ADDENDUM

p 45 line 11: After "et al., 1999),", insert "adenosine A_{2B} receptor (Beukers et al., 2004),"

p 125 line 17: Add at the end of paragraph 2: "To ascertain the mode of receptor activation by brucine, preliminary interaction studies in Ca^{2+} mobilisation assays between brucine and the orthosteric antagonist, atropine, were performed (data not shown). These experiments revealed that the interaction between brucine and atropine was non-competitive, and that atropine was thus a negative modulator of the efficacy of brucine-stimulated Ca^{2+} mobilisation. This finding confirmed that brucine-induced M_3 $K^{7.32}E$ mAChR-mediated Ca^{2+} mobilisation was indeed mediated via an allosteric site.

p 145 line 12: Add at the end of paragraph 1: "Conversely, the discrepancy seen between the yeast and mammalian data in Chapter 2 may be due to enhanced coupling efficiency in CHO cells, which has been shown to be a general characteristic of immortalised cell lines (Eglen et al., 2008).

p 154 line 3: New paragraph: "Beukers MW, van Oppenraaij J, van der Hoorn PP, Blad CC, den Dulk H, Brouwer J and AP IJ (2004) Random mutagenesis of the human adenosine A2B receptor followed by growth selection in yeast. Identification of constitutively active and gain of function mutations. *Mol Pharmacol* **65**:702-710."

p 157 line 4: New paragraph: "Eglen RM, Gilchrist A and Reisine T (2008) The use of immortalized cell lines in GPCR screening: the good, bad and ugly. *Comb Chem High Throughput Screen* **11**:560-565."

Copyright Notices

Notice 1

Notice 2

Pharmacological profiling of G protein-coupled receptors using *Saccharomyces cerevisiae*

Gregory David Stewart

Submitted in total fulfillment of the requirements of the degree of Doctor of Philosophy

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Department of Pharmacology

Monash University

Produced on archival quality paper

Abstract

It is commonly accepted that the nature of drug efficacy is not a linear concept, but rather a multi-dimensional signalling event. This gives rise to the potential for ligands to be functional selectivity, which is preferential activation of a set of signalling cascades at the exclusion of others. This idea poses certain problems and provides new approaches for drug discovery programs with regard to choice of assay format and generation of pathway-selective compounds, respectively. In light of this, the majority of drug discovery programs that search for functionally selective ligands, combat this issue by using multiple endpoint assays, which vary depending on the targeted pathway. However, this approach is sometimes difficult to interpret for a number of reasons, such as the fact that many pathways are mediated by more than one upstream effector, the experimental conditions may vary between assay formats, for example, buffer composition and kinetics of activation. Moreover, using many functional endpoints is often expensive and inefficient. In this vein, this thesis discusses the development of a novel approach to screening for functional selective ligands, using a *Saccharomyces cerevisiae* expression system, which has been hitherto overlooked for this purpose. The ability of this system to detect functional selectivity of ligands within a Gα protein family ($G\alpha_{i/o}$), and across multiple Gα proteins ($G\alpha_{q}$, $G\alpha_{i1/2}$, $G\alpha_{12}$), was assessed. There was evidence that both orthosteric and allosteric ligands that bind M_3 muscarinic acetylcholine receptors display functional selectivity, which was predicted using the yeast system; whilst the capability of the yeast system to predict selectivity between $G\alpha_{i/o}$ subunits was comparatively reduced. In addition, the capacity of the yeast system to predict ligand parameters such as affinity and efficacy was also investigated. It was found that from data obtained in yeast, accurate affinity estimates could be generated. Furthermore, a potential use for yeast in estimating conformation-specific affinity

values for agonists was revealed. Taken together, the evidence in this thesis suggests that the yeast signalling assays is a valuable and tractable platform for detecting pharmacological characteristics of existing and novel ligands.

Declaration

In accordance with Monash University Doctorate Regulation 17/Doctor of Philosophy and Master of Philosophy(MPhil) regulations the following declarations are made:

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

This thesis includes 1 original paper published in a peer-reviewed journal The core theme of the thesis is "Pharmacological profiling of G protein-coupled receptors using *Saccharomyces cerevisiae*". The ideas, development and writing up of all papers in the thesis were the principal responsibility of myself, the candidate, working within the Department of Pharmacology under the supervision of Profs. Arthur Christopoulos and Patrick Sexton.

The inclusion of co-authors reflects the fact that the work came from active collaboration between researchers and acknowledges input into team-based research.

In the case of Chapter 2 my contribution to the work involved the following:

Thesis chapter	Publication title	Publication status	Nature and extent of candidate's contribution
2	"Determination of adenosine A_1 receptor agonist and antagonist pharmacology using *Saccharomyces cerevisiae*: Implications for ligand screening and functional selectivity"	In Press	Performed experiments & data analysis. Manuscript preparation and revision. (80%)

Signed:…….. Date:……...

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Abbreviations

AA – Arachidonic acid

ACh – Acetylcholine

ADA – Adenosine deaminase

ADAM – A disintergrin and metalloprotease

AMP – Adenosine monophosphate

ATCM – Allosteric ternary complex model

ATP – adenosine triphosphate

ATSM – Allosteric two-state model

BCA – Bicinchoninic acid

BRET – Bioluminescence resonance energy transfer

BSA – Bovine serum albumin

CaM – Calmodulin

CaMK – Calmodulin kinase

cAMP – 3'-5'-cyclic adenosine monophosphate

Ca_v – Voltage-operated Ca^{2+} (channel)

CCh – Carbachol

CCK – Cholecystokinin

CHO – Chinese Hamster Ovary

CNS – Central nervous system

DAG – Diacylglycerol

DMEM – Dulbecco's modified Eagle's medium

DOI – (±)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminpropane

DPCPX – 8-cyclopentyl-1,3-dipropylxanthine

D-Trp-ODE – D-Tyr-Gly-[(Nle28,31, D-Trp30)cholecystokinin-26-32] phenethyl ester

ECL – Extracellular loop

EDTA – Ethylenediaminetetraacetic acid

ERK1/2 – Extracellular signal-regulated kinases 1 and 2

FBS – Foetal bovine serum

FDG – Fluorescein di(β-d-galactopyranoside)

FRET – Förster (fluorescence) resonance energy transfer

FSCPX – 8-cyclopentyl-3-(3-((4(fluorosulfonylbenzoyl)oxy)propyl)-1-propylxanthine

GABA – γ-amino butyric acid

GAP – GTPase-activating protein

GDP – Guanosine diphosphate

GEF – Guanine nucleotide exchange factor

GIRK – G protein-coupled inward rectifying potassium (channel)

GPCR – G protein-coupled receptor

GPS – GPCR proteolytic domain

GRK – GPCR kinase

GTP – Guanosine triphosphate

GTPase – Guanine triphosphatase

GTPγS – Guanosine 5'(γ-thio)triphosphate

HEPES – 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

IP_3 – Inositol 1,4,5-trisphosphate

JNK – c-Jun N-terminal kinase

Kir – Inward-rectifying potassium (channel)

mAChR – muscarinic acetylcholine receptor

MAPK – Mitogen-activated protein kinase

MLCK – Myosin light-chain kinase

MLCP – Myosin light-chain phosphatase

MMP – Matrix-metalloprotease

NCX – Na^+/Ca^{2+} exchangers

NHERF – Na^+/H^+ exchange regulatory factor

NMS – N-methylscopolamine

OTR – Oxytocin receptor

PBS – Phosphate buffered saline

PCR – Polymerase chain reaction

PDE – Phosphodiesterase

PGD_2R – prostaglandin D_2 receptor

PH – Pleckstrin homology (domain)

