states with equal affinity and switch the receptor to an inactive state, little change in the pEC₅₀ compared to the K_i would be predicted for either mutant or wild-type receptor. A species of receptor that is inactive but still binds G-proteins is one of the key features of the cubic ternary complex model (Weiss *et al.* 1996a, b, c) and is one of the features that distinguish it from the extended ternary complex model. Although the data presented here cannot definitively distinguish between the different modes of inverse agonism, they strongly suggest that the antagonists may exhibit similar functional effects with quite different preferential binding or receptor conformations. Different types of inverse agonists (selective and non-

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selective) have also been observed at the type-1 parathyroid hormone receptor (Carter *et al.*, 2001).

4.5.3. ^{N514Y}M₃ mACh receptor structural instability

Despite equivalent plasmid loading, the ^{N514Y}M₃ receptor was expressed at significantly lower levels than the M₃-WT receptor; furthermore we have shown that the relatively low expression level of the ^{N514Y}M₃ receptor can be increased by pre-incubation with any of the inverse agonists tested. There is some debate in the literature as to the precise mechanism of constitutively active mutant receptor up-regulation and whether this is an inverse-agonist-specific effect (Miserey-Lenkei *et al.*, 2002; Parnot *et al.*, 2002; Zeng *et al.*, 2003). It is appropriate briefly to comment on these differences so the remainder of this discussion can be placed in context.

For some receptors the degree of up-regulation appears to be positively correlated both to the degree of constitutive activity in the system and also to the ability of a ligand to decrease this basal activity (presumably by stabilizing the inactive conformation of the receptor). The up-regulation of a spontaneously active H₂ histamine receptor has been demonstrated by pre-incubation with ligands, such as cimetidine and ranitidine, which were characterized as inverse agonists by their ability to decrease basal and forskolin-induced cyclic AMP production. Burimanimide, which behaved as a neutral antagonist in cyclic AMP assays, did not affect the level of the H₂ histamine receptor expression (Smit *et al.*, 1996). This suggests that the receptor is down-regulated directly as a consequence of productive G-protein coupling. If a constitutively active mutant functions in a similar way to its cognate agonist-induced wild-type counterpart, we would predict that only inverse-agonists would cause up-regulation by virtue of stabilizing the inactive conformation (Miserey-Lenkei *et al.*, 2001)

However, other studies (Gether *et al.*, 1997b; McLean *et al.*, 1999; Pauwels and Tardif, 2000) have shown that the creation of constitutively active mutants removes stabilizing constraints within the receptor. This leads to an inherently unstable receptor conformation that is more susceptible to denaturation or proteolytic degradation (Stevens *et al.*, 2000).

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As such, the expression level of these mutants is increased by any ligand regardless of its efficacy, presumably by stabilizing the structure as result of docking in the receptor ligand-binding pocket.

The three antagonists classified as inverse agonists from their ability to decrease basal [³H]-InsP_x accumulation were able to up-regulate the expression of the ^{N514Y}M₃ receptor in time- and concentration-dependent manner. This effect was specific to the mutant ^{N514Y}M₃ receptor as no significant effect on the receptor density could be detected for either the M₃-WT, or the small endogenous HEK-M₃ receptor population. The concentrations required for half-maximal up-regulation of receptor by atropine and pirenzepine were in good agreement with EC₅₀ values obtained for the decrease of basal [³H]-InsP_x accumulation. This would appear to suggest that the up-regulation of the ^{N514Y}M₃ mACh receptor by the inverse agonists is a result of the stabilization of the same inactive conformation that enables these agents to decrease spontaneous G-protein coupling. However, 4-DAMP despite being a full inverse-agonist with respect to decreasing basal [³H]-InsP_x accumulation, was only 50% as efficacious as atropine with respect to receptor up-regulation, with a significantly lower potency than that obtained for other functional assays. This is suggestive of a more complex mechanism of receptor up-regulation for this agent.

One of the functions of the endoplasmic reticulum (ER) is to act as a quality control point to ensure that correctly folded proteins reach the cell-surface. The physiological effect of mutations in many receptors has shown to result in the retention of the receptors in the ER even though the mutations themselves may cause only minor functional effects (Kim and Arvan, 1998). Petaja-Repo *et al.* (2002) demonstrated that membrane-permeable opioid ligands were able to facilitate the ER export of the δ-opioid receptor to the cell-surface, thereby acting as ‘molecular chaperones’. 4-DAMP carries a permanent positive charge due to the presence of a quaternary ammonium moiety, which greatly reduces its permeability through membranes (**Fig. 4.11**). It is therefore possible that full receptor up-

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regulation requires a component of intracellular ‘ligand-rescue’ in addition to receptor stabilization at the cell-surface, the latter component only being evident with 4-DAMP. Alternatively, though less likely, 4-DAMP may only be able to permeate the cell membrane at relatively high concentrations, and therefore the concentration-response appears to be shifted to the left.

Li *et al.* (2001) compared the up-regulation properties of naloxone and naloxone methiodide at a constitutively active rat μ -opioid receptor and found that the charged nature of naloxone methiodide reduced its efficacy with respect to its ability to up-regulate this receptor (in comparison to levels seen with naloxone) by 50%. They suggested that the inherently unstable mutant μ -opioid receptor was constitutively internalized and trafficked through early and late endosomes before degradation by lysosomes and proteasomes. Although they suggested that naloxone acts to up-regulate/stabilize the receptor at both intracellular and cell-surface locations, no one precise locus of action was suggested. Chaipatikul *et al.* (2003) used a fluorescently tagged μ -opioid, trafficking-deficient receptor mutant to demonstrate ligand-dependent rescue of cell-surface μ -opioid receptor expression directly from the ER. In this present study we have shown that ^{N514Y}M₃ mACh receptor up-regulation was still occurring linearly after 24 h, it remains to be established whether the ligands are also stabilising newly synthesized receptors which are delivered to the membrane without chaperoning. The precise molecular mechanism underlying ^{N514Y}M₃ mACh receptor up-regulation requires further detailed analysis, in order to investigate from which intracellular components (e.g. the ER or endosomes) the inverse-agonists may rescue mutant ^{N514Y}M₃ receptors. Further studies are also required to clarify if the up-regulation is especially related to inverse-agonist properties, or whether any ligand (such as agonists) that can stabilize the structure will also be as effective.

4.5.4 Constitutive receptor phosphorylation

Although we have speculated that the conformation of the ^{N514Y}M₃ mACh receptor might resemble the agonist-bound conformation of the M₃-WT receptor, the demonstration that

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the ^{N514Y}M₃ is constitutively phosphorylated is good evidence that other proteins in the signalling pathway specifically recognize its conformation. Pei *et al.* (1994) demonstrated that a β_2 -adrenoceptor constitutively active mutant (β_2 -CAM) underwent constitutive phosphorylation in a reconstituted system that was free of cognate G-protein. Furthermore, the quantified level of β_2 -CAM agonist-independent phosphorylation was comparable to that seen for the β_2 -WT with agonist stimulation. This was taken as good evidence that the mutant possessed a conformation that resembled the agonist-bound β_2 -WT state.

The ^{N514Y}M₃ mACh receptor induces a level of constitutive phosphorylation that is approximately 50% higher than that seen for the M₃-WT receptor. This raises the intriguing possibility that the ^{N514Y}M₃ receptor is also constitutively desensitised and therefore the absolute degree of constitutive activity may well be underestimated. Atropine is able to reduce the ^{N514Y}M₃ agonist-independent phosphorylation to levels similar to that quantified for the M₃-WT basal. Interestingly, both the ^{N514Y}M₃ and the M₃-WT receptors produced similar levels of agonist-stimulated phosphorylation, despite the previous observation that methacholine elicited a higher intrinsic activity (in terms of d.p.m) in the [³H]-InsP_x accumulation assay. It has been previously demonstrated that the magnitude of phosphorylation is related to the efficacy of the agonist for some GPCRs (Benovic *et al.*, 1988), and therefore we might have expected a greater magnitude of phosphorylation for the ^{N514Y}M₃ receptor. However, it is possible that the agonist-induced phosphorylation of the M₃-WT represents the maximum. It would be interesting to see if the ^{N514Y}M₃ receptor is able to attain maximum phosphorylation at lower concentrations of agonist. In addition, although the extents of agonist-induced phosphorylation appear similar, we cannot discount the possibility that the ^{N514Y}M₃ receptor is phosphorylated at different sites to the M₃-WT receptor.

When assessed for their ability to decrease the agonist-independent [³H]-InsP_x accumulation at the ^{N514Y}M₃ receptor, all four antagonists behaved as 'full-inverse agonists. Interestingly, when the ability of the inverse-agonists to decrease the basal levels of ^{N514Y}M₃ phosphorylation was quantified, methoctramine failed to cause a

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statistically significant attenuation. As all of the inverse agonists were assessed on the same assay plate, subjected to electrophoresis on the same polyacrylamide gel, and data collated and quantified from at least four separate autoradiograms, I am confident that the failure of methoctramine to reduce agonist-independent phosphorylation is a real event.

Thomas *et al.* (2000) presented evidence that an analogue of angiotensin II, [Sar¹,Ile⁴,Ile⁸]AngII, was unable to produce inositol phosphate signalling, but promoted a robust phosphorylation of the AT_{1A} receptor. In addition, constitutively active AT_{1A} receptors elicited robust agonist-independent and -dependent inositol phosphate production, but did not exhibit any basal or agonist-promoted phosphorylation. Finally the internalization of both wild-type and constitutively active AT_{1A} receptors was induced by angiotensin II, but not [Sar¹,Ile⁴,Ile⁸]AngII. This, and additional evidence from μ -opioid receptors (Blake *et al.*, 1997; Yu *et al.*, 1997; Chakrabarti *et al.*, 1998), is highly suggestive of multiple conformational states, each of which relates to a separate stage in the receptor activation process. Within this framework a different (but potentially overlapping) conformation of a receptor is required for basal, G-protein signalling, phosphorylation and internalization and agonists may promote one of these pathways without the other. Therefore, in contrast to the sequential activation of processes previous suggested, an agonist may promote effective phosphorylation with little or no G-protein coupling. This is at the core of 'ensemble theory' discussed previously in section 1.4.4.2. There is also no reason *a priori* to assume there is a single inactive receptor conformation. Using the framework of Thomas *et al.* (2000) an inverse-agonist, such as methoctramine, could stabilize a conformation of the receptor which was unproductive for G-protein coupling, but allowed (partial) phosphorylation of the receptor.

In this Chapter I have presented evidence from binding, G-protein binding, regulation of receptor number and phosphorylation assays that suggests that the actions of inverse agonists at the mutant N^{514Y}M₃ mACh receptor are more complex than the simple stabilization of an inactive conformation (**R**) of the receptor. Whilst the evidence for these multiple conformations of the N^{514Y}M₃ receptor are suggestive, they require further examination. To this end we attempted to create an homologous point mutation in the M₁

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mACh receptor, in order to compare and contrast the same antagonist and agonists at a different receptor. Preliminary data for the M_1 receptor mutant are reported in the next Chapter.

Chapter Five.

Creation and characterisation of a constitutively active, mutant M_1 mACh receptor

5.1 Introduction

In the previous Chapter the point mutation of amino acid residue 514 in the M₃ mACh receptor, elicited robust constitutive activity. Furthermore, the differences in the affinity, potency and efficacy of inverse agonists in binding and functional assays, suggested that the simplest mode of inverse agonism (the stabilization of the inactive, **R** receptor conformation) was not able to explain fully all of the experimental observations. Atropine and 4-DAMP showed decreased affinity in binding assays coupled with a decrease in potency when tested in functional assays at the ^{N514Y}M₃ receptor with respect to M₃-WT. This lead to the hypothesis that there maybe two types of inverse-agonists: those that show differential affinity for the **R** and **R*** conformations (e.g. atropine) and those that operate thorough another mechanism (e.g. pirenzepine). The simplest approach to test this idea was to create a homologous mutation within the M₁ mACh receptor and to characterize the pharmacological properties of a the same ligands.

Fig. 5.1 shows a partial sequence alignment of all 5 subtypes of mACh receptors, showing the high degree of structural homology within this region. The proposed homologous mutation in the M₁ mACh receptor, based on this alignment, is **S388**. Although the asparagine residue (**N514** in the M₃) in not fully conserved amongst the subtypes, it is encouraging that a homologous mutation in the M₅ mACh receptor (**S465**) produced a robust level of constitutive activity (Spalding *et al.*, 1997). This Chapter therefore details the creation and pharmacological characterization of a mutant ^{S388Y}M₁ mACh receptor.

Figure 5.1 Partial sequence alignment of the mACh receptors near the junction of TM6 and the third extracellular (e3) loop

Subtype	TM6	3 rd extracellular loop	TM7
M ₁	T W T P Y N I M V L V S T F C K D C V P E T L W E L G Y W		
M ₂	T W A P Y N V M V L I N T F C A P C I P N T V W T I G Y W		
M ₃	T W T P Y N I M V L V N T F C D S C I P K T F W N L G Y W		
M ₄	T W T P Y N V M V L V N T F C Q S C I P D T V W S I G Y W		
M ₅	T W T P Y N I M V L V S T F C D K C V P V T L W H L G Y W		

5.2 Methods

For details of the methodologies used in this Chapter please refer to **Chapter 2 –**

Materials and Methods:

2.2 Transfection procedures

2.3 Cell membrane preparation

2.4.1.1 [³H]-NMS radio ligand saturation binding to membrane preparations

2.4.1.2 [³H]-NMS radio-ligand saturation binding to plated whole cells

2.4.1.3 [³H]-NMS saturation binding to plated whole cells pre-treated with antagonists

2.4.2.1 [³H]-NMS displacement binding

2.4.2.2 Effects of GTP on [³H]-NMS displacement binding

2.7 [³H]-inositol phosphate accumulation

5.3 Data analysis

All data are shown as means $\pm$ S.E.M. for at least three separate experiments performed in duplicate except where indicated.

[³H]-NMS binding for each individual experiment was analysed by non-linear regression using a commercially available programme (GraphPad Prism version 3.0/4.0; GraphPad Software, San Diego, CA, USA) to give B_{max} and K_D estimates. Displacement binding data were fitted to sigmoidal (variable slope) curves using the same analysis package, IC₅₀ values were converted to pK_i values using the method of Cheng and Prusoff (1973).

All concentration response curves, from either [³H]-InsP_x accumulation or receptor upregulation data were fitted using the sigmoidal (variable slope) curve-fitting function of the same programme. All data were compared for a one- and two-site fit and only the most statistically significant value is quoted (F-test, GraphPad Prism).

5.4 Results

5.4.1. Part I - Sub-cloning of the M₁ mACh receptor gene

A pcDNA construct encoding the full-length human M₁ mACh receptor was a kind gift from Dr. A.B. Tobin. To ensure comparable transfection efficiencies between the M₁ and M₃ constitutively active receptor mutants the M₁ receptor gene was sub-cloned from the pcDNA vector into pcDNA3.

5.4.1.1. Primer design

The M₁ receptor gene was sub-cloned from pcDNA using the following primers:

Sense (Primer 1)

5' – CCC CCC **GGA TCC** ATG AAC ACT TCA GCC CCA CCT GCT GTC AGC CCC AAC – 3'

Antisense (Primer 2)

5' – CCC CCC **CTC GAG** TCA GCA TTG GCG GGA GGG AGT GCG GTG CAC GGA GCC – 3'

The residues in **BLUE** provide spacer nucleotides to allow for efficient restriction enzyme digestion; those in **RED** encode the restriction enzyme recognition sequences for *Bam* HI and *Xho* I in primer 1 and primer 2, respectively. The restriction enzyme recognition sequences are included to assist ligation via 'sticky ends' into the new vector.

5.4.1.2. Polymerase chain reaction

The m1 receptor gene was amplified from the pcDNA template using a NovaTaqTM DNA polymerase kit using the following recipes:

1 µl	Template pcDNA-M ₁ DNA – (50 ng)
1 µl	dNTP mix (10 mM each)
3.1 µl	Primer 1 (200 µg ml ⁻¹)
3.1 µl	Primer 2 (200 µg ml ⁻¹)
5 µl	10 x NovaTaq Buffer with MgCl ₂
0.25 µl	NovaTaq DNA polymerase (1.25 U)
36.55 µl	PCR-grade H ₂ O

Four PCR reactions were set up and the contents were gently mixed in thin-walled PCR tubes and subjected to the following thermal cycling: 30 cycles 1 min at 94°C, 1 min at 60°C, 2 min at 72°C followed by a final extension for 5 min at 72°C. Samples were held at 4°C before proceeding. A negative control was performed using an identical recipe lacking only the template. Before proceeding with the PCR purification, a DNA gel on the reaction products was performed using 10 µl of the PCR reaction and 2.5 µl of 5 x DNA loading buffer to check the size of the amplified fragment. (For DNA gel electrophoresis method, see Section 4.4.1.9).

Fig. 5.1A shows the respective DNA gel, the m1 receptor PCR product (approx. 1,400 bp) is shown running just below the 1,500 bp marker.

5.4.1.3. Purification of PCR products

The PCR product was purified, to remove unwanted amplification products and impurities, using a SpinPrep[™] PCR clean-up kit as recommended by the manufacturer. Briefly the four PCR reactions (360 µl) were pooled together and 1440 µl of SpinPrep-Bind buffer added. This mixture was added to a SpinPrep PCR filter and centrifuged at 10,000 xg in a microcentrifuge. The supernatant was discarded and the PCR products washed with 400 µl SpinPrep-Bind buffer and centrifuged as before. After discarding the supernatant, the filter was washed once more with 500 µl SpinPrep wash buffer and centrifuged as before. The SpinPrep filter was transferred to a fresh Eppendorf tube and 50 µl of pre-warmed SpinPrep-Elute buffer added. The tube was warmed for 3 min at 50°C before immediate centrifugation at 10,000 xg to collect the purified PCR product.