PI3K – phosphoinositide 3-kinase

PIP_2 – Phosphatitylinositol 4,5-bisphosphate

PKA – cAMP-dependent kinase

PKC – Protein kinase C

PLA – Phospholipase A

PLC – Phospholipase C

PLD – Phospholipase D

PM – Plasma membrane

PTX – pertussis toxin

RGS – Regulator of G protein signalling

RH – RGS homology (domain)

ROCK – Rho kinase

R-PIA – (-)-N^6-(2-phenylisopropyl)adenosine

RTK – Receptor tyrosine kinase

sAC – Soluble adenylate cylase

Sos – Son of sevenless

STIM1 – Stromal interaction molecule 1

TM – Transmembrane

tmAC – Transmembrane adenylate cyclase

TRPC – Canonical transient receptor potential

VCP-189 – 5'-deoxy-N^6-(endo-norborn-2-yl)-5'-(2-fluorophenylthio)adenosine

Publications arising from this thesis

Peer-reviewed articles

Stewart G.D., Valant C, Dowell S.J., Mijaljica D., Devenish R.J., Scammells P.J., Sexton P.M., and Christopoulos A. (2009) Determination of adenosine A_1 receptor agonist and antagonist pharmacology using *Saccharomyces cerevisiae*: Implications for ligand screening and functional selectivity. J. Pharmacol. Exp. Ther. (In Press)

Werry T.D., **Stewart G.D.**, Crouch M.F., Watts A., Sexton P.M. and Christopoulos A. (2008) Pharmacology of 5HT(2C) receptor-mediated ERK1/2 phosphorylation: agonist-specific activation pathways and the impact of RNA editing. Biochem. Pharmacol. 76(10):1276-87

Book Chapters

Stewart G.D., Sexton P.M. and Christopoulos A.

Book chapter on *Inverse agonists* for the Encyclopaedia of Psychopharmacology (2009), Springer publishing

Published abstracts

Stewart G.D., Sexton P.M. and Christopoulos A.
Using *Saccharomyces cerevisiae* to identify functional selectivity of orthosteric and allosteric ligands at the M_3 muscarinic acetylcholine receptor. *15th Annual Society for Biomolecular Sciences Conference and Exhibition*, Lille, France, April 2009.

Stewart G.D., Sexton P.M. and Christopoulos A.
Using *Saccharomyces cerevisiae* to identify pathway selectivity of allosteric compounds at the M_3 muscarinic acetylcholine receptor. *3rd British Pharmacological Society Cell Signalling meeting*, Leicester, UK, April 2009.

Stewart G.D., Sexton P.M. and Christopoulos A.
Characterisation of the M_3 muscarinic acetylcholine receptor in *Saccharomyces cerevisiae*. *5th Molecular pharmacology of G protein-coupled receptors meeting*, Sydney, Australia, November 2008.

Keov P., **Stewart G.D.,** Sexton P.M. and Christopoulos A.
The M_3 muscarinic acetylcholine receptor mediates Ca^{2+} extrusion in 3T3 fibroblast cells, but not SH-SY5Y neuroblastomas. *5th Molecular pharmacology of G protein-coupled receptors* meeting, Sydney, Australia, November 2008.

Stewart G.D., Scammells P.J., Sexton P.M. and Christopoulos A.
Characterisation of the adenosine A_1 receptor in *Saccharomyces cerevisiae*. *Experimental biology*, San Diego, USA, April 2008.

Stewart G.D., Sexton P.M. and Christopoulos A.
Pharmacological characterisation of the M_3 muscarinic acetylcholine receptor in *Saccharomyces cerevisiae*. *Recent advances in muscarinic receptor pharmacology and therapeutics* colloquium, Satellite meeting to Experimental biology, San Diego, USA, April 2008.

Stewart G.D., Scammells P.J., Sexton P.M. and Christopoulos A.
Pharmacological characterisation of the adenosine A_1 receptor in *Saccharomyces cerevisiae*. Presented as a poster communication at the *4th Molecular pharmacology of G protein-coupled receptors meeting*, Melbourne, Australia, July 2007.

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CHAPTER 1

GENERAL INTRODUCTION

1.1 Phylogeny and generic structure of GPCRs

Guanine nucleotide-binding protein- (G protein) coupled receptors (GPCRs) are a superfamily of plasma membrane spanning proteins that are activated by extracellular stimuli and, classically, transduce their signals to intracellular effectors via G proteins. According to the International Union of Pharmacology, Committee on Receptor Nomenclature and Drug classification, mammalian GPCRs can be divided into three main classes (1-3) or families (A-C), with a separate class defined for the Frizzled family (Foord et al., 2005). However, based on phylogenic analysis undertaken by Fredriksson et al. (2003), five main classes of human GPCRs have been identified: glutamate, rhodopsin-like, adhesion, frizzled/taste2 and secretin, also termed the 'GRAFS' classification system. Structurally, GPCRs are defined by a common architecture composed of an extracellular N-terminal domain, an intracellular C-terminal domain, and 7 transmembrane (TM) domains linked by three extracellular and three intracellular loops. Despite this common architecture, GPCRs exhibit a remarkable diversity in the array of ligands that they recognise such as large peptide hormones, lipids, small peptides, amines, amino acids, ions and photons (Lagerstrom and Schioth, 2008).

Evolutionarily, the oldest GPCRs are those from the adhesion family (Nordstrom et al., 2009). To date, there are 33 members of this family and possess a proline- and glycosylation-rich N-terminal region of between 200-2800 amino acids (Fredriksson et al., 2003; Lagerstrom and Schioth, 2008). These receptors also contain a GPCR proteolytic domain (GPS); where cleavage of the N-terminal is believed to be a part of receptor post-processing and allows fusion of the receptor with its cognate adhesion molecule (Krasnoperov et al., 2002). Some members of the adhesion receptor family

include the latrotoxin receptors, lectomedin receptors and epidermal growth factor-like module containing mucin-like receptor protein 2.

The secretin family of receptors also possess a large N-terminal region in excess of 115 amino acids and contain six cysteine residues that form three intramolecular disulphide bonds that are conserved across the family (Miller et al., 2007). These receptors bind peptide hormones, which interact with the N-terminal domain and extracellular loops. The binding and activation mechanism of secretin family receptors is believed to be a complex, two-step mechanism of ligand binding and insertion of the ligand/N-terminal complex into the transmembrane domains to elicit activation (Hoare, 2005). The secretin family of receptors include the secretin, calcitonin, glucagon and vasoactive intestinal peptide receptors (Sexton et al., 2006).

The frizzled/taste2 are the newest family of receptors to be classified, and include 11 frizzled receptors and 25 taste2 receptors (Lagerstrom and Schioth, 2008). The frizzled receptors recognise many ligands, the most characterised of which is the lipoglycoprotein Wnt ligands (Schulte and Bryja, 2007). Frizzled receptors are believed to be involved in aspects of cell polarity, cell adhesion and development (Fredriksson et al., 2003). The taste2 receptors, expressed in the tongue and palate epithelium are thought to be responsible for the ability to taste bitter molecules; a single nucleotide polymorphism in this receptor family is the single determinant of whether one can detect bitterness (Lagerstrom and Schioth, 2008).

Another family of GPCRs which possesses a large N-terminal domain, consisting of approximately 280-580 amino acids, is the glutamate family of receptors (Fredriksson et al., 2003). However, these N-terminal domains are structurally conserved across this family, and form a structure known as the venus fly-trap domain, which was first

confirmed for the metabotropic glutamate receptor (Kunishima et al., 2000). This domain consists of a bi-lobate structure and a hinge region that form a pocket that closes around the endogenous ligand upon binding (Pin et al., 2004b). Additionally, these receptors appear to exist as constitutive dimers, either homodimers, e.g., metabotropic glutamate and Ca^{2+} sensing receptors (Jensen et al., 2002; Pin and Acher, 2002), or obligatory heterodimers of the γ-amino butyric acid type B_1 and B_2 ($GABA_{B1}$ and $GABA_{B2}$) receptors (Galvez et al., 2001; Duthey et al., 2002; Pin et al., 2004a). The glutamate family of receptors includes eight metabotropic glutamate receptors, two $GABA_B$ receptors, a calcium-sensing receptor, and sweet and umami taste receptors (Lagerstrom and Schioth, 2008).

The best studied GPCR family is the rhodopsin-like receptor family, with approximately 670 known members (Lagerstrom and Schioth, 2008). Generically, they possess short N-terminal domains, seven transmembrane-spanning domains that are well conserved between receptor subtypes and a short C-terminal domain (with some exceptions). There are also key amino acid residues in the TM helices that are conserved across all rhodopsin-like GPCRs. Two of particular note are the Glu(Asp)-Arg-Tyr (E(D)RY) motif at the bottom of TMIII and the Asp at the base of TMVI. Additionally, there are a number of conserved prolines located within TMV, VI and VII, which introduce kinks in these α-helical TM domains and are believed to be pivotal in facilitating receptor activation (Bhattacharya et al., 2008). On the extracellular face of the rhodopsin family of receptors, there is also a pair of cysteine residues, which is conserved across many rhodopsin family GPCRs, that can form a disulphide bridge between extracellular loop two (ECL2) and the extracellular face of TMIII (Eilers et al., 2005). This is thought to contribute to the stability of the receptor and reduce spontaneous activation.

Determination of the structural characteristic of GPCRs is paramount to the understanding of the site and mode of ligand binding in the ongoing search for new therapeutics. Approximately a decade ago, Palczewski et al. (2000), reported on the first high resolution crystal structure of a mammalian GPCR, publishing a 2.8Å model of inactive bovine rhodopsin with the endogenous, covalently bound, inverse agonist, retinal. Some years later, the same group published a structure of active bovine rhodopsin, however the poor resolution (4.15Å) of this structure was not conducive to making any solid deductions about the mode of receptor activation (Salom et al., 2006). Mammalian rhodopsin signals through the G protein, transducin, which is a structural homologue of the $G_{i/o}$ family of proteins. However, squid rhodopsin couples to G_q-like proteins; when the crystal structure of squid rhodopsin was published it revealed some very distinct features (Shimamura et al., 2008). The most noteworthy feature was that of the extended TMV and TMVI helices into the cytoplasmic space, which possibly offers new insight into the structural determinants required for G_q coupling compared to $G_{i/o}$ coupling.

In addition to these, there has been an influx of crystal structures being reported for other rhodopsin family receptors, including the human β_2- and turkey β_1-adrenoceptors, and the human adenosine A_{2A} receptor (Rasmussen et al., 2007; Jaakola et al., 2008; Warne et al., 2008). These structures revealed distinct structural similarities to the rhodopsin crystal structure, but also some remarkable differences, such as the position of ECL2 of the β_2-adrenoceptor compared to that of rhodopsin; and the adenosine A_{2A} receptor possesses two extra disulphide bonds on the extracellular surface, compared to the other crystal structures of GPCRs.

1.2 Mechanisms of GPCR activation

1.2.1 Structural determinants of GPCR activation

The structural basis of GPCR activation is complex and heavily dependent on the receptor subtype. The difficulty in determining the dynamic changes that a receptor undergoes upon activation is concomitant with the difficult nature of solving active GPCR crystal structures. Nonetheless, some key observations regarding rhodopsin family GPCR activation have been made through mutagenesis studies at some GPCRs (for examples, see Scheer et al., 1996; Ballesteros et al., 1998; Ballesteros et al., 2001). These studies converge on the same idea, that the interaction between TMIII and TMVI via the conserved E(D)RY motif and the formation of an ionic lock is critical for activation of rhodopsin family receptors. Studies performed at the β_2-adrenergic receptor, using homology modelling, have identified another activation switch, termed the 'rotamer toggle switch', that involves an alteration in the proline-induced kink in TMVI, resulting in movement of the cytoplasmic end of TMVI (Shi et al., 2002). These findings are now starting to be further corroborated by the elucidation of new agonist-bound β_2-adenoceptor crystal structures (Kobilka, 2009).

1.2.2 Conceptual and theoretical frameworks of drug action

In the absence of detailed biochemical or biophysical data explaining the mechanisms of GPCR activation, the modes of drug action and receptor activation were first theorised based on empirical pharmacological evidence. Accordingly, pharmacological properties of GPCR ligands have traditionally been described in terms of two parameters, affinity and intrinsic efficacy. Affinity is the attraction between the ligand and receptor and is influenced by the reciprocal electrochemical forces exerted by both species. Intrinsic

efficacy is the ability of a ligand to engender or reduce a stimulus at a receptor, and is governed by the propensity of the ligand to alter the receptor conformation between quiescent and active states.

The 'two-state model' is the most common descriptor of the interaction between ligand (A) and the inactive (R) and active (R*) receptor states. The affinity of the ligand for the receptor is defined by the ligands affinity constant, K_A. The propensity of the receptor to transition between R and R* is governed by the isomerisation constant, L, and the influence of the ligand on the transition is α (Figure 1.1).

In this model, agonists are ligands that promote an active conformation of a GPCR and hence promote receptor signal transduction. The difference between 'full' and 'partial' agonists is their degree of efficacy, which is governed by the ligand's affinity for R* over R.

Antagonists are compounds that block the actions of an agonist, and this is true for neutral antagonists, which merely occlude the binding pocket with limited effects on receptor conformation. However, GPCRs are highly dynamic structures and the ability of receptors to become active in the absence of agonist has been well documented (Costa and Cotecchia, 2005). From this, it was found that some ligands that are able to stabilise GPCRs in an inactive conformation, therefore reducing constitutive basal tone; these ligands are termed inverse agonists. The detection of inverse agonism relies on the signal pathway being measured, the sensitivity of the assay and the propensity of the receptor to undergo spontaneous activation. In terms of the two-state model, inverse agonists display preferred affinity for R over R*.

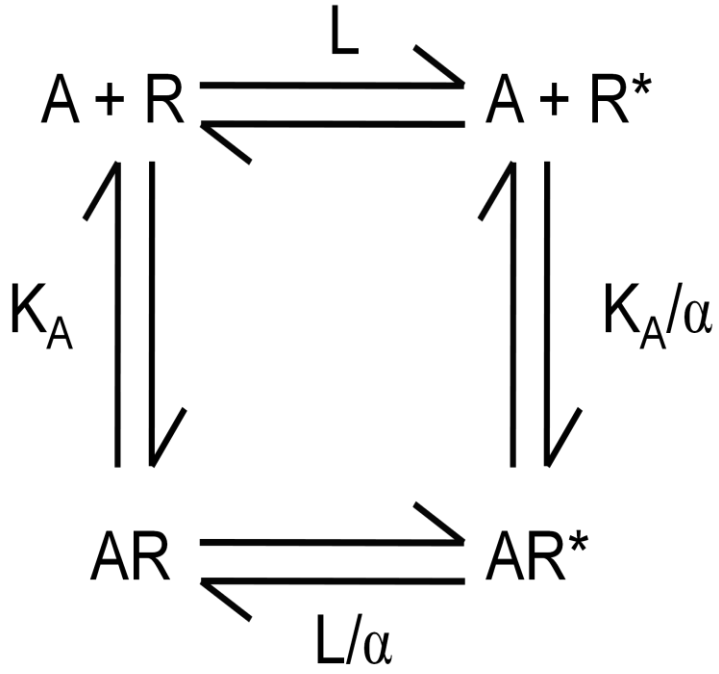

Figure 1.1 Two-state model used to define ligand-receptor interactions. This model describes the interaction of the ligand (A), governed by its affinity (K_A), with the receptor in inactive (R) and active (R*) states, and incorporates spontaneous receptor isomerisation (L) and how that is affected by ligand binding (α).