5.4.1.4. Digestion of pcDNA3 and the PCR product

To enable ligation of the m1 PCR product into the pcDNA3 vector, both were double digested with *Bam* HI and *Xho* I to form 'sticky-ends'. Restriction digests were carried out using the recipes below followed by incubation at 37°C for 2 h.

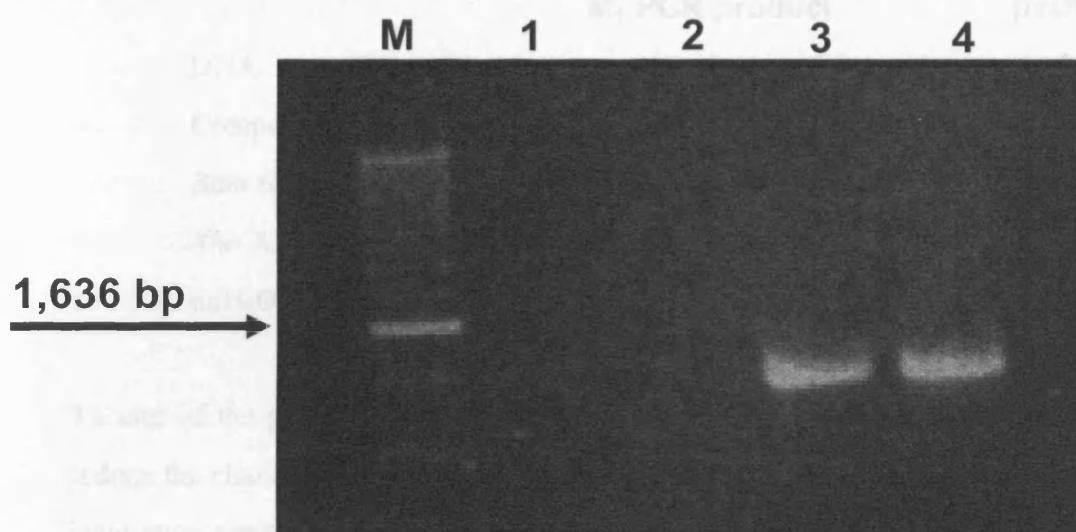


Fig. 5.1A DNA gel of M_1 PCR products

Negative control reactions (lanes 1 and 2) and M_1 mACh receptor PCR products prepared as per Section 4.2.9. Lanes 3 and 4 indicate the production of a length of DNA with a mobility just below 1,500 bp. An Invitrogen 1 kb ladder was used.

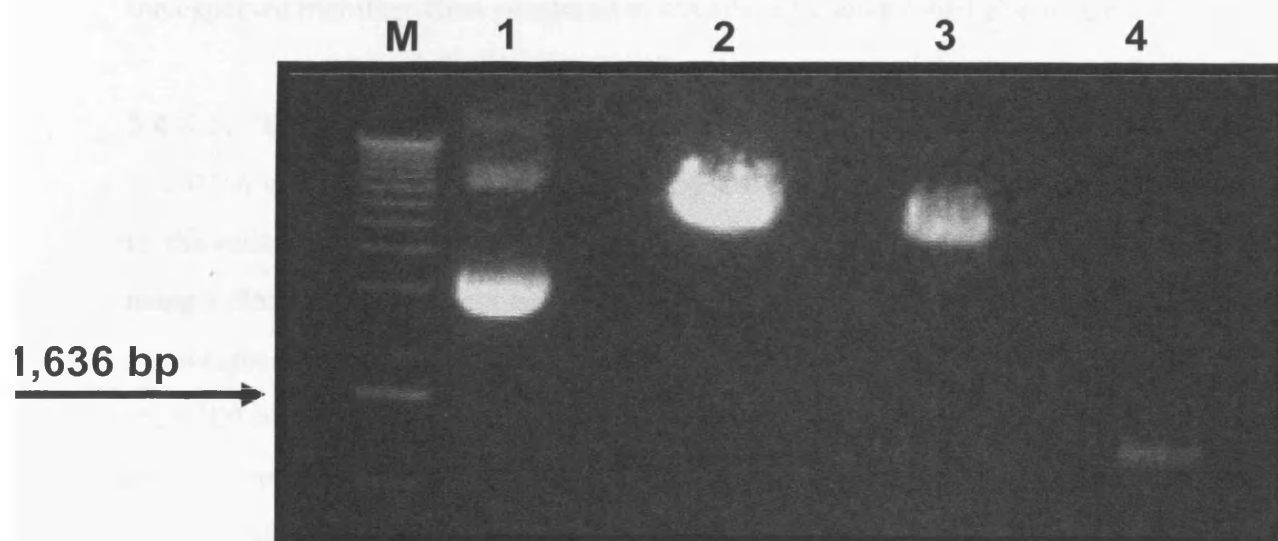


Fig. 5.1B Restriction enzyme analysis of pcDNA3 and M_1 PCR product

Restriction enzyme analysis, using *Bam*HI and *Xho*I, of pcDNA3 (lane 2), pcDNA3 with alkaline phosphatase treatment (lane 3) and M_1 mACh receptor PCR product (lane 4). All bands are of the expected size when compared to the Invitrogen 1 kb ladder. An undigested pcDNA3 control is included (lane 1) to show successful digestion of the vector.

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	M ₁ PCR product	pcDNA3
DNA	15 µl	5 µl
Compatible 10 x buffer	2 µl	2 µl
<i>Bam</i> HI	1 µl	1 µl
<i>Xho</i> XI	1 µl	1 µl
ddH ₂ O	1 µl	11 µl

To one of the pcDNA3 restriction samples 1 µl of alkaline phosphatase was added to reduce the chances of self-ligation, 30 min prior to the completion of the digest. After the incubation period, DNA gels performed as per section 4.4.1.9 to check for the presence of a PCR fragment of the correct mobility.

Fig. 5.1B shows the *Bam* HI and *Xho* XI, the pcDNA3 is clearly linearized when compared to the uncut vector. Both the digested vector and PCR product are running at the expected mobility when compared to markers of known molecular weight.

5.4.1.5. Extraction of DNA from agarose gel

The DNA was recovered from the agarose gel using a SpinPrep™ gel DNA kit according to the manufacturers instructions. Briefly, DNA fragments were excised from the gel, using a clean scalpel taking care to minimize the excess gel. Each slice was transferred to pre-weighed Eppendorf tubes and re-weighed to calculate the weight of each slice. For each 100 mg of gel 300 µl of SpinPrep gel-melt solution was added and the slice melted by incubating for 10 min at 50°C. This solution was added to a SpinPrep filter and centrifuged at 10,000 xg in a microcentrifuge. The supernatant was discarded and washed with 400 µl SpinPrep gel-melt solution and centrifuged at 10,000 xg. This procedure was repeated twice using 650 µl SpinPrep wash buffer. The SpinPrep filter was transferred to a clean Eppendorf tube, 50 µl of pre-warmed SpinPrep elution buffer was added and the tubes incubated for 3 min at 50°C. The purified DNA was eluted by centrifugation at 10,000 xg in a microcentrifuge.

5.4.1.6. Ligation of DNA

The result of a ligation reaction depends on the relative concentrations of the DNA in the solution, if the vector is too dilute/too concentrated, concatamers or circular molecules may be formed (Powell and Gannon, 2000). To this end the concentration of vector is kept constant and the varying amounts of PCR reaction are added. Ligation was performed using a ClonablesTM kit according to the manufacturers instructions.

The following equation was used to calculate pmol DNA

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

mass of DNA was determined by absorbance of $\lambda=260/280$ nm using a spectrophotometer.

The following ligation recipes were prepared:

Insert:vector ratio	1:2	1:3	1:4
pcDNA3 vector	1 μ l (0.025 pmol)	1 μ l (0.025 pmol)	1 μ l (0.025 pmol)
PCR insert	1.8 μ l (0.050 pmol)	2.7 μ l (0.075 pmol)	3.6 μ l (0.10 pmol)
nuclease-free H ₂ O	2.2 μ l	1.3 μ l	0.4 μ l
2 x ligation mix	5 μ l	5 μ l	5 μ l

Duplicate reactions were carried out containing the alkaline phosphatase-treated vector to try to optimize the ligation conditions. The ligation mix, containing appropriate buffer and DNA ligase, was added last and the reaction carried out for 15 min at 16°C. Following incubation the sample reactions were stored at 4°C until ready to proceed.

5.4.1.7. Transformation of ligation products

Transformations using NovaBlue Strain SinglesTM competent cells were carried out using the manufacturers protocol. For each transformation reaction, 50 μ l of competent cells were thawed for 5 min on ice. For each transformation 1 μ l of the ligation was added

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directly to the cells, the tubes were gently flicked and incubated for 5 min on ice. The cells were then heat-shocked by incubation at 42°C for 30 sec after which the tubes were returned on ice for a further 2 min. To each tube 250 μ l of SOC (pre-warmed to room temperature) was added and the tubes incubated for 30 min at 37°C with shaking at 250 r.p.m.

Ten μ l and 90 μ l of each sample transformation was plating out onto LB-amp plates (prepared as per section 4.4.1.5). The plates were left for 30 min to allow excess liquid to be absorbed before inverting and incubating at 37°C for 20-24 h. We have found that the ligation reactions take significantly longer to form visible colonies than standard/mutagenesis transformations.

5.4.1.8. Small-scale preparation of DNA

Ligation reactions are generally of poor efficiency and across all 6 plates only 16 colonies were visible after a 20 h incubation period. Therefore, to maximize the probability of a successful ligation clone, all 16 colonies were picked for small-scale preparation of DNA (see section 4.4.1.7 for small scale preparation of DNA method).

5.4.1.9. Restriction digest

Double restriction digest were carried out using the recipes below followed by incubation at 37°C for 2 h.

	M_1-WT
10 x compatible buffer	2 μ l
<i>Bam</i> HI enzyme	1 μ l
<i>Xho</i> XI	1 μ l
DNA	5 μ l
ddH ₂ O	11 μ l

5.4.1.10. Gel electrophoresis

The DNA gel was performed as per section **4.4.1.9.** and the respective image is shown in **Fig. 5.2.** Clones taken as a successful indicator of ligation are those which produced two bands, one at approx. 5,500 bp corresponding to the pcDNA3 vector and one at approx. 1.5 kb corresponding to the M₁ receptor fragment. On the basis of this restriction analysis DNA from clones 2, 3, 4, 5, 8, 10 and 11 were selected. To ensure the presence of functional M₁ receptor, DNA from these clones was transiently transfected into CHO cells plated on 24-well plates (0.2 µg DNA per well) and the expression of M₁ mACh receptor verified by [³H]-NMS binding.

5.4.1.11. Large-scale preparation of DNA

On the basis of cell-surface expression and restriction fragment analyses, clones 3 and 4 were selected for further analysis. Large-scale preparation of DNA was performed as per section **4.4.1.10..**

Fig. 5.3 shows the restriction analysis, using *Bam* HI and *Xho* XI, of the two selected m1 receptor pcDNA3 clones. Both were verified by sequencing analysis prior to further experimentation.

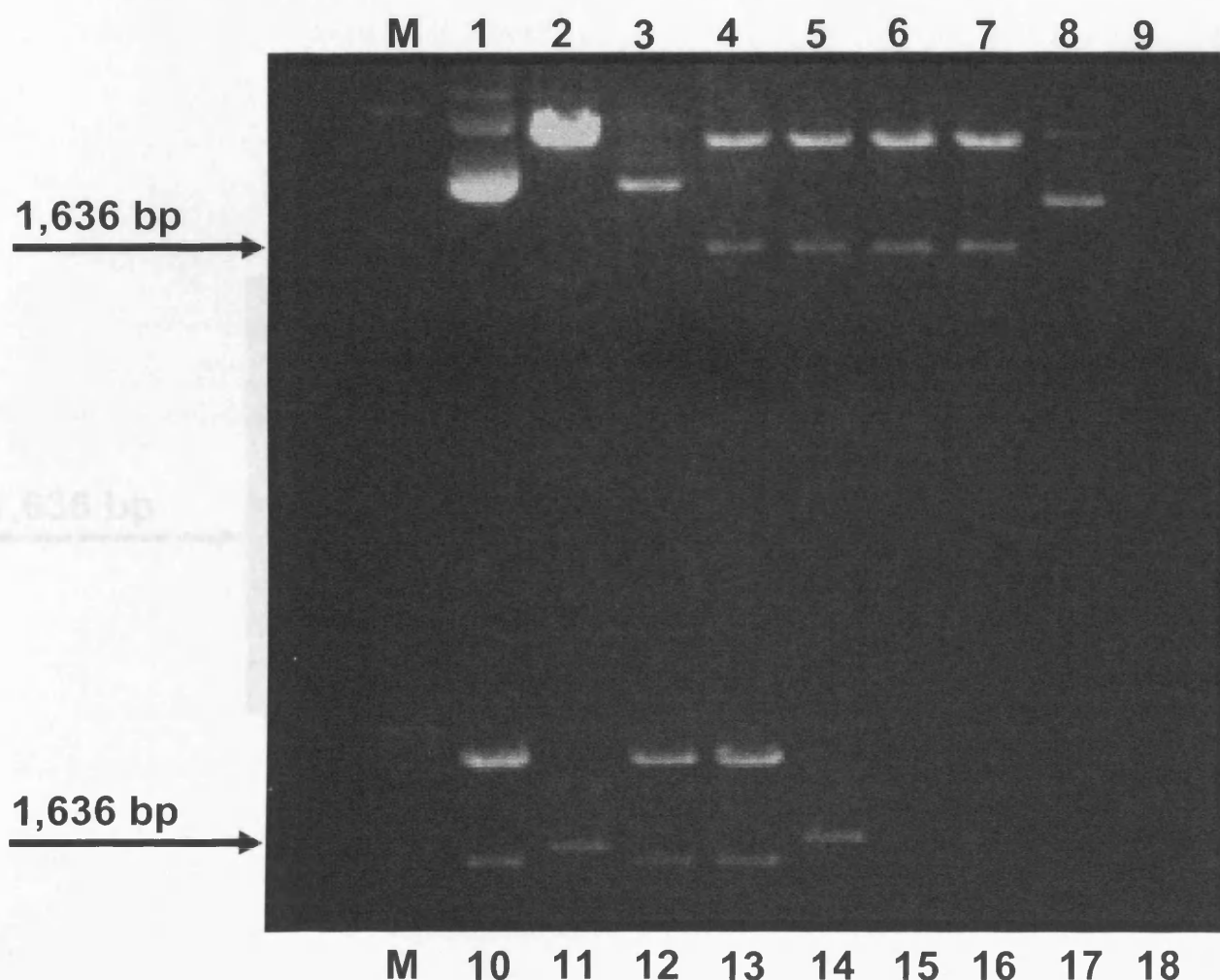


Fig. 5.2 Restriction enzyme analysis of DNA ligation preparations

Restriction enzyme analysis, using *Bam* HI and *Xho* I, of pcDNA3 (lane 2) and ligation clones 1 – 16 (lanes 3-18 respectively). Double digests were performed with *Bam* HI and *Xho* I, an undigested pcDNA3 (lane 1) was included as a digestion control. pcDNA3 contains a restriction site for each enzyme separated by only a few base pairs and therefore runs as a single band of linearised DNA. Clones with successful ligation of vector + insert show bands running at approximately 1.5 and 5 kb (e.g. lanes 12 and 13). An Invitrogen 1 kb ladder was used.

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5.4.2. Part II - Creation of the M_1 mACh mutant receptor

5.4.2.1. Primer design

A pDNA construct encoding the full-length human M_1 mACh receptor, *hM1*, was sub-cloned into pcDNA3 and sequenced (see 5.4.1 above). Mutation was carried out using the QuikChange site-directed mutagenesis kit (Stratagene) using the

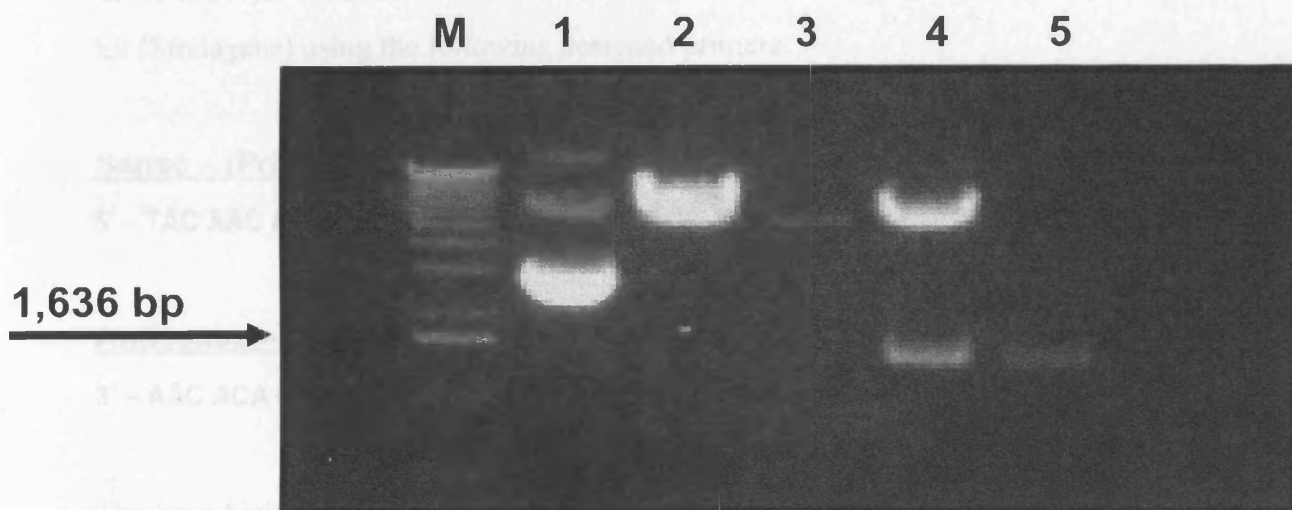


Fig. 5.3 Restriction enzyme analysis of DNA maxi-prep clones

Restriction enzyme analysis using, *Bam* HI and *Xho* I, of pcDNA3 (lane 2) and ligation clones 3 and 4 (lanes 3 and 4) respectively). An undigested pcDNA3 vector (lane 1) was included as a digestion control. pcDNA3 contains a restriction site for each enzyme separated by only a few base pairs and therefore runs as a single band of linearised DNA. Clones with successful ligation of vector + insert show bands running at approx. 1.5 and 5 kb (e.g. lanes 12 and 13). An Invitrogen 1 kb ladder was used. Lane 5 contains the M_1 PCR product used a positive control to confirm the motility of the lower weight product from clones 3 and 4.