1.3 Promiscuous coupling of GPCRs

1.3.1 Composition and regulation of heterotrimeric G proteins

Heterotrimeric G proteins consist of an α subunit and a membrane-tethered, fused βγ subunit, both of which have the capacity to signal to various effectors. There are twenty-eight distinct subtypes of α subunits, six subtypes of β and twelve isoforms of γ subunits; from these many different combinations of heterotrimers can be attained, which produce the many signalling pathways required to maintain the specificity and fidelity of cellular systems (Kristiansen, 2004). The α subunits are guanine nucleotide binding proteins of between 39 to 45kDa in mass, which rest in an inactive, guanosine diphosphate- (GDP) bound state. The β subunits are approximately 35 to 39kDa in mass and are tightly associated with the considerably smaller γ subunits (6 to 8kDa), both of which are membrane-tethered via post-translational prenylation (Hamm, 1998).

Upon GPCR activation, a subsequent conformational change promotes a high affinity receptor state for the α subunit to bind, and subsequently alters the α subunit's conformation. This decreases the affinity of GDP for the α subunit, the GDP dissociates and the resultant complex is a high-affinity, nucleotide free GPCR-G protein complex. However, in a physiological system the nucleotide-free state of a Gα protein is transient as guanosine triphosphate (GTP) rapidly binds, consequently reducing the affinity of the α subunit for the receptor and βγ subunit and resulting in the dissociation of the αβγ complex. The dissociated, GTP-bound α subunit has a much higher affinity for its cognate effectors, compared to the GDP-bound or nucleotide-free form, allowing the propagation of the signal cascade (Hamm, 1998). In addition to this, the liberated βγ subunits are also capable of promoting activation of their affiliated effectors.

The activated state of the Gα subunit is also transient, as the α subunit possesses intrinsic guanosine triphosphatase (GTPase) activity, which catalyses the hydrolysis of GTP to GDP, returning the α subunit to the ground state, which consequently results in the re-association of the heterotrimer. Interestingly, however, it was recognised that the kinetics of the Gα subunit's intrinsic GTPase activity were too slow to explain the rapid deactivation of GPCR signalling observed in physiological settings (Neubig and Siderovski, 2002). One example of this is rhodopsin and its cognate G protein, transducin, which exhibits a GTP hydrolysis rate (measure of GTPase activity) of 1-2 per minute in a cell-free system, however the signalling of rhodopsin displays rapid recovery (~200ms) of G protein signalling in retinal-rod photoreceptor cells (Arshavsky and Pugh, 1998). This suggested involvement of other mediators, which have since been discovered and termed the regulator of G protein signalling (RGS) proteins; they exhibit Gα-specific GTPase-activating protein (GAP) activity (Zhong and Neubig, 2001). There are approximately 30 identified RGS proteins, all of which possess an RGS domain of ~120 amino acids in conjunction with various other domains, such as PDZ-binding domains or β-catenin-binding domains (Hepler, 1999; Bansal et al., 2007). Interestingly, some proteins known for other functions, such as GPCR kinase (GRK) 2 and p115-RhoGEF, also possess RGS activity, and in the case of p115-Rho guanine-nucleotide exchange factor (GEF) this activity is exclusively extended to $G\alpha_{12/13}$ proteins, whilst maintaining its activity as a $G\alpha_{12/13}$ protein effector (Suzuki et al., 2009). Numerous studies using overexpression systems of RGS proteins have resolved that they are a major negative-regulator of GPCR signalling in a G protein and tissue specific manner, hence making them an attractive target for drug discovery programs (Neubig and Siderovski, 2002; Gu et al., 2009).

1.3.2 G protein effectors and signalling paradigms

The twenty-eight Gα subunits have been divided into four major subtype groups, $G\alpha_s$, $G\alpha_{i/o}$, $G\alpha_{q/11}$ and $G\alpha_{12/13}$, each being classified on their sequence homology and the signalling cascade they promote. However, there is a multitude of evidence suggesting that no single G protein exclusively activates a single type of effector (reviewed in Hur and Kim, 2002). For example $G\alpha_{12/13}$ proteins activate RhoGEFs, but also certain phospholipase C (PLC) isoforms, and $G\alpha_{q/11}$ also activates PLC in addition to certain RhoGEFs (Zeng et al., 2002; Lutz et al., 2005; Worzfeld et al., 2008). It is for this reason that the following sections are divided into effectors, rather than G proteins. Table 1.1 provides examples of different G proteins and their various effectors.

1.3.2.1 Adenylate cyclases

Historically, $G\alpha_s$ proteins were the first G proteins to be discovered and thus named for their ability to stimulate adenylate cyclase (AC), which then catalyses the conversion of adenosine triphosphate (ATP) to the prototypical second messenger, 3',5'-cyclic adenosine monophosphate (cAMP; Cooper, 2003). The increase in cytosolic cAMP concentration can lead to activation of various effectors, such as cAMP-dependent protein kinase (PKA), cyclic nucleotide-dependent gated ion channels and cAMP response element binding proteins (Kamenetsky et al., 2006; Sands and Palmer, 2008)

Two types of ACs are known, a transmembrane isotype (tmAC), of which there are nine, and a G protein-insensitive, soluble form (sAC), of which there is one (Sunahara et al., 1996). The amino-acid sequencing and cloning of a bovine tmAC1 revealed that it consisted of twelve membrane-spanning domains, two homologous ATP-binding regions and long C- and N-termini (Krupinski et al., 1989). Since then, eight more isozymes have been cloned and all display similar structure to tmAC1 (reviewed in Willoughby and Cooper, 2007). One interesting feature of these enzymes is that they

can be positively and negatively regulated by a vast array of interacting proteins and complexes, such as Ca^{2+}/Calmodulin (Ca^{2+}/CaM), Gβγ subunits or some protein kinases (Sunahara and Taussig, 2002), and all are activated by $G\alpha_s$ proteins (Willoughby and Cooper, 2007). ACs can be divided into three main groups, βγ-activated (AC2, 4 and 7), those that are sensitive to inhibition by Ca^{2+} and protein kinases (AC5 and 6), and Ca^{2+}/CaM-sensitive isoforms (AC1, 3 and 8; Sunahara and Taussig, 2002).

Adenylate cyclase isozymes 2, 4 and 7 are positively modulated Gβγ subunits, and act synergistically in the presence $G\alpha_s$ (Tang and Gilman, 1991; Sunahara et al., 1996). This allows Gβγ subunits liberated from $G\alpha_{i/o}$ proteins to activate these isoforms, where it was commonly thought that $G\alpha_{i/o}$ solely inhibited the function of ACs (Cooper, 2003). Interestingly, AC2 and 7, but not 4, are also activated by phosphorylation by protein kinase C (PKC) (Jacobowitz et al., 1993; Jacobowitz and Iyengar, 1994).

Conversely, AC isozymes 5 and 6 are sensitive to inhibition by Gβγ subunits, in addition to being inhibited by PKA, $Ca^{2+}$and $G\alpha_{i/o}$ proteins (Beazely and Watts, 2006). Interestingly, a recent study has suggested that AC5 may be activated not only by $G\alpha_s$ and some PKC isoforms (Sunahara and Taussig, 2002), also by stromal interaction molecule 1 (STIM1) after it translocates to the plasma membrane (PM) upon depletion of store-Ca^{2+} in the sarco(endo)plasmic reticulum (Lefkimmiatis et al., 2009).

The prototypical AC isoform, AC1, shares homology and functional characteristics with AC3 and 8, in that they are able to be activated by Ca^{2+}/CaM complexes, but are inhibited by CaM kinase (CaMK; Sunahara and Taussig, 2002).

Table 1.1 Gα protein subtypes and examples of their cognate effectors and signalling molecules.

Gα protein families and subtypes	Effector and signalling pathway
$G\alpha_s$ Family $G\alpha_s$ $G\alpha_{olf}$	**Adenylate cyclase stimulation** cAMP, PKA
$G\alpha_{i/o}$ Family $G\alpha_{i1}$ $G\alpha_{i2}$ $G\alpha_{i3}$ $G\alpha_{oa}$ $G\alpha_{ob}$ $G\alpha_{t1}$ $G\alpha_{t2}$ $G\alpha_z$	**Adenylate cyclase inhibition** cAMP, PKA
$G\alpha_{q/11}$ Family $G\alpha_q$ $G\alpha_{11}$ $G\alpha_{14}$ $G\alpha_{15}$ $G\alpha_{16}$	**Stimulation of Phospholipase C** IP_3 release, DAG, Ca^{2+}, PKC
Ga_{12} Family $G\alpha_{12}$ $G\alpha_{13}$	**Activation of Rho** DAG, PKC
Gβγ Units	**Stimulation of Phospholipase C** Inhibition of ion channels (Kir3 and Ca_v2) Activition of MAPK, PI3K, adenylate cyclase

1.3.2.2 Phosphodiesterases

Phosphodiesterases (PDEs) catalyse the hydrolysis of cyclic nucleotides (cAMP and cyclic 3',5' guanosine monophosphate, cGMP). There are eleven different PDE families, eight of which are capable of hydrolyzing cAMP (Houslay and Milligan, 1997). The change in substrate concentration alters activation of PDEs but, depending on the isoform, they are also modulated by other factors, such as Ca^{2+}/CaM and other cyclic nucleotides (Bender and Beavo, 2006). Intriguingly, the role of PDEs is not only to regulate global concentrations of cyclic nucleotides, but also to modulate the spatiotemporal signalling of cyclic nucleotides in microenvironments and, indeed, PDEs are often compartmentalised with ACs and PKA to tightly regulate cAMP signalling (Baillie et al., 2005).

1.3.2.3 Phospholipases

There are four main groups of phospholipases (PLs): phospholipase A, B, C and D, all of which catalyse the hydrolysis of phospholipids. Given the lack of data surrounding PLB, this subclass will not be discussed.

The phospholipase A (PLA) family is divided into two subtypes, PLA_1 and PLA_2 . The function of PLA_1 is largely unknown, however it is known that there are at least nine isoforms, six of them are extracellular and three intracellular (Aoki et al., 2007). Both forms share little sequence homology and their functions appear to be distinct. The role of PLA_2, however, has been studied in greater depth, being involved in production of inflammatory mediators from arachidonic acid phospholipid cleavage (AA; Chakraborti, 2003). There are three main groups of PLA_2 enzymes, cytosolic, secretory and intracellular, all of which have AA as a substrate with varying specificity. PLA_2-mediated cleavage of AA phospholipid cleavage from the plasma membrane allows the enzymes cyclooxygenase and lipoxygenase to further cleave AA into prostaglandins and

cysteinyl-leukotrienes, hence PLA_2 enzymes presents a useful target for anti-inflammatory therapeutics (Yedgar et al., 2000).

The PLC subclass of phospholipases is that which cleaves phosphoinositides from the plasma membrane into products that mediate intracellular Ca^{2+} release (Fain et al., 1988; Berridge and Irvine, 1989). To date, there are thirteen isoforms of PLC that have been identified, PLCβ(1-4), δ(1,3,4), γ(1,2), η(1,2), ξ and ε (Suh et al., 2008). All share a general architecture of two catalytic subunits (X and Y domains), between a Ca^{2+}-binding-elongation factor (EF)-hand motif and C2 domain, flanked by various other domains, for example pleckstrin homology domains or RasGEFs. Additionally, all PLCs have the ability to cleave phosphatitdylinositol 4,5-bisphosphate (PIP_2) from the plasma membrane, resulting in the products, inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG), IP_3 then binds with IP_3 receptors on the sarco(endo)plasmic reticulum to evoke intracellular Ca^{2+} mobilisation; DAG is an activator of protein kinase C (PKC), which can phosphorylate an large array of protein targets.

PLC-β is the largest of the of the subgroups of PLCs. PLCβ1 and PLCβ3 are widely expressed throughout the body, whereas PLCβ2 appears to be restricted to haemopoetic cells, and PLCβ4 is localised to retinal cells and certain types of neurons (Rhee, 2001). All PLCβ isoforms are activated by members of the $G\alpha_{q/11}$ family of G proteins and Gβγ subunits with varying efficiencies, and their deactivation is dependent on restoration of the G protein to the ground state (Katan, 1998; Rhee, 2001). Moreover, PLCβ2 is not exclusively activated by $G\alpha_{q/11}$ and Gβγ subunits, but can also be activated by Rac (Bunney and Katan, 2006). In addition to this, PLCβ1 possesses GAP activity for $G\alpha_q$ proteins. Interestingly, all PLCβs contain PDZ-binding motifs, which have been shown to interact with proteins such as Na^+/H^+ exchange regulatory factor (NHERF) and NHE3 kinase A regulatory factor (Hwang et al., 2000; Suh et al., 2001).

PLCξ is believed to be exclusively expressed in spermatozoa and in the testes, and there is evidence that it is involved in Ca^{2+}-mediated ovum activation after gamete fusion (Swann et al., 2006). PLCη is found largely in the brain and kidneys, whilst PLCγ1 is expressed nearly exclusively in the brain, and PLCγ2, like PLCβ2, nearly exclusively in haemopoetic cells (Stewart et al., 2007; Suh et al., 2008). PLCδ isoforms are expressed in skeletal muscle, heart and the reproductive system, but have the highest expression in the brain.

The PLCε subclass of phospholipases possess an intriguing feature, in that they are preferentially activated (in contrast to PLCβ2), by Rho GTPases, but not Rac or Cdc42 (Bunney and Katan, 2006). Some interesting points of PLCε, with regard to its structure, are that it possesses a Cdc25 domain (RasGEF) and two Ras association (RA) domains (Rossman et al., 2005), and it has been suggested that PLCε, further to Rho GTPases, is also an effector of Ras GTPases (Kelley et al., 2001). Given that Ras is an effector of receptor tyrosine kinases (RTK) and Rho is an effector of $G\alpha_{12/13}$, the effects of PLCε dysfunction have potential to lead to a vast array of pathologies. Indeed, mutagenesis studies have shown that deletion of PLCε in mice resulted in malformed aortic and pulmonary cardiac valves, increased propensity of cardiac hypertrophy in response to chronic cardiac stress and, in fact, a naturally occurring truncation of PLCε and a Ser^{1484}Leu mutation in humans led to early-onset nephrotic syndrome (Bunney and Katan, 2006).

Phospholipase D enzymes are known to catalyse the hydrolysis of phosphatidylcholine into phosphatidic acid and choline and, to date, two isoforms have been identified (Vorland et al., 2008). Similarly to some PLC isoforms, PLD can also be activated by small GTPases, however, PLD is preferentially activated by ARF proteins. The effects of PLD are thought to be mediated by phosphatidic acid, which plays key roles in

vesicle transport and cytoskeletal rearrangement, in addition to modulation of some enzymes (Vorland et al., 2008), PLCγ and ξ, NADPH oxidase and Raf-1 kinase (Jones and Carpenter, 1993; Ghosh and Bell, 1997; Gomez-Cambronero and Keire, 1998).