5.4.2. Part II - Creation of the M₁ mACh mutant receptor

5.4.2.1. Primer design

A pcDNA construct encoding the full-length human M₁ mACh receptor, cloned within *Bam* HI and *Xho* XI, was sub-cloned into pcDNA3 and sequence verified (see section 5.4.1 above). Mutation was carried out using the QuikChange® site-directed mutagenesis kit (Stratagene) using the following designed primers:

Sense – (Primer 1)

5' – TAC AAC ATC ATG GTG CTG GTG TAC ACC TTC TGC AAG GAC TGT GTT - 3'

Antisense – (Primer 2)

3' – AAC ACA GTC CTT GCA GAA GGT GTA CAC CAG CAC CAT GAT GTT GTA - 3'

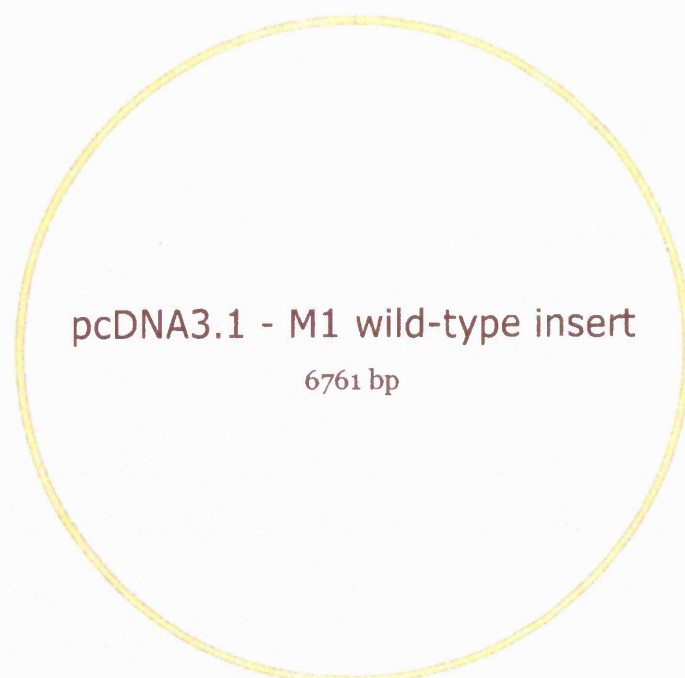
The base highlighted in **RED** encodes the amino acid residue switch from Ser to Tyr at position 388 (S388Y). This particular base change substitution was chosen as it fortuitously produces an unique restriction recognition site for the restriction enzyme *Ssp* BI that is not present in the wild-type sequence, see **Fig. 5.4**.

5.4.2.2. Mutant strand synthesis (thermal cycling)

Complementary oligonucleotide primers were diluted to a concentration of 125 µg ml⁻¹ in ddH₂O. The following reactions were prepared on ice in thin-walled PCR tubes

Mutagenesis control reaction:	5 µl	10x reaction buffer
	2 µl	pWhitescript 4.5-kb control plasmid
	1.25 µl	control primer #1
	1.25 µl	control primer #2
	1 µl	dNTP mix
	39.5 µl	ddH ₂ O

A.



B.

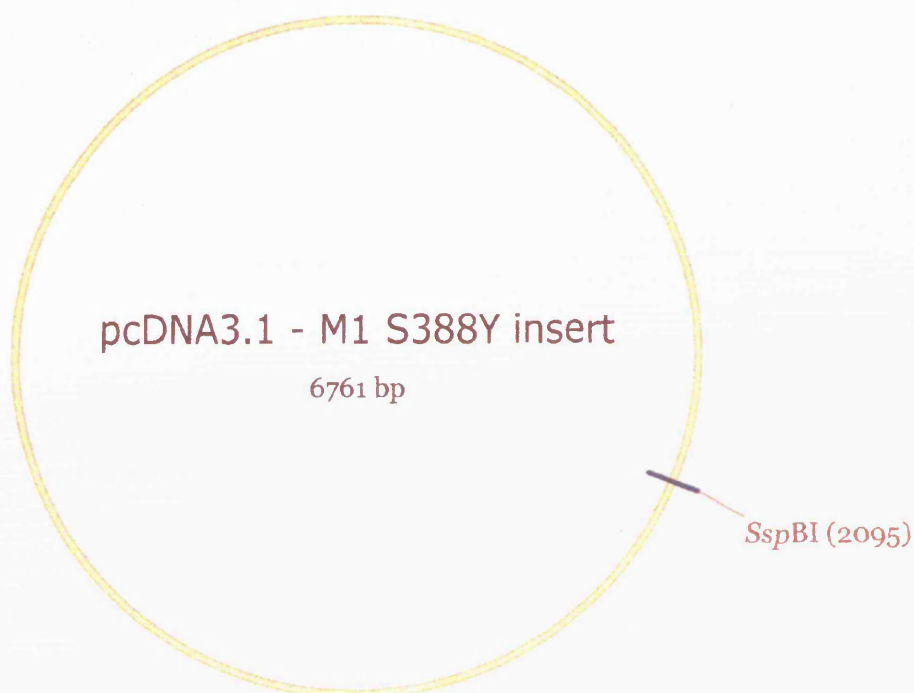


Fig. 5.4 *Ssp* BI restriction map and digestion fragments

The expected restriction digest maps of *Ssp* BI on M₁-WT and ^{S388Y}M₁ mACh receptor cloned into pcDNA3. With the incorporation of a restriction site in ^{S388Y}M₁ the restriction fragments produced by the digest of *Ssp* BI on the two plasmids can clearly be differentiated.

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$S^{388Y}M_1$ sample reaction:	5 μ l	10x reaction buffer
	2 μ l	pcDNA + M_1 -WT template (at 25 μ mol ml ⁻¹)
	1.25 μ l	sense primer
	1.25 μ l	anti-sense primer
	1 μ l	dNTP mix
	39.5 μ l	ddH ₂ O

Each sample mixture was gently mixed and then 1 μ l of pfu Turbo DNA polymerase added. The samples were then subjected to the following thermal cycle: 1 cycle 30 sec at 95°C; and 16 cycles of 30 sec at 95°C, 1 min at 55°C, and 7.5 min at 68°C. Samples were then held at 4°C before proceeding.

5.4.2.3. *Dpn* I digestion of the PCR products

Dpn I digestion was carried out as described in section 4.4.1.3.

5.4.2.4. Transformation of XL1-Blue supercompetent cells

Transformations of XL1-Blue supercompetent cells were carried out as described in section 4.4.1.4.

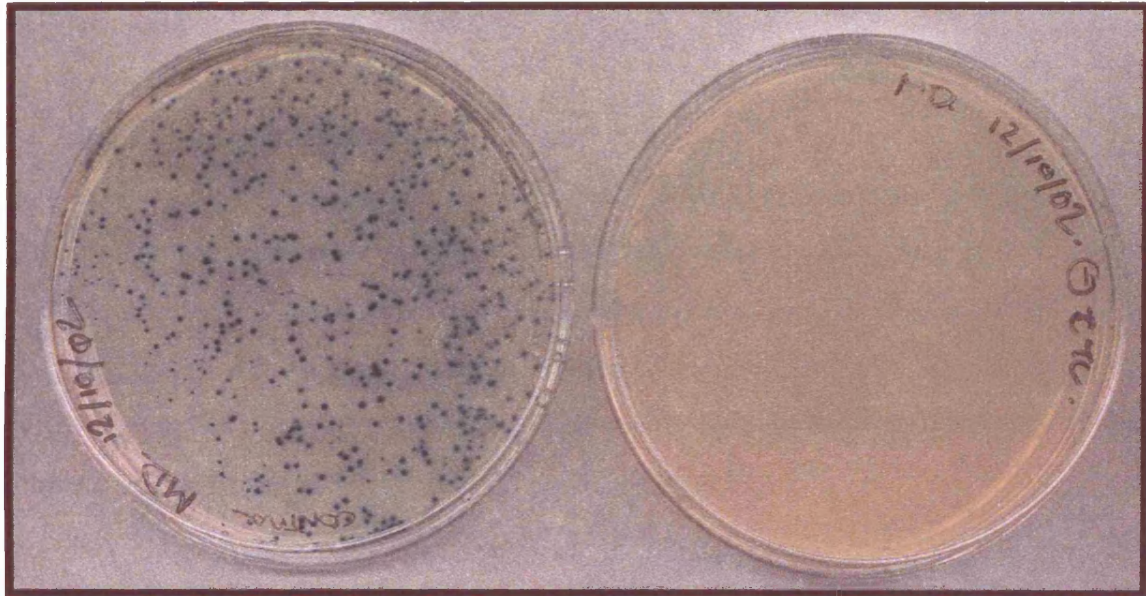
5.4.2.5. Plate preparation

Plates were prepared as described in section 4.4.1.5.

5.4.2.6. Colony screening

Colonies were screened as described in section 4.4.1.6.

Fig. 5.6 shows the colony plates after a 20 hour incubation, indicating mutagenesis efficiency of almost 100%.



**B. pWhitescript 4.5kB
mutagenesis control**

A. Untransformed bacteria

Fig. 5.5 Colony growth after 16 hour incubation

A. - untransformed bacteria (negative control) indicating all growth on subsequent plates was due to successful transformation with ampicillin-resistant plasmids. **B.** – mutagenesis plates, blue colonies indicate successful control mutagenesis.

5.4.2.7. Small-scale preparation of DNA

Eighteen well-separated colonies were picked for small-scale DNA preparation, which was carried out as detailed in section 4.4.1.7.

5.4.2.8. Restriction digest

The incorporation of the silent mutation into the ^{S388Y}M₁ mACh receptor gene produces a different restriction fragment pattern compared to the wild-type M₁-mACh receptor when digested with *Ssp* BI. Therefore, restriction digest followed by DNA gel electrophoresis allows for an initial assessment of successful mutagenesis clones before large scale-DNA preparation and sequencing. Restriction digest were carried out using the recipes below followed by incubation at 37°C for 2 h.

	M₁-WT	^{S388Y}M₁
10 x supplied buffer	2 µl	2 µl
<i>Ssp</i> BI enzyme	1 µl	1 µl
DNA	1 µl	10 µl
ddH ₂ O	16 µl	7 µl

5.4.2.9. Gel electrophoresis

DNA gels were cast and as detailed in section 4.4.1.9. **Fig. 5.6A** shows a selection of some of the 18 digested clones. Due to the presence of coiled and supercoiled DNA, undigested DNA runs with at least two mobilities. M₁-WT contains no restriction site for *Ssp* BI and therefore runs similarly to undigested DNA. ^{S388Y}M₁ DNA contains a unique restriction site for *Ssp* BI and therefore runs as a single fragment of linear DNA. On the basis of the digestion clones 4 and 5 were chosen for large scale DNA preparation.

5.4.2.10. Large-scale preparation of DNA

Large-scale preparation of DNA from clones 4 and 5 was carried out as detailed in section 4.4.1.10.

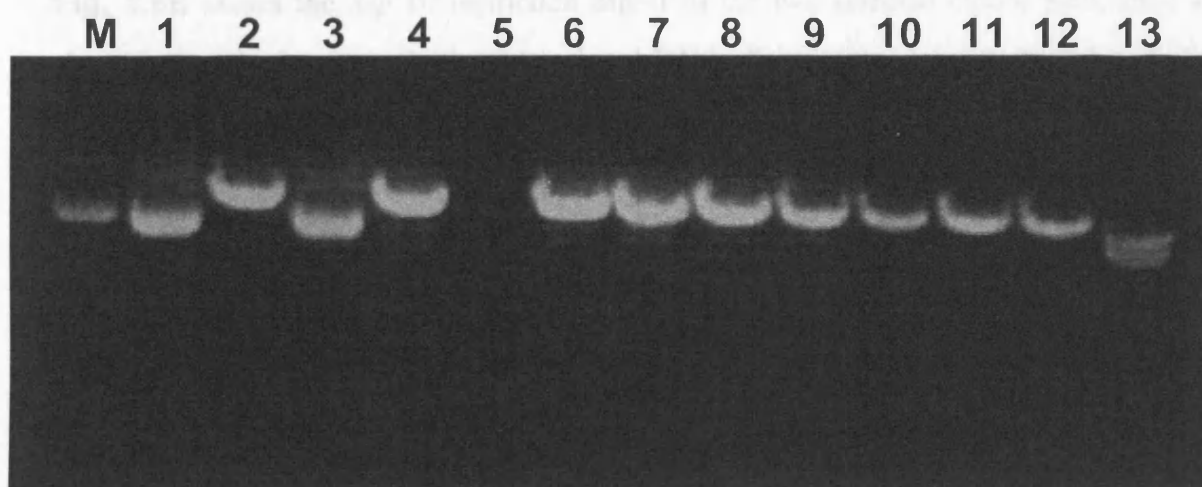


Fig. 5.6.A Restriction enzyme analysis of DNA mini-prep clones

Restriction enzyme analysis, using *Ssp* BI, of M_1 -WT (lane 1) and $S^{388Y}M_1$ mACh receptor clones (lanes 6-13). Undigested DNA (lane 3) runs at least two weights (coiled and supercoiled DNA). pcDNA3 cut with *Bam* HI and *Xho* I, to linearized the DNA was used as controls (lanes 2 and 4 respectively). Successful mutagenesis of the m1 DNA will produce clones that run as single linear bands of DNA (e.g. lanes 6-12).

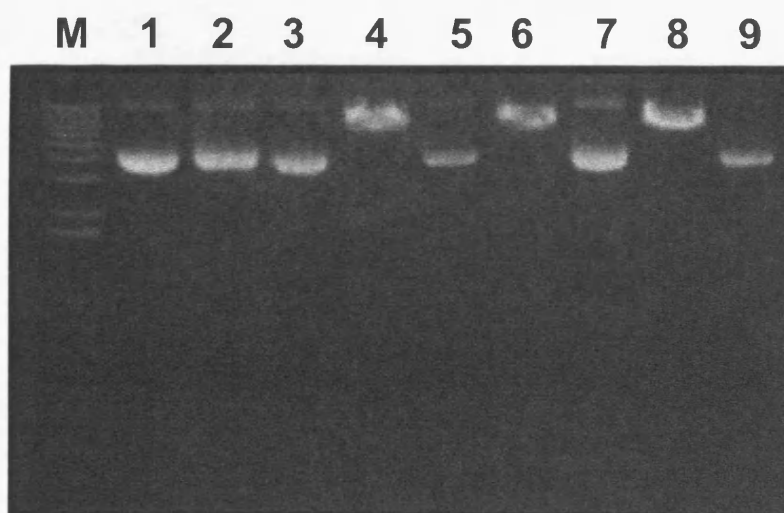


Fig. 5.6.B Restriction enzyme analysis of DNA maxi-prep clones

Restriction enzyme analysis, using *Ssp* BI, of M_1 -WT (lanes 1-4) and $S^{388Y}M_1$ receptor clone 4 and 5 (lanes 5-9). Lane 1 shows uncut pcDNA3 + M_1 -WT insert running identical to *Ssp* BI digested pcDNA3 + M_1 -WT (lanes 2 and 3). Lane 4 contains M_1 -WT digested with *Bam* HI, known to linearize the vector, as a control. Lane 5 & 6 and 7 & 8 contain clones 4 and 5, respectively, +/- *Ssp* BI. Lane 9 contains the M_1 PCR product as a molecular marker.

Fig. 5.6B shows the *Ssp* BI restriction digest of the two selected clones performed as above, except only using 2 µl of the clonal DNA. Prior to experimentation successful mutagenesis was confirmed by sequencing.

5.4.3. Part III - Pharmacological characterization of a constitutively-active, mutant M₁ mACh receptor

5.4.3.1. Binding studies

To allow comparative analysis between the M₁ and M₃ mACh receptors HEK-293 cells were selected for transient transfection experiments. Initial experiments on plated whole-cell monolayers revealed that the expression level of the ^{S388Y}M₁ receptor was consistently 2 fold less than that of the M₁-WT receptor when equivalent quantities (μg DNA well⁻¹) were transfected. When appropriate, this difference was corrected for in experiments allowing a relatively accurate matching of mutant/WT receptor densities.

The expression level of the small endogenous M₃ mACh receptor population is approx. 50 fmol mg⁻¹. The same rationale, detailed in section 4.4.2.1.1, is applicable to the transfected M₁ mACh receptors and therefore two controls were always utilized in binding experiments: untransfected and empty-vector transfected cells.

5.4.3.1.1. Expression levels and [³H]-NMS affinity

As the ^{N514Y}M₃ mACh receptor displayed significantly reduced [³H]-NMS affinity when compared to M₃-WT, initial experiments were conducted to determine the K_D of NMS at both M₁ receptor types. **Fig. 5.7A, B** shows representative saturation binding curves for the M₃-WT (**A**) ^{S388Y}M₁ (**B**) receptors, respectively. Protein concentration was adjusted such that maximal binding for each concentration did not exceed more than 5% of that added (in terms of d.p.m.). When protein concentration was accounted for the expression of the M₁-WT receptor was between 1200-2500 fmol mg⁻¹ protein, whereas that of the ^{S388Y}M₁ mACh receptor was between 800-1500 fmol mg⁻¹ protein. These preparations, with the indicated receptor expression levels, were used for all the detailed binding experiments.

Across all the membrane preparations the K_D of [³H]-NMS was significantly greater at the ^{S388Y}M₁ receptor when compared to the M₁-WT receptor (WT, 148 ± 5; mutant, 291 ± 40 pM, *p*<0.05; paired Student's *t*-test). The difference in [³H]-NMS K_D values for the

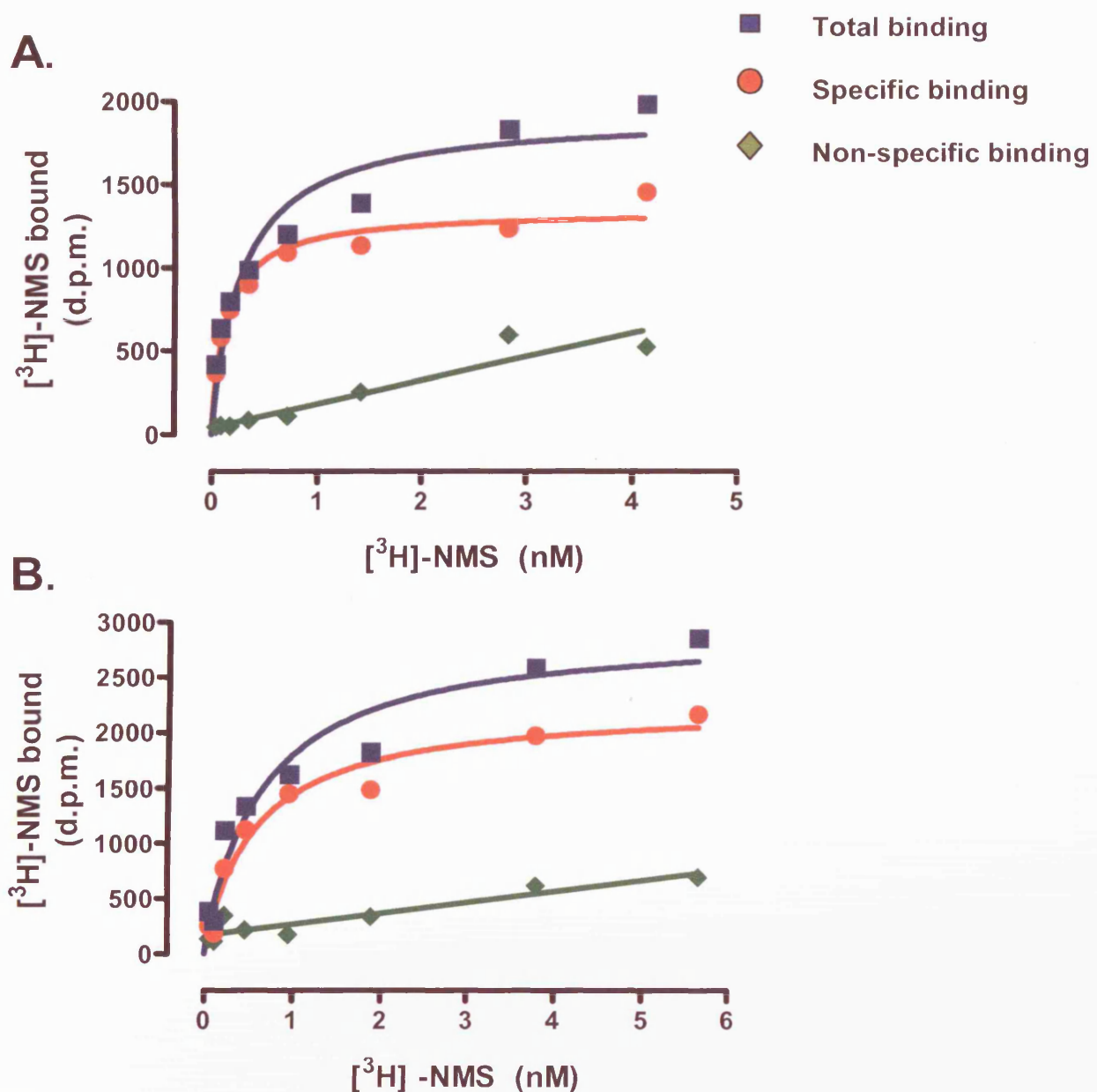


Fig. 5.7 $[^3H]$ -NMS binding to human mACh receptors in membranes prepared from transiently transfected HEK cells.

Membranes were prepared from HEK-cells transiently transfected with either **A.** M_1 -WT or **B.** $S^{388Y}M_1$ mACh receptor cDNA, were incubated in the presence or absence of atropine for 60 min. All experiments were performed in duplicate and the graphs above are representative of between 6-8 separate experiments. Receptor densities and dissociation constants for NMS are summarized in the main text (section 5.4.3.1.1).

Chapter 5: Creation and characterization of a constitutively-active, mutant M₁ mACh receptor

M₁-WT and ^{S388Y}M₁ mACh receptors can also be observed in the Rosenthal/Scatchard analysis shown in **Fig. 5.8**. These data are included for informative purposes only and were not used to calculate the affinity values for [³H]-NMS.

5.4.3.1.2. Affinity of mACh receptor antagonists

The same four antagonists (shown in **Fig. 4.11**) employed in the M₃ study (see Chapter 4) were also used here to assess affinity differences at the two M₁ mACh receptors. **Figs. 5.9** and **5.10** illustrate the [³H]-NMS displacement curves by the various antagonists and the corrected pK_i values are summarized in **Table 5.1**. Although all antagonists tested show modest reductions in affinity at the ^{S388Y}M₁ receptor, all WT-mutant differences were significant except that for methoctramine. The rank order of affinity reduction at the ^{S388Y}M₁ receptor (c.f. cognate M₁-WT value) was: pirenzepine>4-DAMP=atropine>>methoctramine. All values for antagonists at the M₁-WT mACh receptor are in good agreement with those from the literature (Caulfield and Birdsall, 1998). All slope factors were not significantly different from unity, except those obtained from methoctramine (see section **4.4.2.1.2.** for a discussion of the possible reasons for this relatively high slope factor).

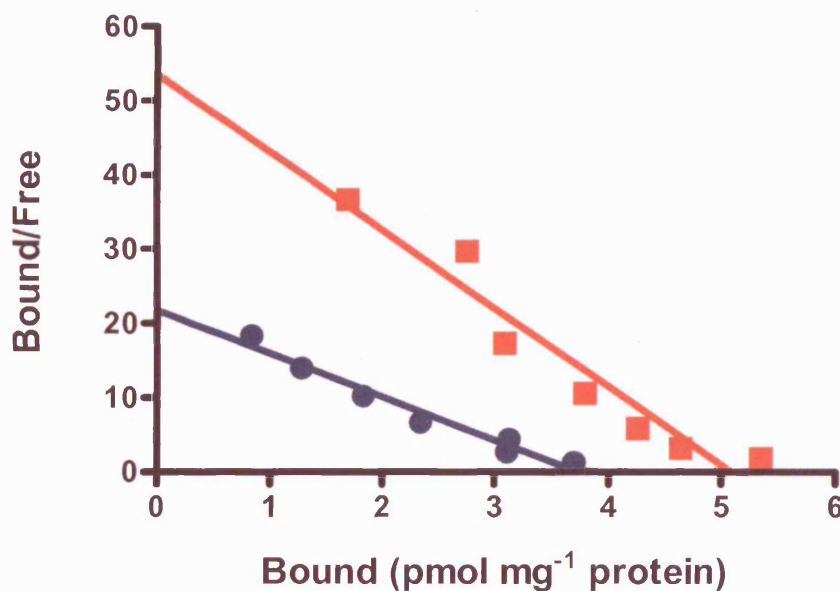


Fig. 5.8 Scatchard transformation of [³H]-NMS binding data for human mACh receptors in membranes prepared from transiently transfected HEK cells.

Membranes were prepared from HEK-cells transiently transfected with **A.** M_1 -WT and **B.** $S^{388Y}M_1$ receptor cDNA were incubated in the presence or absence of atropine for 60 min. Data obtained has been transformed into a Scatchard plot to demonstrate the different affinity (K_D) of [³H]-NMS for the two receptor forms. Data shown are representative of at least 6-8 separate experiments performed in duplicate.

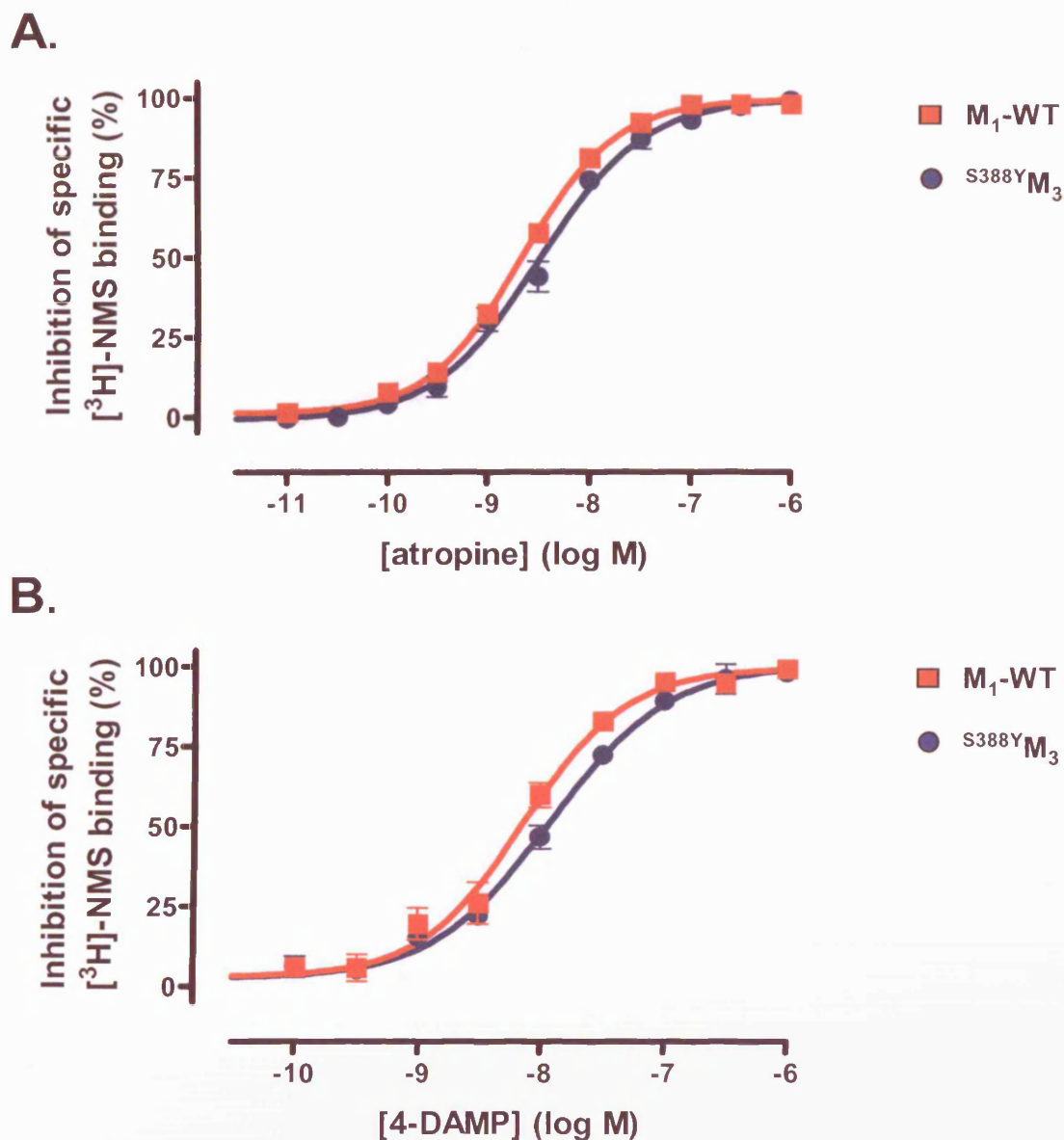


Fig. 5.9 Antagonist displacement of $[^3\text{H}]\text{-NMS}$ binding to human mACh receptors in membranes prepared from transiently transfected HEK cells.

Inhibition binding experiments on $M_1\text{-WT}$ (■) or $S388Y M_1$ (●) mACh receptors, performed on membrane preparations obtained from transiently transfected HEK293 cells, in the presence of 300-500 pM $[^3\text{H}]\text{-NMS}$. Data shown represent the mean $\pm$ S.E.M from at least three experiments performed in duplicate. Summary data for the antagonists are shown in

Table 5.1.

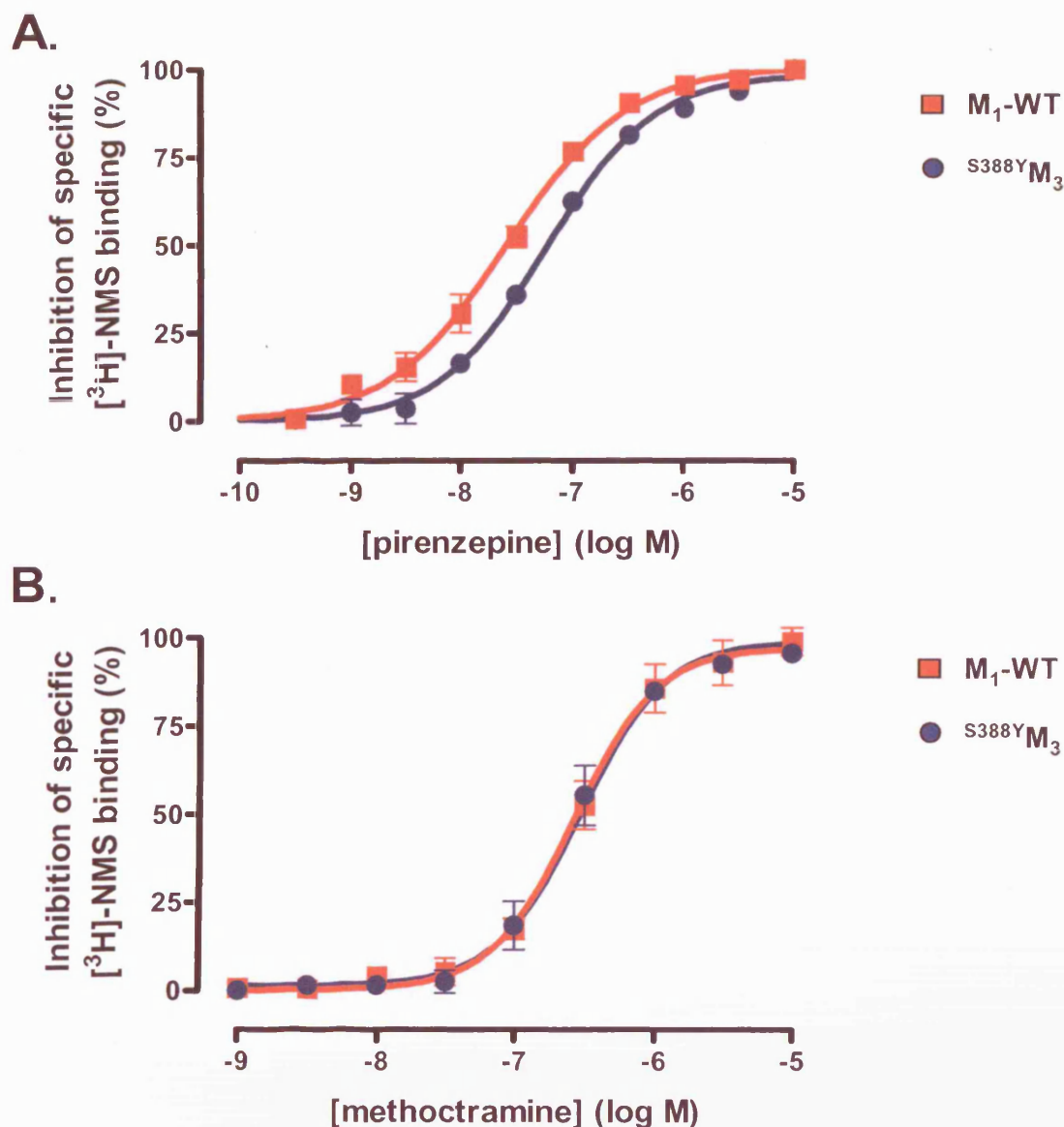


Fig. 5.10 Antagonist displacement of [3 H]-NMS binding to human mACh receptors in membranes prepared from transiently transfected HEK cells.

Inhibition binding experiments on M_1 -WT (■) or $S388Y M_1$ (●) mACh receptors, performed on membrane preparations obtained from transiently transfected HEK293 cells, in the presence of 300-500 pM [3 H]-NMS. Data shown represent the mean $\pm$ S.E.M from at least three experiments performed in duplicate. Summary data for the antagonists are shown in **Table 5.1**.