1.3.2.4 Phosphoinositide 3-kinase

To date, there have been three distinct classes of phosphoinositide 3-kinases (PI3K), I, II and III, all of which possess a homologous catalytic core domain, linked to a PI kinase homology domain and a C2 domain (Vanhaesebroeck et al., 2001). PI3Ks are activated by RTKs, Ras and Gβγ subunits liberated by activation of $G\alpha_{i/o}$-coupled GPCRs, and is thought to be one of the main effectors of the Akt pathway, but there is also evidence that it can recruit various PLC isoforms and tyrosine kinases (Landry et al., 2006; Franke, 2008). There is evidence that PI3K and Akt activation plays key roles in oncogenesis, whereby the tumour suppressor phosphatase and tensin homologue, a phosphatase that inactivates products of PI3K and inhibits Akt activation, is often mutated in some cancers (Li et al., 1997; Maehama and Dixon, 1998).

1.3.2.5 Modulation of ion channels and transporters by GPCRs

1.3.2.5.1 Sodium transporters

GPCRs are known to interact either directly or indirectly with a variety of ion channels to modulate membrane potential and ionic concentration (Mahaut-Smith et al., 2008). As previously alluded to, GPCR-mediated activation of PLCβ can promote an interaction with NHERF, which can then interact with Na^+/H^+ exchangers to pump H^+ into the extracellular space, as has been shown for the endothelin-1A receptor (Horinouchi et al., 2008); this is in addition to endothelin-1A receptor-mediated activation of Na^+/Ca^{2+} exchangers (NCXs) to decrease levels of intracellular Ca^{2+} whilst

pumping Na^+ into the cytosol (Horinouchi et al., 2007). In addition, there is also evidence that GPCR-mediated activation of NCXs occurs for purinergic receptors (Hansen et al., 2009), β_1-adrenoceptors (Janowski et al., 2009; Tsang et al., 2009) and the bradykinin B_1 receptor (Ifuku et al., 2007).

1.3.2.5.2 Potassium channels

G protein-coupled inward rectifying potassium (GIRK) channels or inward-rectifying potassium type 3 (Kir3) channels are expressed in the neuronal, cardiac and endocrine tissues and can be activated by both Gα and Gβγ subunits (Peleg et al., 2002; Landry et al., 2006; Doupnik, 2008). Activation of Kir3 channels plays an important physiological role by causing cellular hyperpolarisation, thus increasing the polarisation threshold of excitatory tissues, such as neurons. The activation of Kir3 channels is predominantly controlled by pertussis toxin-sensitive G proteins, and is synergistically enhanced by the presence of PIP_2 (Wickman and Clapham, 1995; Hilgemann et al., 2001). The deactivation of Kir3 channels is nearly entirely dependent on the restoration of the G proteins to the inactive state, however, the deactivation of Kir3 channels was found to be much more rapid than the intrinsic GTPase activity of the Gα subunit, thus suggesting a pivotal role of RGS proteins in directly GPCR-dependent membrane potentials (Mark and Herlitze, 2000).

1.3.2.5.3 Calcium channels

Ionic calcium is arguably the most important component of signal transduction, as it plays a role in nearly all physiological processes. GPCRs are also known to regulate the function of mechanisms to inhibit and promote Ca^{2+} entry in the cell (Landry et al., 2006; Abramowitz and Birnbaumer, 2009). The modulation of voltage-operated Ca^{2+}

(Ca_v) channels is an important component in controlling neurotransmission, and, similarly to Kir3 channels, are inhibited by Gβγ subunits of pertussis toxin-sensitive G proteins in presynaptic neurons (Herlitze et al., 1996). Presynaptic Ca_v channels, P/Q- and N-type or $Ca_v2.1$ and $Ca_v2.2$ respectively, like all Ca_v channels consists of a pore forming α_1 subunit and accessory α_2, β, δ and γ subunits (Dolphin, 2006). The α_1 subunit consists of four modules of six transmembrane-spanning domains and the Gβγ subunit is thought to bind on the intracellular loop connecting the first and second module (Catterall, 2000). In addition to $Ca_v2.1$ and $Ca_v2.2$, GPCRs can also indirectly activate L-type or Ca_v1 family calcium channels via phosphorylated PKA or PKC, following Gα subunit-mediated signalling (Landry et al., 2006). The desensitisation of Ca_v channels, again, like Kir3 channels, is dependent on the Gα-GDP sequestration of the Gβγ subunits, furthermore, RGS proteins play a critical role in reversing the inhibition of Ca_v channels (Mark et al., 2000).

Another fundamental component of Ca^{2+} signalling is influx of extracellular Ca^{2+}, which is predominantly mediated by the canonical transient receptor potential (TRPC) channels (Abramowitz and Birnbaumer, 2009). TRPC channels can be activated by GPCR-mediated PLCβ or RTK-mediated PLCγ activation, where the DAG generated from phosphoinositide hydrolysis is capable of activation TRPC channels (Birnbaumer et al., 1996; Onohara et al., 2006). This response is potentiated by the depletion of intracellular Ca^{2+} stores from the sarco(endo)plasmic recticulum and the translocation of STIM1 to the plasma membrane (Wang et al., 2008). STIM1 associates with the TRPC channel, which forms complex with regulatory Orai proteins, to furnish an active, low capacitance Ca^{2+} channel complex (Liao et al., 2008). Many GPCRs have been shown to activate TRPC channels, such as the $G\alpha_q$-coupled muscarinic acetylcholine, angiotensin II type 1, endothelin-1A and the serotonin 5-HT_2 receptors, and given the

wide expression of TRPC channels and receptors that activate TRPC channels, their role in the modulation of the Ca^{2+} response adds a further degree of fine-tuning to Ca^{2+} signal transduction (Abramowitz and Birnbaumer, 2009).

1.3.2.6 Small G proteins

The Ras family of G proteins consists of three subtypes, N-Ras, H-Ras and K-Ras, of which the latter is alternatively spliced to yield four isoforms of K-Ras (Buday and Downward, 2008). In addition, four subfamilies of RasGEF have been identified, son-of-sevenless (Sos), RasGRF, RasGRP and CNRasGEF, plus the aforementioned RasGEF domain of PLCε. Activation of RasGEFs by GPCRs occurs in a variety of ways, including Ca^{2+}/CaM and DAG activation of RasGRF and cAMP-mediated activation of CNRasGEF (Amsen et al., 2006; Werry et al., 2006; Buday and Downward, 2008). Furthermore, the Gβγ-mediated activation of c-Src and PI3K also has the potential to stimulate Ras via RasGRF or the recruitment of Sos/Grb2 complexes to the plasma membrane (Luttrell et al., 1996; Lopez-Ilasaca et al., 1997). Ras is thought to be a major mediator in synaptic plasticity and also perform critical roles in oncogenesis due its ability to activate mitogen-activated protein kinase (MAPK) pathways (Wang et al., 2004; Giehl, 2005).

The Rho family of proteins encompasses Rho, Rac and Cdc42, and together these proteins perform vital functions in mediating cytoskeletal sub-structural changes, adhesion and migration (Burridge and Wennerberg, 2004). Since their discovery in 1992 by Ridley and Hall, the functions of Rho and Rac, with regard to cytoskeletal changes, have been quite disparate; Rho induces stress fibre formation, and Rac, membrane ruffling (Ridley and Hall, 1992; Ridley et al., 1992). Whilst this postulate is

largely correct, with the advent of more sensitive probes and new techniques, it is now possible to show that signalling of Rho and Rac is inter-connected in both stress fibre formation and membrane ruffling responses, and the observed outcome is largely dependent on the cell type used (Hong-Geller and Cerione, 2000; Salhia et al., 2005; Pertz et al., 2006; Ridley, 2006; Meller et al., 2008). Furthermore, both Rac and Rho are proposed to work together in secretory responses, however this is thought to be independent of actin polymerisation (Price et al., 1995b; Norman et al., 1996). Both Rho and Rac activation, in conjunction with Cdc42 result in the recruitment and activation of various regulatory and scaffold proteins to the plasma membrane, such as vinculin, focal adhesion kinase, cofilin, WAVE and WASP (DeMali and Burridge, 2003).