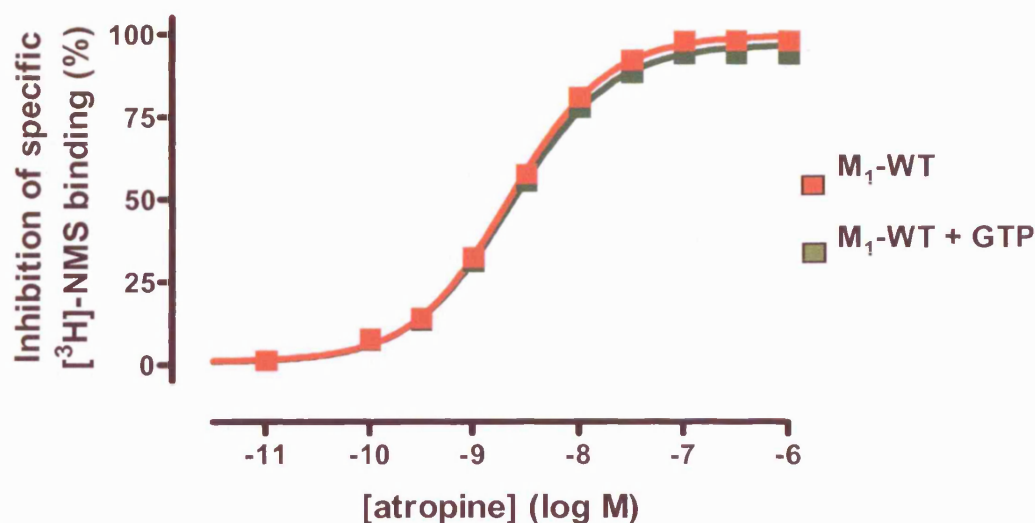
Table 5.1 Affinity constants for antagonists at M_1 -WT and $S^{388Y}M_1$ receptors

	M_1 -WT		$S^{388Y}M_1$		
antagonist	corrected pK_i	Hill slope	corrected pK_i	Hill Slope	fold-change
atropine	9.25 ± 0.03	0.96 ± 0.01	8.90 ± 0.06	0.91 ± 0.02	2.2*
4-DAMP	8.81 ± 0.03	1.06 ± 0.10	8.36 ± 0.02	0.94 ± 0.06	2.8*
pirenzepine	8.25 ± 0.09	0.86 ± 0.10	7.70 ± 0.04	0.88 ± 0.06	3.5*
methoctramine	7.24 ± 0.10	1.58 ± 0.22	7.15 ± 0.09	1.41 ± 0.25	1.2

Data are from assays shown in **Figs. 5.9** and **5.10**. Corrected IC_{50} values (pK_i values) were calculated according to the method of Cheng and Prusoff, (1973), using the appropriate [3H]-NMS K_D value. Fold changes represent the lower/higher affinity of the $S^{388Y}M_1$ receptor (obtained by dividing the pK_i of mutated receptor by the pK_i of the wild-type receptor for antagonists). Data represent the mean $\pm$ S.E.M. for at least three experiments. *indicates $S^{388Y}M_1$ corrected affinity is statistically different to that obtained for the M_1 -WT receptor ($p < 0.05$; unpaired Student's t -test).

As discussed in section **4.4.2.1.2**. GTP reduces the affinity of agonists by decreasing the proportion of receptors (pre)-coupled to G-proteins. If we assume that an inverse agonist has higher affinity for the **R** state, then we might expect an increase in its affinity with the inclusion of GTP. **Fig. 5.11** illustrates the effects of supplementing the binding medium with GTP (100 μ M) for both M_1 -WT and $S^{388Y}M_1$ mACh receptors. Under the binding conditions employed GTP had no significant on the pIC_{50} values derived from the atropine curves (M_1 -WT, 8.65 ± 0.04 and 8.63 ± 0.08 ; $S^{388Y}M_1$, 8.48 ± 0.05 and 8.49 ± 0.04 in the absence and presence of GTP, respectively). Additionally no significant differences were seen for the other three antagonists tested (data not shown). As with the

A.



B.

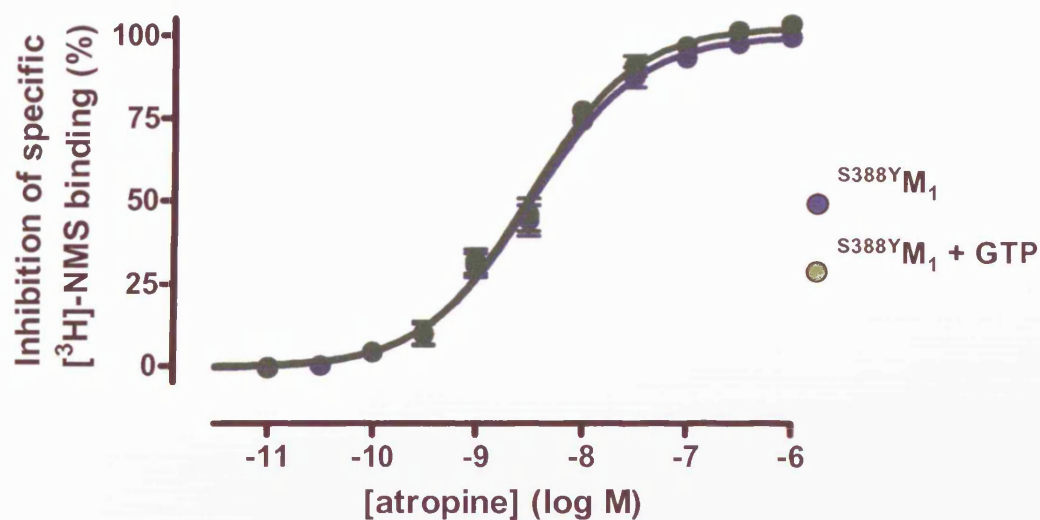


Fig. 5.11 Effect of GTP on antagonist displacement of [3 H]-NMS binding to human mACh receptors in membranes prepared from transiently transfected HEK cells.

Inhibition binding experiments on M_1 -WT (■) or $S388Y M_1$ (●) mACh receptors, performed in the absence or presence (■ / ●) of 100 μ M GTP, on membrane preparations obtained from transiently transfected HEK293 cells, in the presence of 300-500 pM [3 H]-NMS. Data shown represent the mean $\pm$ S.E.M from at least three experiments performed in duplicate. Summary data for the antagonists are shown in **Table 5.1**.

effects of GTP on the M₃ mACh receptor antagonist affinity curves, this could reflect the difficulty in quantifying an expected small increase in affinity, or possibly a more complex interaction with receptor conformations.

5.4.3.1.3. Affinity of mACh receptor agonists

The extended version of the ‘allosteric ternary complex model’ assumes that only the **R*** conformation can efficiently interact with G proteins, and agonists increase the population of receptors that reside in this active state (Cotecchia *et al.*, 2003). In a system where the proportion of the receptors residing in the active conformation is increased, the prediction is that agonists will display a higher affinity. **Figs. 5.12** and **5.13** show [³H]-NMS displacement curves for three agonists, the structures of which have been previously shown in **Fig. 3.8**. **Table 1.2** summarizes the corrected pK_i values and slope factors obtained for the three agonists.

Methacholine displays a significantly increased affinity at the ^{S388Y}M₁ receptor compared to M₁-WT (pK_i values, 5.12 ± 0.05 and 4.39 ± 0.10 , respectively, $p < 0.05$; unpaired Student’s *t*-test). Interestingly, the slope factor for the ^{S388Y}M₁ receptor was significantly reduced compared to M₁-WT (0.59 ± 0.03 and 0.80 ± 0.04 , respectively, $p < 0.05$; unpaired Student’s *t*-test). Pilocarpine (a partial agonist) similarly exhibited increased affinity at the ^{S388Y}M₁ compared to M₁-WT receptor, albeit modestly compared to methacholine (pK_i values, 5.15 ± 0.05 and 5.60 ± 0.04 , respectively, $p < 0.05$; unpaired Student’s *t*-test). There was no significant difference between the slope factors obtained for pilocarpine at the ^{S388Y}M₁ and M₁-WT receptors (0.76 ± 0.04 and 0.84 ± 0.04 , respectively, $p > 0.05$; unpaired Student’s *t*-test).

Inhibition binding experiments on M₁-WT (■) or S^{388Y}M₁ (●) mACh receptors, performed on membrane preparations obtained from transiently transfected HEK293 cells, in the presence of 300-500 pM [³H]-NMS. Data shown represent the mean ± S.E.M from at least three experiments performed in duplicate. Summary data for the antagonists are shown in **Table 5.2.**

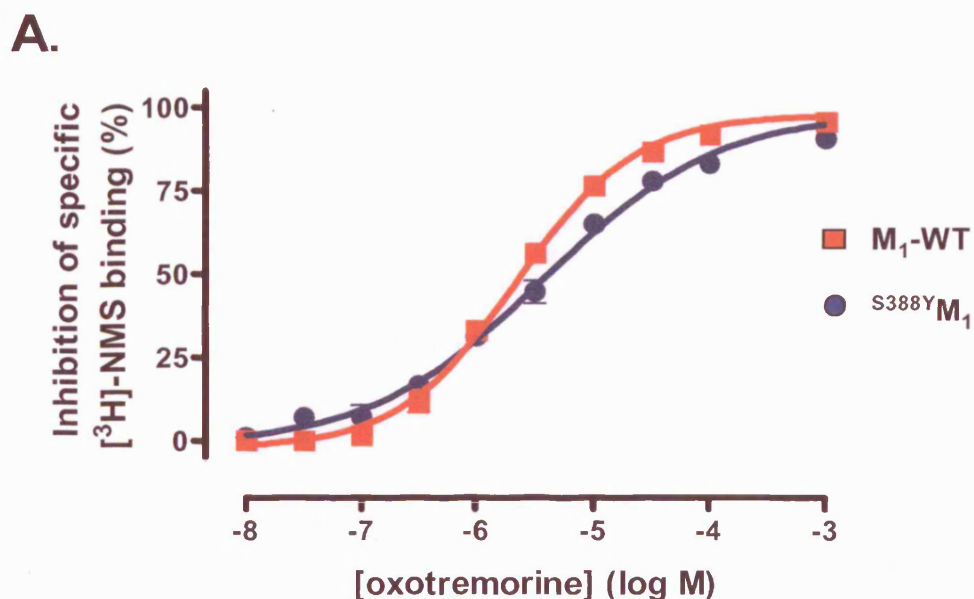


Fig. 5.13 Agonist displacement of $[^3H]$ -NMS binding to human mACh receptors in membranes prepared from transiently transfected HEK cells.

Inhibition binding experiments on M_1 -WT (■) or $S388Y M_1$ (●) mACh receptors, performed on membrane preparations obtained from transiently transfected HEK-293 cells, in the presence of 300-500pM $[^3H]$ -NMS. Data shown represent the mean $\pm$ S.E.M from at least three experiments performed in duplicate. Summary data for the antagonists are shown in **Table 5.2**.

Table 5.2 Affinity constants for agonists at M_3 -WT and $N^{514Y}M_3$ receptors

	M_3 -WT		$N^{514Y}M_3$		
agonist	corrected pK_i	Hill slope	Corrected pK_i	Hill Slope	fold-change
methacholine	4.39 ± 0.10	0.80 ± 0.04	5.12 ± 0.10	$0.59 \pm 0.03^{*a}$	5.4*
oxotremorine	6.30 ± 0.16	0.86 ± 0.16	6.05 ± 0.10	$0.58 \pm 0.08^{*a}$	n/a
pilocarpine	5.15 ± 0.05	0.84 ± 0.04	5.60 ± 0.04	0.76 ± 0.04	2.8*

Data are taken from data shown in **Figs. 5.12** and **5.13**. Corrected IC_{50} values (pK_i values) were calculated according to the method of Cheng and Prusoff, (1973), using the appropriate [3H]-NMS affinities. Fold change represents the lower/higher affinity of the $N^{514Y}M_3$ receptor (obtained by dividing the pK_i for the mutated receptor by the pK_i for the wild-type receptor). Data represent the mean $\pm$ S.E.M for at least three experiments. *indicates $N^{514Y}M_3$ pK_i is statistically different to that obtained for the M_3 -WT receptor ($p < 0.01$, unpaired Student's t -test). *i indicates $S^{388Y}M_1$ slope factor is statistically different to that obtained for the M_1 -WT receptor ($p < 0.05$; unpaired Student's t -test).

Oxotremorine is reported to be of higher efficacy than pilocarpine at the M_1 mACh receptor (Richards and Van Giersbergen, 1995b) and therefore it was expected that the extent of its affinity shift would lie somewhere between methacholine and pilocarpine. However, the affinity of oxotremorine was actually less at the $S^{388Y}M_1$ receptor when compared to the M_1 -WT receptor, although this did not reach significance (pK_i values, 6.05 ± 0.10 and 6.30 ± 0.16 , respectively, $p > 0.05$; unpaired Student's t -test). However, the slope factor of the oxotremorine-displacement isotherm for the $S^{388Y}M_1$ receptor was significantly reduced compared to that obtained for the M_1 -WT (0.58 ± 0.08 and 0.86 ± 0.10 , respectively, $p < 0.05$; unpaired Student's t -test). The significant differences in slope factors may represent a more complex interaction of agonists with receptor conformations at the mutant M_1 mACh receptor.

The predicted effects of GTP on agonist binding at wild-type and constitutively-active, mutant receptors has been thoroughly discussed in section 4.4.2.1.3. **Fig. 5.14** shows the effect 100 μ M GTP on methacholine-elicited [³H]-NMS displacement curves at M₁-WT and ^{S388Y}M₁ receptors. No significant effects of GTP were detected in M₁-WT receptor preparations with similar IC₅₀ values are slope factors (pK_i values, 4.41 ± 0.04 and 4.36 ± 0.05 ; slope factors, 0.83 ± 0.05 and 0.77 ± 0.04 ; in the absence and presence of GTP, respectively). However there was a significant effect of GTP on ^{S388Y}M₁ receptor preparations, with a decrease in MCh affinity and an increase in the slope factor (pK_i values, 5.21 ± 0.02 and 4.88 ± 0.04 ; slope factors, 0.60 ± 0.04 and 0.86 ± 0.07 ; in the absence and presence of GTP, respectively, $p < 0.05$, unpaired Student's *t*-test). Therefore, there appears to be a GTP-sensitive proportion of the high-affinity binding, which is only seen for the mutant ^{S388Y}M₁ receptor. Despite this, GTP-sensitive components for the binding curves for oxotremorine and pilocarpine could not be detected (data not shown).

5.4.3.2. Functional studies

5.4.3.2.1. Assessment of [³H]-InsP_x accumulation

The M₁/M₃/M₅ mACh receptors have been demonstrated to elicit robust agonist-mediated phosphoinositide hydrolysis (Wojcikiewicz and Nahorski, 1993). We therefore quantified [³H]-InsP_x accumulation (in the presence of millimolar Li⁺) in order to assess the effects of both agonists and antagonists on (any) constitutive activity at the M₁-WT and ^{S388Y}M₁ mACh receptors.

The optimization of this assay was reported for the M₃ mACh receptor in sections 4.4.2.2.1 – 4.4.2.2.3, and therefore an identical experimental protocol was used for both agonists and antagonists at both M₁-WT and ^{S388Y}M₁ mACh receptors. Note that preliminary experiments confirmed that these conditions produced robust, reproducible signals (data not shown). For agonist studies, cell monolayers in twenty-four well plates were transfected with 0.2 μ g and 0.6 μ g of M₁-WT and ^{S388Y}M₁ DNA, respectively. This gave an approx. equal expression of the receptors (1.2 – 2.0 pmol mg⁻¹ protein). For experiments looking at the effect of antagonists on basal [³H]-InsP_x accumulation, the M₁-

Inhibition binding experiments on M₁-WT (■) or S^{388Y}M₁ (●) mACh receptors, performed in the absence or presence (■ / ●) of 100 μM GTP, on membrane preparations obtained from transiently transfected HEK-293 cells, in the presence of 300-500 pM [³H]-NMS. Data shown represent the mean ± S.E.M from at least three experiments performed in duplicate.

WT was relatively over-expressed to maximize the chance of detecting any constitutive activity. The maximum expression of the M₁-WT receptors was achieved by transfection of 0.5 µg DNA well⁻¹, which yielded expression levels of 3-5 pmol mg⁻¹ protein. Similar to the N^{514Y}M₃ receptor, the maximum expression of the S^{388Y}M₁ receptor was considerably lower than its wild-type counterpart, with 0.6 µg DNA well⁻¹ yielding expression levels between 1.2 and 2 pmol mg⁻¹ protein.

5.4.3.2.2. Antagonist effects on basal [³H]-InsP_x accumulation

Fig. 5.15. shows the effect on maximal concentrations of antagonists (as assessed from binding experiments) initially for the M₁-WT (**A**) and S^{388Y}M₁ (**B**) mACh receptors run under the standard antagonist protocol established in Chapter 4. For all functional experiments two controls were run: (1) Untransfected wells to assess the contribution from the endogenous M₃ mACh receptor population (2) Wells transfected with empty vector to assess any variability due to the transfection process. For the same reasons as discussed in section 4.4.2.2.1 we are confident that the contribution of the minor endogenous M₃ population does not make a significant contribution to the data presented.

Despite a receptor density of approx. twice that of the S^{388Y}M₁, the M₁-WT receptor produced a basal [³H]-InsP_x accumulation that was 2-fold lower than for S^{388Y}M₁ (agonist-independent [³H]-InsP_x accumulations, 13604 ± 478 and 27114 ± 1487 d.p.m. mg⁻¹ protein, for M₁-WT and S^{388Y}M₁, respectively). The basal level of [³H]-InsP_x accumulation for the M₁-WT mACh receptor was not significantly elevated above levels measured for untransfected cells. This probably indicates that the M₁-WT receptor, even when over-expressed in HEK-293 cells, has a low propensity spontaneously to adopt an active conformation. This is exemplified by the finding that none of the four antagonists tested were able to inhibit significantly agonist-independent [³H]-InsP_x accumulation.