The regulation of Rho is predominantly by RhoGEFs, activated by $G\alpha_{12/13}$, and RhoGAPs, of which there are many types, including p115-RhoGEF, PDZ-RhoGEF, leukaemia-associated RhoGEF (LARG) and the chimaerin family of proteins (RhoGAPs; Siderovski and Willard, 2005; Yang and Kazanietz, 2007). The two main effectors of Rho are Rho kinase (ROCK) and mDia, which work synergistically in producing stress fibres (Burridge and Wennerberg, 2004). On the one hand, ROCK facilitates the phosphorylation of myosin light-chain phosphatase (MLCP), rendering it inactive, and pushing the equilibrium of myosin light-chain into the active, phosphorylated state (Noda et al., 1995). On the other hand, mDia promotes actin nucleation and polymerisation, which results in actin fibre formation that is enhanced by the activity of ROCK (Narumiya et al., 2009).

Regulation of Rac, like Rho, is dependent on RacGEFs such as DOCK180 (when in a complex with ELMO), however, it has also been shown to be positively (Fujii et al., 2005; Yuan et al., 2006) or negatively (Herroeder et al., 2009) regulated by $G\alpha_{12/13}$

subunits. Similarly to Rho, Rac is known to mediate cytoskeletal changes, such as membrane ruffling and lammelepodia formation (Brown et al., 2006; Cote and Vuori, 2007). Interestingly, the activation of Rac extends beyond the cytoskeleton, as Rac forms part of the active, membrane-associated enzyme, NADPH oxidase, and is associated with signalling down extracellular signal-regulate kinases 1 and 2 (ERK1/2) and c-Jun N-terminal kinase (JNK) pathways (Abo et al., 1991; Clerk et al., 2001).

1.3.2.6 Transactivation of receptor tyrosine kinases by GPCRs

The transactivation of RTKs by GPCRs in many cells performs a key role in regulation of cell proliferation, growth and survival, mainly due to their strong linkage to MAPK pathways, and three mechanisms of transactivation of RTKs have been identified (Delcourt et al., 2007). The first mechanism requires the GPCR-mediated accumulation of second messengers, Ca^{2+} and reactive oxygen species, for example, or activation of protein kinases (Tsai et al., 1997; Eguchi et al., 1998; Frank et al., 2003). The second mechanism is direct protein-protein interaction between c-Src, resulting in the activation of Pyk2 and phosphorylation of the RTK (Ohtsu et al., 2006). The third is the activation of matrix-metalloproteases (MMPs) or a disintergrin and metalloproteases (ADAMs), which results in shedding of ectodomain-tethered RTK ligand precursors from the plasma membrane (Delcourt et al., 2007). Indeed, many GPCRs have been shown to transactivate RTKs via this mechanism, such as the serotonin 5-HT_{2C}, vasopressin V_2 and the orphan GPCR, GPR30 (Filardo, 2002; Charest et al., 2007; Werry et al., 2008). Moreover, ADAMs and MMPs have been implicated in many different pathologies involving hypertrophy or hyperplasia in various tissues, and may offer a valuable drug target (Huovila et al., 2005).

1.3.2.7 Non-G protein-mediated signalling

In addition to signalling via G proteins, GPCRs are also capable of signalling via non-G protein-mediated mechanisms, such as direct activation of c-Src and recruitment of arrestin adapter proteins (Werry et al., 2006). Arrestins -2 and -3 (β-arrestin1 and -2) were initially identified as adapter molecules involved in inhibiting G protein binding at the β_2-adrenoceptor which led to homologous desensitisation (Lohse et al., 1990), (which will be further discussed in the next section). However, the concept of arrestins as signalling molecules in their own right is now well established as a non-G protein-mediated pathway that can lead to activation of MAPKs, Akt and JNK3, for example (DeWire et al., 2007). The first identification of arrestins, in particular the cytosolic arrestin, arrestin3, as signalling molecules was observed in systems incapable of receptor internalisation, either by chemical inhibition or inhibition by dominant negative proteins, which led to the discovery that arrestin3 recruitment to the GPCR, in turn, led to the recruitment of c-Src to the receptor/arrestin3 complex (Luttrell et al., 1999; DeFea et al., 2000). It was then found that proteins such as Raf-1 and MAPK/ERK kinase (MEK) were recruited to this complex, resulting in the phosphorylation of ERK1/2 (Luttrell et al., 2001). Similarly, the dopamine-induced, dopamine D_2 receptor-mediated formation of an arrestin3/Akt/phosphatase 2A complex, led to activation of the Akt signalling pathway, although this was not evident in the arrestin3 deficient system (Beaulieu et al., 2005). It is in these ways that the GPCR activation/desensitisation event can lead to further 'fine-tuning' of signalling, independently of G proteins.

1.3.3 Lipid rafts and caveolae

For a long while, GPCRs, G proteins and their effectors, were considered as entities moving through a fluid plasma membrane, and that their coupling (and thus signalling), was due to random collision events (Ostrom et al., 2000). However, this scenario does not account for the rapid kinetics of signalling seen in many systems. It was this discrepancy that led to the hypothesis that GPCRs, G proteins and effectors may in fact, be restricted in lipid rich domains, non-coated pits or caveolae (Neubig, 1994). GPCRs have since been shown to compartmentalise in these domains, and it is this restriction of movement throughout the plasma membrane which allows rapidity and fidelity of signal transduction, maintains the spatiotemporal control of second messengers and is potentially a cellular means of restricting promiscuous signalling (Ostrom et al., 2000). The major protein constituent of caveolae is caveolin, of which there are three known isoforms (-1, -2 and -3), all consisting of cytoplasmic N- and C-terminal domains, membrane-associating palmitoylation sites and a scaffolding domain that interacts with compartmentalised proteins. The caveolin scaffolding domain (DGIWKASFTTFTVTKYWFYR) interacts with many membrane-associated proteins that contain a caveolin-binding motif, either: φXXXXφXXφ, φXφXXXXφ or φXφXXXXφXXφ, where φ is an aromatic amino acid and X is any other amino acid (Okamoto et al., 1998). Many signalling molecules have the predisposition to be sequestered into caveolae; a few examples are H-Ras, Src, PDEs, PKA, NCX channels and G proteins (Sargiacomo et al., 1993; Li et al., 1995; Mineo et al., 1996; Calaghan et al., 2008). Perhaps the signal transduction pathway that requires the most rapid activation and deactivation involves the operation of ion channels and, indeed, many ion channels, exchangers and related effectors, such as TRPC and L-type Ca^{2+} channels, are restricted in caveolae in many systems to maintain rapid kinetics and fidelity of the

signal (Li et al., 1997; Darby et al., 2000; Gosens et al., 2007b; Balijepalli and Kamp, 2008). The disruption of caveolae resulted in impaired muscarinic acetylcholine receptor-mediated Ca^{2+} release in airway smooth muscle cells (Gosens et al., 2007b), although it did not impede the phosphorylation of ERK1/2 by the same receptors in the same cell type (Gosens et al., 2007a). These studies suggest that caveolae perform an important function in controlling cell signalling effectors and potentially restricting promiscuous effector coupling of GPCRs.

1.3.4 Mechanisms of GPCR regulation and trafficking

Beyond activation and inactivation of G proteins, there are additional feedback mechanisms in place to arrest signalling of GPCRs that act on the receptor itself. The first step of GPCR desensitisation is receptor phosphorylation, which comes in two forms, homologous (agonist-dependent) and heterologous desensitisation (Kelly et al., 2008). Both processes involve the phosphorylation of serine and threonine residues at various sites on the intracellular face of the receptor, primarily thought to be the intracellular 3rd loop and the C-terminal tail (Tobin et al., 2008). The addition of phosphate groups to the receptor promotes the association of adapter proteins such as arrestins, which sterically interdicts the re-association of the G protein to the receptor (Lefkowitz, 1998). Heterologous desensitisation is desensitisation of a receptor or receptor signalling pathway, and may involve phosphorylation of the receptor by second messenger kinases PKA and PKC, for example, and/or alterations in regulation of downstream effectors (Kelly et al., 2008). Furthermore, heterologous desensitisation is not necessarily a direct result of agonist-stimulated receptor activation, but may be caused by activation of a shared signalling pathway by different GPCR subtype, this

mode of desensitisation is particularly common among PLC-coupled GPCRs (Werry et al., 2003).