All four antagonists tested were able to reduce significantly the increased basal level of [³H]-InsP_x accumulation at the S^{388Y}M₁ mACh receptor. All antagonists reduced basal accumulations to those seen without Li⁺ addition (atropine, 14362 ± 1710; pirenzepine, 14640 ± 1375; 4-DAMP, 13586 ± 1462; methoctramine, 13242 ± 1136 d.p.m. mg⁻¹

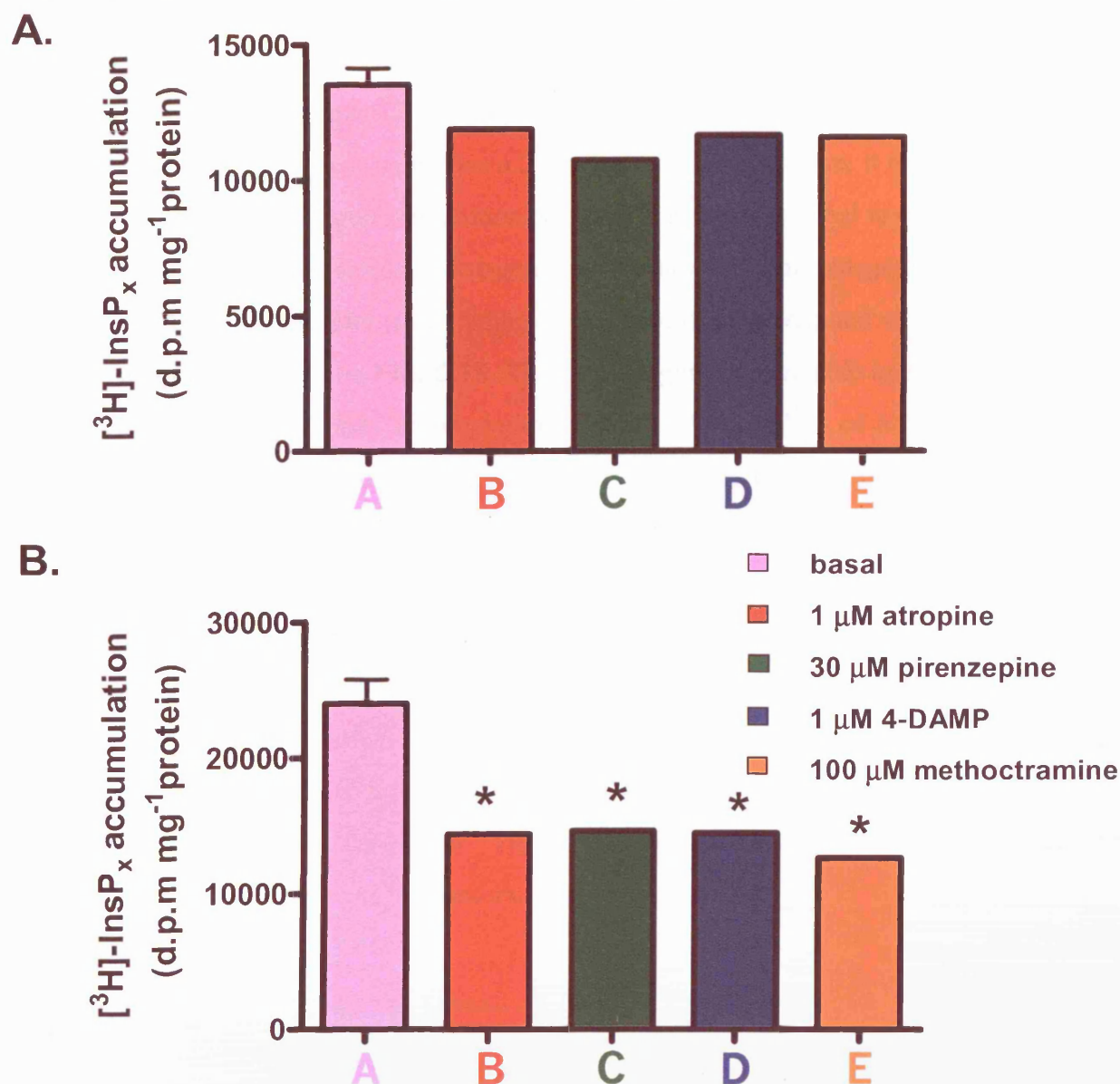


Fig. 5.15 Antagonist inhibition of agonist-independent $[^3\text{H}]\text{-InsP}_x$ accumulation in the presence of Li^+ .

Antagonist-mediated decreases in phosphoinositide hydrolysis at M_1 -WT (**A**) or $S^{388Y}M_3$ (**B**) mACh receptors transiently transfected into HEK-293 cells. Monolayers were incubated as per the standard protocol described in section 4.4.2.2.3. $[^3\text{H}]\text{-inositol}$ phosphates were extracted as described in section 2.7. Data represent the means $\pm$ S.E.M from at least three experiments performed in duplicate.

protein). Therefore, all the antagonists tested can be classified as full inverse agonists with respect to [³H]-InsP_x accumulation. The agonist-independent increase in basal [³H]-InsP_x accumulation (approx. 13000 d.p.m. above the -Li⁺ value) seen for the mutant S^{388Y}M₁ mACh receptor, only represents a 2:1 noise ratio, and therefore it proved difficult to obtain reliable inverse agonist inhibition curves. Thus, it was decided to concentrate on obtaining data for a non-selective antagonist (atropine) and one antagonist exhibiting selectivity for the M₁ receptor (pirenzepine). The concentration-response curves for these two antagonists are shown in **Fig. 5.16**. The two antagonists were able to concentration-dependently inhibit [³H]-InsP_x accumulation, yielding pEC₅₀ values of 8.43 ± 0.03 and 7.68 ± 0.02 for atropine and pirenzepine, respectively. The comparison between these values and binding affinities are discussed further in section **5.5**.

5.4.3.2.3. Agonist effects on basal [³H]-InsP_x accumulation

The effect of the three agonists used in binding studies were assessed for their ability to increase [³H]-InsP_x accumulation, as they have been reported to elicit a spectrum of efficacies and potencies at the M₁ mACh receptor. **Figs. 5.17** and **5.18** show the concentration-dependent increases in [³H]-InsP_x accumulation for the three agonists at both M₁-WT and S^{388Y}M₁ mACh receptors.

All three agonists had increased potency at the S^{388Y}M₁ receptor compared to M₁-WT, with the smallest increase being seen for oxotremorine (pEC₅₀ values: methacholine, 5.39 ± 0.14 and 6.21 ± 0.14; pilocarpine, 5.36 ± 0.21 and 5.99 ± 0.32; oxotremorine, 6.66 ± 0.14 and 7.04 ± 0.03 at the M₁-WT and S^{388Y}M₁ mACh receptors, respectively - see **Table 5.3**). The increase in oxotremorine potency is perhaps surprising considering that this agonist displayed no increased binding affinity for the S^{388Y}M₁ receptor and may be an indication of a more complex interaction between agonists and receptors than just the stabilization of a single active state.

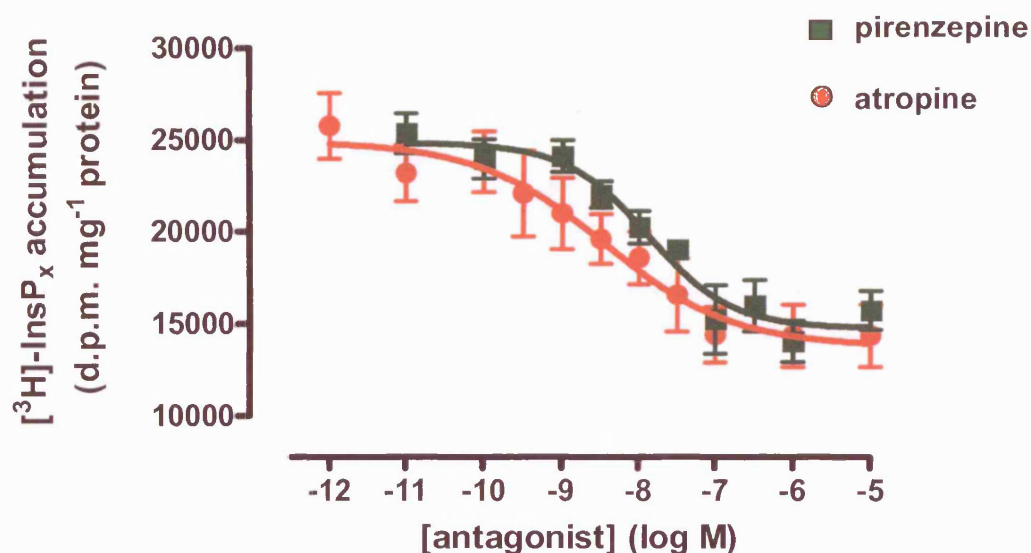


Fig. 5.16 Concentration-dependent antagonist inhibition of basal $[^3\text{H}]\text{-InsP}_x$ accumulation.

Antagonist-mediated decreases in phosphoinositide hydrolysis for $^{N514Y}M_3$ mACh receptors transiently transfected into HEK293 cells. Monolayers were incubated as per the standard protocol described in section 4.4.2.2.3. $[^3\text{H}]\text{-inositol}$ phosphates were extracted as described in section 2.7. Data represent the means $\pm$ S.E.M from at least three experiments performed in duplicate.

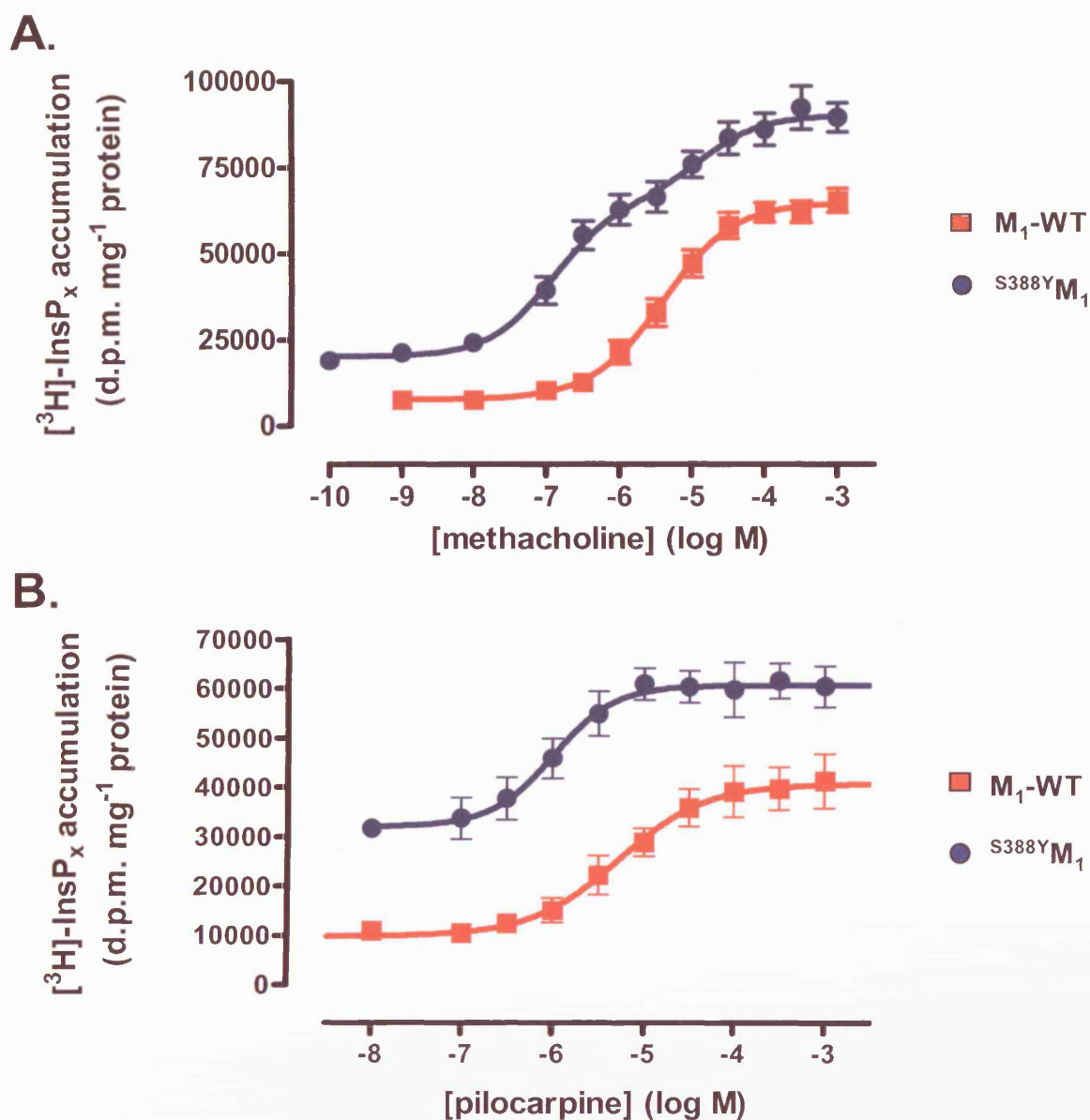


Fig. 5.17 Concentration-dependence of agonist-stimulated increases on $[^3\text{H}]\text{-InsP}_x$ accumulation.

Agonist-mediated increases in phosphoinositide hydrolysis for $M_1\text{-WT}$ (■) or $S388Y M_1$ (●) mACh receptors transiently transfected into HEK293 cells. Monolayers were incubated as per the standard protocol described in section 4.4.2.2.3. $[^3\text{H}]\text{-inositol}$ phosphates were extracted as described in section 2.7. Data represent the means $\pm$ S.E.M from at least three experiments performed in duplicate. Data are summarized in Table 5.3.

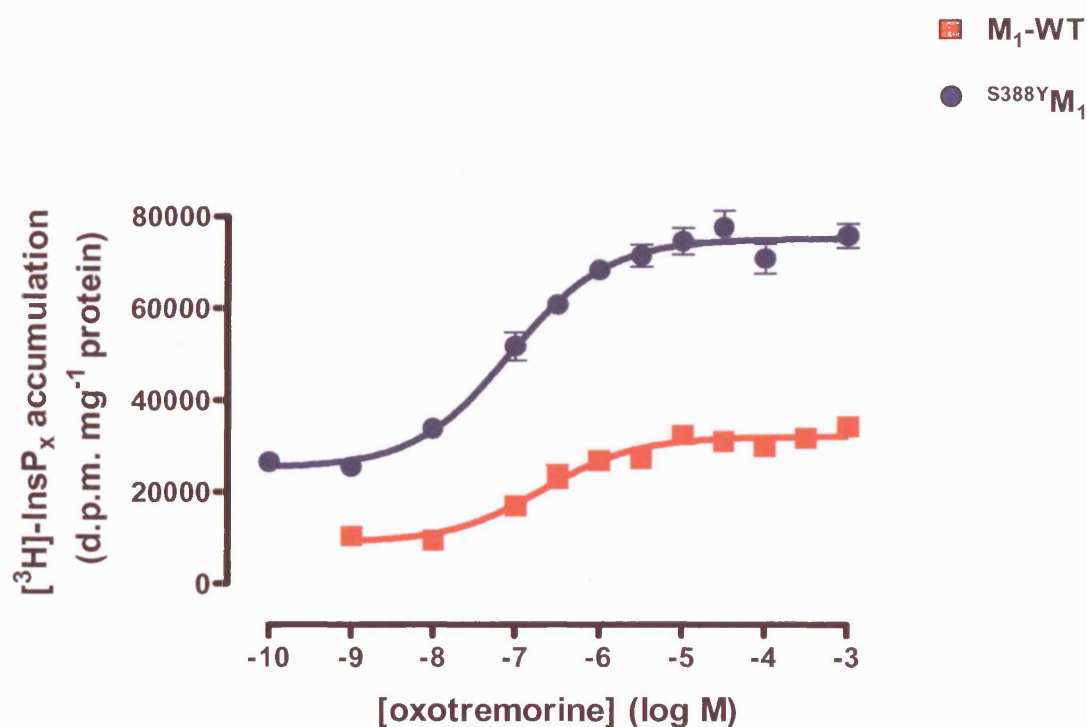


Fig. 5.18 Concentration-dependence of agonist-stimulated increases in $[^3\text{H}]\text{-InsP}_x$ accumulation.

Agonist-mediated increases in phosphoinositide hydrolysis on $M_1\text{-WT}$ (■) or $S388Y M_1$ (●) mACh receptors transiently transfected into HEK293 cells. Monolayers were incubated as per the standard protocol described in section 4.2.2. $[^3\text{H}]\text{-inositol}$ phosphates were extracted as described in section 2.7. Data represent the means $\pm$ S.E.M from at least three experiments performed in duplicate. Data are summarized in Table 5.3.

Table 5.3 Comparative agonist effects on basal [3H]-InsP_x accumulation

	M_1 -WT		$S^{388Y}M_3$		
Agonist	pEC ₅₀	E _{max}	pEC ₅₀	E _{max}	fold difference
methacholine	5.39 ± 0.14	100%	K _H 7.00 ± 0.19 (56 ± 6%) K _L 4.85 ± 0.22	100%	17
oxotremorine	6.66 ± 0.15	40%	7.04 ± 0.03*	66%	15
pilocarpine	5.36 ± 0.21	54%	5.99 ± 0.22*	47%	14

Data are from experiments shown in **Figs. 5.17** and **5.18**. Efficacy values are percentage maximal [3H]-InsP_x accumulation compared to that obtained for methacholine (1 mM) for each receptor type. Fold change represents the higher pEC₅₀ of the mutated receptor to increase [3H]-InsP_x accumulation (obtained by dividing the pEC₅₀ of the $S^{388Y}M_1$ receptor by the M_1 -WT). Data represent the means ± S.E.M for at least three experiments. *indicates $N^{514Y}M_3$ pEC₅₀ is statistically different to that obtained for the M_3 -WT receptor ($p < 0.01$, unpaired Student's *t*-test). Only a two site fit was statistically valid for methacholine at the $S^{388Y}M_1$ (F -test value < 0.01), however the fold difference has been calculated using the EC₅₀ of the one-site fit of the $S^{388Y}M_1$ receptor.

Although the slope factors of all three agonists were lower for the $S^{388Y}M_1$ receptor, only the value for methacholine achieved statistical significance (slope factors, 0.95 ± 0.10 and 0.53 ± 0.09 , for M_1 -WT and $S^{388Y}M_1$, respectively, $p < 0.05$ unpaired Student's *t*-test). When comparing one- and two-site fits all four of the individual experimental datasets for the $S^{388Y}M_1$ receptor, all were fit best by two-potency fractions ($F < 0.05$). This analysis yielded a pEC_{50H} of 7.00 ± 0.19 and a pEC_{50L} of 4.85 ± 0.22 , with the high affinity fraction constituting $56 \pm 6\%$ of the total specific binding. This raises the possibility that the $S^{388Y}M_1$ receptor can cause PIP₂ hydrolysis via the activation of different Gα subtypes with varying potency.

5.4.3.3 Antagonist effects on receptor expression levels

The lower receptor expression levels of constitutively-active mutants in comparison to wild-type counterparts have been suggested to be due in part to agonist-independent receptor internalization and down-regulation (Milligan, 2003a). To test this we incubated HEK293 cells transfected with either 0.2 µg M₁-WT or 0.6 µg ^{S388Y}M₁ DNA with various concentrations of antagonists for 24 h before assessing membrane receptor densities using [³H]-NMS binding as detailed in section 2.4.1.2. Using these concentrations of receptor DNA produced basal receptor expression levels between 1.2–2.0 pmol mg⁻¹ protein.

Fig. 5.19A shows the receptor expression levels after 24 h incubation with maximal antagonist concentrations (as determined from binding experiments). Under these conditions the receptor expression levels of the endogenous M₃ mACh receptors or HEK293 cells transfected with M₁-WT DNA showed no significant differences in receptor expression levels with any of the antagonists tested ($p > 0.05$ unpaired Student's *t*-test) (data not shown). With cells transfected with ^{S388Y}M₁ mACh receptor DNA the increases in receptor levels after incubation with 1 µM atropine, 10 µM pirenzepine and 1 µM 4-DAMP were $54 \pm 8\%$, $51 \pm 7\%$ and $14 \pm 1\%$, respectively.

To compare the activity of antagonist-mediated receptor up-regulation with both binding affinities and decreases in basal [³H]-InsP_x accumulation, full concentration-response curves were constructed. The relatively modest up-regulation seen with 4-DAMP incubation meant that an accurate determination of its potency was not possible. **Fig. 5.19B** shows full concentration-response curves for atropine and pirenzepine. Under these conditions pEC₅₀ values were 8.54 ± 0.19 and 7.29 ± 0.33 for atropine and pirenzepine, respectively.

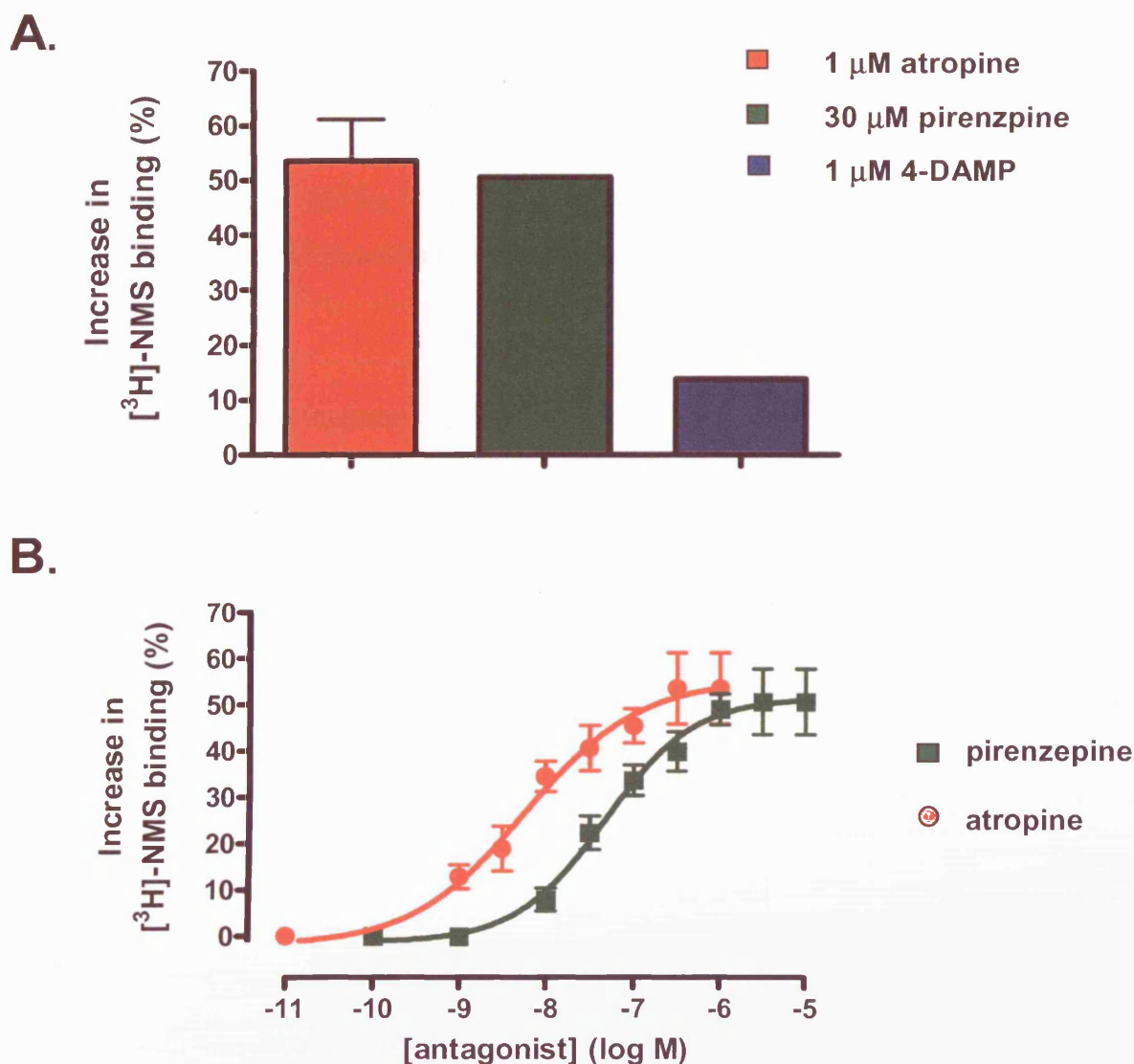


Fig. 5.19 Effect of inverse-agonist pre-incubation on $[^3\text{H}]\text{-NMS}$ binding to $S^{388}Y M_1$ mACh receptors expressed in HEK293 cells

(A) HEK-cell monolayers were incubated with the indicated concentration of each inverse agonist, 24 h post-transfection ($0.4 \mu\text{g DNA well}^{-1}$). After 24 h cells were thoroughly washed and $[^3\text{H}]\text{-NMS}$ binding completed as per section 2.4.1.3. **(B)** Concentration-dependency of atropine and pirenzepine. Data shown are a representative of at least three separate experiments performed in triplicate.

5.5. Discussion

A point mutation homologous to that created in the M₃ mACh receptor was introduced into the M₁ mACh receptor, to compare the pharmacology of the same subset of muscarinic ligands. Although the level of constitutive activity was found to be modest compared to that seen for the M₃ mACh receptor, a number of observations allow the molecular effects of the point mutation on ligand affinity and G-protein coupling to be inferred.

5.5.1 Level of constitutive activity

As depicted by the sequence alignments in **Fig. 5.1**, M₁ and M₃ mACh receptors show a high degree of sequence homology in regions proximal to the proposed location of the S388Y mutation. In Chapter 4 we proposed that the mutation in M₃ caused local disruptions to the packing of amino acid residues within transmembrane domain 6 (TM6), which leads to exposure of previously hidden residues within the third intracellular loop (i3). The N- and C-terminal regions of i3 are known to be important for G-protein coupling and are also highly conserved across M₁/M₃/M₅ mACh receptors (Hogger *et al.*, 1995). In particular, an Arg-Ile-Tyr-Lys (RIYK) motif in the N terminal region of i3 is found in both the human M₁ and M₃ mACh receptors. Given this high degree of structural homology we might expect similar functional consequences in response to homologous point mutations.

Although the S^{388Y}M₁ mACh receptor mutation results in constitutive activity, it is modest compared to the level seen for the homologous M₃ mACh receptor mutation. Moreover, agonist-independent responses were quite variable between individual transfections, and this coupled with the modest signal-to-noise ratio made the pharmacological characterization of inverse agonists a difficult task. Differential effects of mutations within these highly homologous regions have been previously reported. Mutation of the Tyr residue (within the RIYK motif) of the M₃ mACh receptor was found to strongly reduce G_{q/11} coupling (Bluml *et al.*, 1994a), whereas only mutation of the Ile residue was found to inhibit phosphoinositide hydrolysis stimulated by the M₁ mACh receptor (Hogger *et al.*, 1995). Although the use of different cell-lines in these studies complicates

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constructive cross-analysis it would appear that despite high sequence homology, individual amino acids may perform different functions even in closely related family members. A further complication is that an E360A point mutation in the M₁ mACh receptor caused agonist-independent activity when expressed in HEK293 cells (Hogger *et al.*, 1995), but was essentially quiescent when the same receptor mutant was transfected into CHO cells (Shockley *et al.*, 1997). Although we have examined mutant/wild-type M₁ and M₃ receptor properties in the same-cell background it is clear that both the cell background and possibly clonal variation can influence the magnitude of functional responses. The M₁ and M₃ experiments were performed some time apart employing different batches of HEK-WT cells, therefore there may be substantial clonal variation between these cell-lines.

Whilst these effects of the M₁ and M₃ mACh receptor mutations were being assessed, a manuscript was published which looked at the effects of a double homologous mutation in all five members of the mACh receptor family (Ford *et al.*, 2002). These mutations corresponded to residues ⁵¹⁴N (→Y) and ⁵¹⁵T (→P) in the M₃ and ³⁸⁸S (→Y) and ³⁸⁹T (→P) in the M₁ mACh receptor, respectively. When assessed in a cellular proliferation assay, the constitutive activity elicited by the M₁ receptor mutation was poorer than for the corresponding M₃ and M₅ receptor mutants (Ford *et al.*, 2002). Although these authors concluded that movement of TM6 might be a general switch for agonist-activation of all mACh receptor subtypes, it was noted that the M₁ mACh receptor might have a lesser propensity to adopt an active conformation.

5.5.2. Mutational effects on (inverse)-agonist affinity and efficacy

All of the antagonists tested, with the exception of methoctramine, exhibited significant, albeit modest decreases in receptor binding affinity. It is interesting to note that, although not completely selective, pirenzepine has relatively high affinity for the M₁ mACh receptor (at least 10-fold selectivity over M₃) and exhibited the greatest fold-change in its affinity between M₁-WT and ^{S388Y}M₁ mACh receptors. This supports the hypothesis, initially developed to accommodate observations obtained with the ^{N514Y}M₃ receptor mutant, that the decrease in binding affinity tends to be greatest for those antagonists that

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possess high affinity/moderate selectivity for the cognate wild-type receptor. In contrast, antagonists with lower affinity/selectivity (e.g. methoctramine) have affinities that are relatively unchanged by the constitutive activity-promoting point mutation. This suggests that the mutation confers a conformation, which (partly) resembles the agonist-bound state and would predict that inverse agonists with relatively high affinity for the inactive conformation, **R**, would exhibit the largest decreases in binding affinity. As with the M_3 mutant receptor there appears to be two types of inverse agonist, those that high differential affinity for the **R** and **R*** conformations and those that do not.

As seen for both the $N^{514Y}M_3$ mutant receptor in the previous Chapter, and a homologous mutation in the M_5 mACh receptor (Spalding *et al.*, 1997) there was no effect of GTP on the inverse agonist receptor binding isotherms. Considering the relatively modest changes in binding affinity the lack of a measurable GTP-effect is perhaps unsurprising. The ability of the inverse agonists to concentration-dependently decrease agonist-independent activity is good evidence that the mutation has conferred a conformation which at least partial resembles the active state. There is, however, a question as to the nature of the conformation that is stabilized by the inverse agonists. If the mutation has induced an 'intermediate' activated receptor state (denoted **R'**) then the conformation stabilized by inverse agonists may not faithfully represent the ground (inactive) state of the cognate wild-type receptor, but some partially activated intermediate that is unable to couple to G-protein. The free energy difference between the inverse agonist-stabilized state and that which couples to G-protein may be similar and provides another possible explanation for the lack of a GTP-shift with either inverse agonists or agonists.

The up-regulation of the $S^{388Y}M_1$ receptor in response to pre-incubation with inverse agonists was again modest relative to that seen with the homologous M_3 receptor mutation. This could be indicative of decreased agonist-independent down-regulation, in comparison to that exhibited by the $N^{514Y}M_3$ receptor, due to the more modest constitutive activity of the $S^{388Y}M_1$ receptor. Interestingly, the up-regulation in response to pre-incubation with 4-DAMP was also partial with respect to other inverse agonists, again, as

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for reasons stated for the ^{N514Y}M₃ receptor, this may represent a more complex interaction of inverse agonists with receptors than simple cell-surface receptor stabilization.

The effect of the M₁ mACh receptor point mutation on the agonist affinity is more complex. Oxotremorine has been reported to possess an intrinsic activity between that of methacholine and pilocarpine when stably expressed in A9L cells, or $\geq$ pilocarpine when stably expressed in CHO cells (Richards and Van Giersbergen 1995, and see Chapter 3). Other studies have reported a direct relationship between the increase in agonist affinity measured for a constitutively active mutant receptor and its efficacy at the cognate wild-type receptor (Samama *et al.*, 1993), including the M₁ mACh receptor (Huang *et al.*, 1998, 1999). The lack of change in affinity for oxotremorine could be interpreted in two ways: (1) When expressed transiently in HEK293 cells, oxotremorine is of lower intrinsic activity than pilocarpine for the M₁-WT receptor (see **Table 5.2**). Considering the modest degree of constitutive activity and the slight fold-change in affinity for pilocarpine, it could be that our system was not sufficiently sensitive to detect the (predicted) modest affinity difference for oxotremorine. Interestingly despite the lack of affinity change there is a significant difference in the Hill slopes obtained for oxotremorine (and methacholine) at the M₁-WT and ^{S388Y}M₁ receptors. This would appear to indicate the mutation has induced a conformation change, which exhibits (at least) two binding affinities for oxotremorine, although a two-site fit of the actual binding curve was not statistically significant. (2) The lack of correlation between changes in efficacy and affinity obtained for the two receptors may indicate that the effect induced by the mutation is more complex than simply increasing the propensity of the receptor to reside in the (partially)-activated state. It is hard to reconcile the effect of the mutation on the binding affinity for the agonists with that seen for functional output. Despite no significant effect on the binding affinity of oxotremorine, the fold-difference between WT and mutant receptor appears to be related to agonist efficacy for methacholine and pilocarpine. Additionally, the increase in efficacy, as assessed by E_{max}, was significantly greater for oxotremorine than pilocarpine. This appears to indicate a distinction between the effects of the mutation on the binding and functional measurements for individual agonists. As detailed in section 1.4.3, we are beginning to appreciate that conformations

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of the same receptor subtype, either stabilized or induced by different agonists, may be sufficiently different to elicit a different G-protein coupling profile.

It has been previously suggested that the simplest model to explain the effect of constitutive active mutation is via changes in **J** (the isomerization which dictates the endogenous levels of active and inactive receptor levels), such that the receptor is found more frequently in the active state (Ford *et al.*, 2002). Although the overall trend of the functional effect of the mutation appears to increase both the potency and efficacy of agonists, the subtle differences cannot be simply explained by an effect on **J**. In the case of methacholine, it would appear that the mutation has an effect on the **R*G** coupling. The differential effects of the same mutation on binding and function for the different agonists may constitute further evidence that the conformations stabilized by these agonists are distinct and further investigation is warranted.

In summary, the level of constitutive activity associated with the S388Y M₁ mACh receptor mutation is modest compared to that seen for the homologous mutation of the M₃ receptor. This has allowed only a partial pharmacological characterization of the M₁ receptor mutant. Despite this limitation, a number of observations have strengthened the hypothesis developed in previous Chapters, especially those concerning the possibility of multiple agonist-receptor conformations, and the mode of inverse agonist binding. The final Chapter will attempt to summarize the data from all the Results Chapters in three sections: (1) agonist-specific receptor conformations (2) receptor activation, and (3) inverse agonist activity.

Chapter Six.

Final discussion

6.0. Final Discussion

6.1. Introduction

The last 25 years have seen a progressive development in the understanding in how the activation of G-protein coupled receptors transmits an extracellular stimulus into a co-ordinated intracellular response. The concept that therapeutic interventions into intracellular signalling networks are restricted to the use of agonists to activate, or antagonists to inhibit, a single cellular response is probably redundant. This Thesis has used a variety of techniques and assays to examine two key observations seen in other GPCR systems, which has necessitated a re-think on the molecular basis of receptor activation and the fidelity of signalling: (1) the active state of a receptor, and consequently its G-protein activation profile, may not be identical for all agonists, and (2) receptors may not be quiescent in the absence of agonist and that some ligands may possess inverse agonist properties.

This section aims briefly to summarize the experimental evidence presented in the preceding Results Chapters with specific inferences to the broader therapeutic relevance of my findings. Whilst some findings have been convincingly demonstrated, others will need further investigation, therefore, proposed future experimental strategies are also detailed.

6.2. Agonist-specific receptor conformations

Incubation of CHO-cell membrane preparations, recombinantly expressing the M₃ mACh receptor, with [³⁵S]-GTPγS and subsequent Gα-specific immunoprecipitation has provided evidence to support the notion of agonist-specific receptor conformations. Whilst methacholine, carbachol and oxotremorine-M appeared to stabilize an active conformation of the receptor that couples to Gα_{q/11} and Gα_{i1-3}, the partial agonists pilocarpine and oxotremorine elicited either comparably poor or no significant Gα_{q/11} coupling above basal. However, both of these agonists elicited relatively robust receptor-mediated [³⁵S]-GTPγS binding to Gα_{i1-3} subunits. Evidence obtained from CHO-cell membranes prepared from PTx-treated cells on the potency and intrinsic efficacy of total

[³⁵S]-GTPγS binding measurements suggested, that at least for full agonists, the M₃ mACh receptor is most productively coupled to Gα_{q/11}. It is therefore tempting to suggest that the partial agonists, oxotremorine and in particular pilocarpine, stabilize a distinct active conformation of the M₃ mACh receptor which is predominantly coupled to activation of Gα_i-containing G proteins. Evidence for the preferential activation of different Gα subunits, for both the M₁ and M₃ mACh receptors, would be considerably strengthened with the construction of full concentration-response profiles for Gα₁₁₋₃ subunits. For the reasons discussed in Chapter 3, this proved experimentally difficult using our current pharmacological tools. Other investigators have reported improved signal-to-noise by assaying specific Gα activations by recombinantly expressing the receptor of interest fused to a particular Gα_i subtype protein which also contains a PTx-resistant mutation (Wise *et al.*, 1999). It is then possible to eliminate background interference from endogenous Gα_{i/o} subunits by the pre-incubation of PTx. Although this appears to be making an already artificial recombinant system even less physiologically relevant, it may be one way in which the agonist-mediated potency and efficacy of Gα_i activation could be assessed.

Although the existence of multiple, active agonist-specific conformations has been proposed for many GPCRs, what is frequently overlooked in these experiments is a structural basis for the agonist-specific differences. A recent elegant study has employed fluorescence spectroscopy to monitor changes in catecholamine-induced conformational changes in the purified β₂-adrenoceptor (Swaminath *et al.*, 2004). These studies have implied that agonist binding and subsequent receptor activation occurs through kinetically-distinct intermediate states. Each of these states is formed sequentially, and each interaction increases the probability of the subsequent step occurring until the active conformation is reached. Furthermore, the conformations induced by the agonists, presumably because of unique interactions between the chemical structure of the agonist and specific amino acid residues within the receptor, appear to be different and may result in the stabilization of active agonist signalling complexes with distinct cellular functions.

If agonist activation proceeds through the stabilization of many intermediate conformations in a so-called ‘multistep process’ (Strange, 1999; Kobilka, 2004), it seems intuitive that agonists with different chemical structures may stabilize subtly different intermediate conformations. However, regardless of these theoretically unlimited intermediates, the physiologically relevant question is whether the ultimate signalling conformation is sufficiently distinct as to cause the activation of different G-proteins or other G-protein-independent pathways. In the case of the M₃ mACh receptor the full agonists (including the endogenous agonist acetylcholine) possess a positively charged quaternary nitrogen ion, and it is therefore tempting to suggest that the presence of this charge could account for the proposed different conformations stabilized by the agonists. A more conclusive study might then consider the possibility of screening a larger number of mACh receptor agonists, with and without quaternary ions, to examine if this property is universal or whether another structural moiety is prevalent.

It is becomingly increasingly appreciated that productive signalling complexes may occur by the simple model of random movement and collision of proteins within the lipid bilayer (Ostrom *et al.*, 2000). Signalling molecules, including G-proteins and many adaptor molecules, are concentrated in microdomains such as ‘lipid-rafts’ and caveolae (Dykstra *et al.*, 2003), additionally receptors are not static within the membrane, but may move in or out of these regions following agonist activation (Ostrom *et al.*, 2000). It has been reported that particular G α proteins are enriched within these signalling regions (Ostrom *et al.*, 2000) and therefore the accessibility of an agonist to these areas, due to its inherent chemical structure, also remains a possibility to explain differential G-protein coupling. The importance of scaffolding proteins in G-protein signalling has recently been demonstrated for the 5-HT_{2A} receptor, where the *in vitro* RNA-interference mediated knock-down of caveolin-1, virtually abolished G α_q -coupling (Bhatnagar *et al.*, 2004)

Another intriguing aspect of agonist activation of the β_2 -adrenoreceptor, monitored by fluorescence spectroscopy (Gether *et al.*, 1997; Ghanouni *et al.*, 2001; Swaminath *et al.*, 2004), or through binding analysis (Liapakis *et al.*, 2004), revealed agonist-specific

differences in not only the actual conformations stabilized, but also the kinetics with which they were formed. Although this has been interpreted as differences in the ability of agonists to induce G-protein coupling (rapid conformational changes) with other processes such as internalization (associated with slower conformational changes), this could also be extended to differential G-protein coupling conformations with different kinetics. Preliminary optimization of the $G\alpha$ -specific immunoprecipitation assay showed that optimal $G\alpha_{q/11}$ activation was obtained after only a 2 min incubation period. The difference kinetics between this immunoprecipitation assay and the filtration assay (45 min optimum time-point) can be partially explained by the higher concentration of [^{35}S]-GTP γ S employed in the former assay (~ 10 nM versus 0.3 nM [^{35}S]-GTP γ S, respectively). However, the possibility remains that optimal conditions were applicable solely to the activation of $G\alpha_{q/11}$ and more detailed time-courses should be repeated to examine the possibility that $G\alpha_{i1-3}$ activation does not yield a different kinetic profile. Additionally, agonist-specific activation of different $G\alpha$ subtypes may also be distinct. Both time and resource constraints meant that full time-course studies for each agonist measured against each $G\alpha$ subtype could not be feasibly completed, although subsequent studies may wish to explore this possibility.

Finally, I have only examined the activation of two $G\alpha$ subtypes employing 'pan' antibodies against $G\alpha_{q/11}$ and G_{i1-3} . Antibodies against individual $G\alpha_i$ are generally prohibitively expensive and/or show some cross-reactivity with other $G\alpha_i$ subunits. Also, not all of the commercially available antibodies can be used successfully in immunoprecipitation experiments. However, even with the employment of limited antibodies, I have demonstrated some interesting agonist-specific differences. Therefore, a natural expansion of this study would be to employ more specific and diverse (e.g. $G\alpha_s$) $G\alpha$ -specific antibodies, which may reveal a greater extent of differences in agonist-G-protein activation profiles than has been reported here.

The need to continue to adapt and develop our understanding of how agonists activate receptors and subsequent signalling pathways is not merely academic, as many of the concepts have important therapeutic aspects. Thus, agonist-specific receptor conformations may lead to a next level of drug-selectivity. Agonists could be engineered that target only one pathway of a receptor's possible complement, whilst avoiding the activation of other potentially harmful routes. Although it has received less attention, the concept of 'strength-of-signal' is also important in its own right. Full agonists may activate all possible pathways associated with a particular receptor, leading to potentially unwanted side-effects. Partial agonists, while not as efficacious may have a limited activation profile, and therefore may be therapeutically advantageous in some cases.

6.3. Receptor activation, constitutive activity and inverse agonism

The constitutive activity elicited by the point mutations, which are proposed to lie at the junction of TM6 and the third extracellular loop, in both the M₁ and M₃ mACh receptors supports evidence that implicates a rotation or tilting of TM6 relative to TM3 in rhodopsin (and other family A GPCRs) is involved in agonist-mediated receptor activation (Farrens *et al.*, 1996; Gether, 2000; Karnik *et al.*, 2003; Kristiansen, 2004). The different degrees of constitutive activity obtained with the two receptors is also evidence that there are subtle differences in the residues involved in receptor activation and G-protein coupling conformations, even between closely-related family members.

The relatively low level of constitutive activity associated with overexpression of the wild-type M₃ mACh receptor, and its complete absence with similar expression levels of the wild-type M₁ mACh receptor, is an indication that the mACh receptors may have a low propensity spontaneously to adopt an active receptor conformation at least in the HEK cell background. Previous studies have shown that substitution of any amino acid other than the endogenous one, at the junction of the third intracellular loop and TM6, elicits some degree of constitutive activity in the β_2 -adrenoceptor (Kjelsberg *et al.*, 1992).

This indicates that there is a strong evolutionary pressure to maintain the receptor in the inactive conformation (Milligan, 2003). The observation that the constitutively-active mutants exhibit increased agonist-independent G-protein coupling, and in the case of the ^{N514Y}M₃ mACh receptor increased basal phosphorylation, is a good indication that the conformation conferred by the mutation is at least recognised by intracellular signalling molecules. However, there is then the question of how useful are constitutively-active mutants as pharmacological tools, and also whether the re-classification of antagonists as inverse agonists has any real therapeutic significance? The next few sections will consider these questions in light of observations from the proceeding Results Chapters.

As explained in section 5.2., agonist-mediated receptor activation is a complex process, involving the release of constraining interactions and the formation of new agonist-mediated ones. The identification of a point mutation that confers agonist-independent activity has been used to unravel the nature of such constraining interactions (Parnot *et al.*, 2002). More detailed analysis on the mACh receptor activation process may be obtained with the substitution of a range of amino acids and subsequent analysis of constitutive activity levels. However, it must be noted that constitutively-active mutations can be found widely within some GPCRs, and therefore identifying the precise residues involved in receptor activation is difficult. Although undeniably useful for identifying which residues are involved in constraining receptor conformation, it is unclear whether constitutively-active mutants recapitulate the different structural modifications found with endogenous agonist-induced conformation(s). The regions involved in the activation of both receptors and G-proteins were reviewed in section 1.3.

I have presented evidence within Chapters 4 and 5 that suggest the constitutively-active mutants may be inherently unstable, due to their significantly lower expression levels compared to their cognate wild-type counterparts. This lower expression level is rescued or increased, to varying degrees, by incubation with putative inverse agonists. However, the exact molecular mechanism through which this receptor up-regulation is achieved has

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not been firmly established. 4-DAMP methiodide, which possesses a quaternary nitrogen ion (and is therefore relatively membrane-impermeable), did not upregulate the ^{S388Y}M₁ or ^{N514Y}M₃ mACh receptors to comparable levels seen for more cell-permeable ligands. This may suggest that inverse agonists are able to act as pharmacological ‘chaperones’, stabilizing and increasing the export of naïve receptor proteins from intracellular compartments to the cell-surface. This mechanism has been recently proposed for the up-regulation of both a wild-type and ER-retained mutant form of the human δ -opioid receptor (Petaja-Repo *et al.*, 2002). If this is the predominant up-regulation mechanism for the mutant mACh receptors, it might be argued that the constitutively-active mutant is merely inherently unstable, rather than accurately reflecting an agonist-bound conformation. However, as the pre-incubation with 4-DAMP elicits significant receptor up-regulation, there might also be a component of cell-surface stabilization of an active conformation of the receptor. In this scenario the mutant receptor could resemble the natural agonist-bound conformation and be subjected to constitutive desensitization, internalization and down-regulation in a similar manner to the cognate agonist-bound signalling conformation. In support of this I have obtained evidence to show that the ^{N514}M₃ receptor is constitutively phosphorylated and therefore it seems reasonable to propose that this receptor is subject to a higher rate of internalisation than its cognate M₃-WT receptor. However more work is required to establish whether the constitutive phosphorylation leads to agonist-independent internalisation, and it also would be interesting to confirm whether the ^{N514}M₃ is phosphorylated at the same residues as M₃-WT. From the experiments I have performed it is hard to reach a conclusion and therefore an extension of this study would include the following experiments:

(1). Agonists, as well as inverse-agonists, have also been shown to up-regulate some mutant GPCRs, due to their ability to stabilize a proposed inherently unstable conformation (Petaja-Repo *et al.*, 2002). A range of agonists and antagonists could be tested for their ability to up-regulate the constitutively-active, mutant M₁ and M₃ mACh receptors.

(2). A correlation between a ligand’s permeability and its ability to cause receptor up-regulation should also be sought. However, it is also important to establish whether

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ligands, such as 4-DAMP methiodide, are essentially impermeable, permeable only at high concentrations or unstable in the assay conditions employed.

(3). Various steps of the receptor re-cycling process could be investigated by employing specific inhibitors which are reported to block the various components, e.g. internalization and lysosomal-mediated downregulation, to establish whether the constitutively-active mutants are subject to these processes in an agonist-independent manner.

As there are potentially many conformations between the ground state of the receptor and the final agonist-bound signalling state(s), there is some debate over the molecular mechanisms of inverse agonists. I have shown, that with respect to phosphoinositide hydrolysis, all the tested mACh receptor antagonists should be classified as inverse agonists. However, the apparent binding affinity (in terms of fold-decrease when compared to wild-type), potency (when compared to binding affinities) and efficacy (receptor upregulation and the inhibition of the basal level of phosphorylation of the ^{N514Y}M₃ mACh receptor) of the inverse agonists have displayed some differences which lead me to propose that the simplest mode of action, i.e. the preferential stabilization of the ground-state of the receptor over the active G-protein signalling conformation, is probably too simplistic to explain all of the pharmacological observations. One possible model to explain the model the molecular nature of inverse-agonists is depicted below:



Within this model, the conformation of the constitutively-active mutant receptor is shown as R^{cam} , this conformation has a greater propensity spontaneously to couple to G-proteins than R (ground-state) due to the proposed more favourable conformation of key residues for coupling. The inverse agonists may exert their effects by stabilizing (having greater affinity for) any of the intermediate conformations R' or R'' , the decrease in the population of R^{cam} therefore decreases the spontaneous agonist-independent G-protein

coupling. For simplicity only two intermediates, R' and R'' are depicted, however, there is no theoretical limit to the number available, and therefore the conformations stabilized by each inverse agonist may be different. By having multiple, intermediate inverse agonist-stabilized conformations, we may predict differences in the potency of inverse agonists based on their preferential affinities for these conformations.

As the conformation(s) stabilized by the inverse agonists will still have the mutation present, the degree to which the intermediate receptor conformation resembles the constrained, native ground-state conformation may be negligible. In this model the inverse agonist is stabilizing an intermediate conformation, which is unfavourable for G-protein coupling. However, as described in the both sections 1.4.3.2 and 5.2, different conformations of the receptors may be stabilized by agonists for different signalling processes, such as phosphorylation and internalization. The same rationale could be applied in this model, in that although the conformation stabilized by the antagonist does not allow for productive G-protein coupling, other signalling process may be unaffected. This may explain why the conformation of the $N^{514Y}M_3$ mACh receptor stabilized by methoctramine decreases agonist-independent total [3H]-inositol phosphate accumulation, but does not affect the basal level of receptor phosphorylation.

This model may also be used to explain the lack of effect of guanine nucleotides on the binding-affinity curves obtained for both agonists and inverse agonists from the constitutively-active, mutant receptors. The increase in agonist, and decrease in inverse agonist binding affinity, coupled with the clear demonstration of agonist-independent G-protein coupling, may lead to an expectation of a GTP-effect on binding isotherms (Strange, 2002). However, the R' conformation stabilized by inverse agonists may be of similar energy as the R^{cam} or $R^{cam}G$, such that a measurable GTP effect is not detected, i.e. neither inverse agonists, nor GTP cause the receptor to reside in a low energy ground-state conformation and therefore no effect of GTP is seen for agonists or antagonists. However, the lack of GTP shift for agonists is also commonly seen for wild-type mACh

receptors and may reflect a high degree of receptor reserve for this pathway (or a low G-protein:receptor ratio). As relatively few receptors need to be occupied, or the pathway is subject to an amplified signal downstream (or in the case of the constitutively-active, mutant receptor spontaneously couple to G-protein) to produce a full-response, the detection of GTP-shifts may be experimentally difficult to resolve .

The agonists effects on both the M_1 and the M_3 constitutively active mutant, in functional assays, suggested that the effect of the mutant changed the **R*G** coupling. The slope factors of [^3H]-InsP_x accumulation were significantly lower at the $N^{514Y}M_3$ receptor when compared to M_3 -WT, and the methacholine [^3H]-InsP_x accumulation at the $S^{388Y}M_1$ receptor could be fitted into two-potency components. Given time, these initial observations would be expanded to look directly at the G-protein coupling at both wild-type and mutant receptors using the [^{35}S]-GTP γ S immunoprecipitation assay.

Constitutively-active mutant receptors are useful tools in understanding the receptor activation process and to reveal both the presence and the molecular mechanism(s) of inverse agonists. However, their therapeutic value is still in doubt as we are currently unsure of the role of constitutive activity in the (patho)physiology of many disease states. However, many antagonists are prescribed long-term for chronic conditions, furthermore, with the relentless re-classification of antagonists as inverse agonists, neutral antagonists are becomingly increasingly rare entities. It is therefore increasingly accepted that many of the currently prescribed antagonists are in fact inverse agonists. Constitutively-active mutants allow us to assess the effects of inverse agonists in an acute setting and some of their effects detailed in this Thesis, such as receptor up-regulation, could potentially result in unwanted side-effects, such as tolerance and hypersensitivity. The next challenge is to understand the degree of constitutive activity manifested by endogenous receptors and whether this changes in various disease states. We should also monitor the effects of long-term inverse agonist therapies at a molecular pharmacological level.

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