Originally, the phosphorylation of GPCRs was thought to be governed mainly by protein kinases such as PKA and PKC (Benovic et al., 1985). However, further investigation of mechanisms of GPCR desensitisation and regulation led to the discovery of a new family of kinases, the G protein-regulated kinases (Ribas et al., 2007). Homologous desensitisation from GRK-mediated phosphorylation is an agonist-dependent event, will only occur on a GPCR in the active state and is not cross-reactive between other GPCR subtypes (Ferguson and Caron, 1998). Figure 1.2 illustrates the desensitisation-arrestin-mediated-internalisation-recycling/degradation pathway.

To date, seven GRK subtypes have been identified (GRK1-7), of which GRK1 and -7 are restricted to the retinal rods and cones, and GRK4 is confined to the cerebellum, testes and the kidneys (Reiter and Lefkowitz, 2006). Conversely, GRK2, -3, -5 and -6 are effectively ubiquitously expressed throughout the body. The seven GRKs can be divided into three families: GRK1 and -7; the PH domain-containing GRK2 and -3, whose membrane translocation is Gβγ- and PIP_2-dependent; and GRK4, -5 and -6, which are constitutively associated with the plasma membrane (Pitcher et al., 1998; Reiter and Lefkowitz, 2006). GRKs possess a conserved catalytic domain of approximately 270 amino acids, flanked by an N-terminal domain (~185 amino acids) and a varied C-terminal domain that ranges from approximately 100 to 230 amino acids, depending on the family (Ribas et al., 2007). The N-terminal domain is believed to be critical in receptor recognition and membrane anchoring. In addition, it contains an RGS homology domain and other structural domains that allow GRK to bind other accessory proteins, such as caveolin, PI3K or α-actinin in a subtype-specific manner. The RH domain has also been shown to interact with the PH domain in GRK2 and -3, in

addition to interacting with the kinase region, suggesting a regulatory role of this domain further to its putative role as an RGS protein (Ribas et al., 2007). The C-terminal domain also contributes to the subcellular localisation and translocation upon agonist activation (Kohout and Lefkowitz, 2003). Intriguingly, there is evidence to suggest that differential phosphorylation affects the kinetics of recruitment of arrestins (Violin et al., 2006), but it has also been suggested that differential phosphorylation may affect arrestin signalling (Tobin et al., 2008). It has been shown for the β_2-adrenergic and vasopressin V_2 receptors that the arrestin-mediated ERK1/2 phosphorylation profile is not altered by inhibition of GRK2 and -3-mediated phosphorylation, but ERK1/2 phosphorylation is reduced by inhibition of GRK5 and -6-mediated receptor phosphorylation, suggesting differential roles for these GRKs (Ren et al., 2005; Shenoy et al., 2006).

As previously mentioned, one of the many roles of arrestins involves signal transduction in a G protein-independent mode. However, their primary identified function was to silence GPCR/G protein signalling. The GRK-mediated phosphorylation of a GPCR results in an increase in affinity of the receptor for arrestins and, indeed, most GPCRs are subject to arrestin binding, with a few exceptions such as the β_3-adrenoceptor and the atypical chemokine receptor, Duffy antigen receptor for chemokines (Liggett et al., 1993; Patel et al., 2009). Arrestin binding promotes the association of adapter proteins AP2, clathrin and dozens of regulatory proteins, together with dynamin, to form clathrin-coated pits, and induce receptor internalisation (Reiter and Lefkowitz, 2006; Hanyaloglu and von Zastrow, 2008; Marchese et al., 2008). Upon internalisation, the endosome can be subject to one of two fates, recycling back to the cell surface or lysosomal degradation (Hanyaloglu and von Zastrow, 2008). The determinants of receptor fate are highly specialised and complex, but generically involve

phosphorylation and ubiquitination of the receptor in discrete patterns (Marchese et al., 2008).

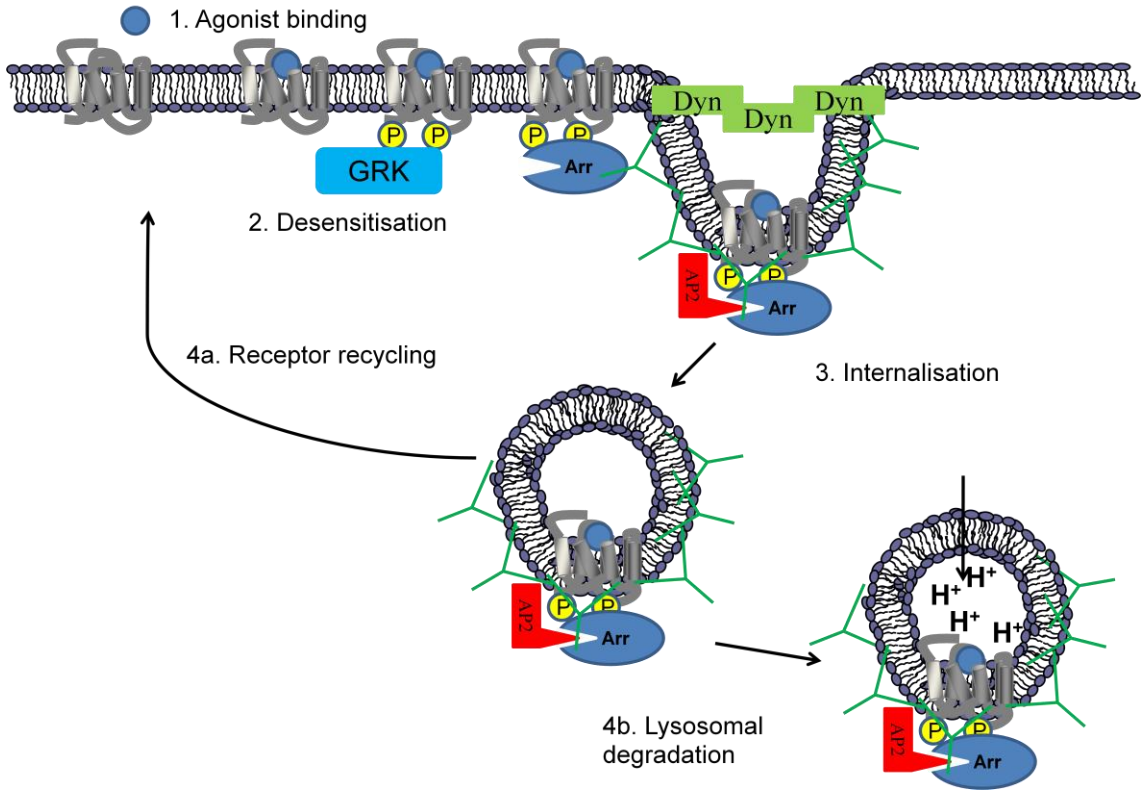

Figure 1.2 Canonical homologous desensitisation-internalisation pathway. Upon agonist binding the receptor is phosphorylated by GRKs; arrestins (Arr) and adapter proteins (AP2) are recruited; clathrin is subsequently recruited to form clathrin-coated pits, and dynamin (Dyn) 'pinches' the membrane to form vesicles. The resultant internalised vesicle may then either be trafficked back to the plasma membrane or undergo lysosomal degradation.

1.4 Novel paradigms of ligand-GPCR interactions

1.4.1 Functional selectivity of GPCR ligands

For many years, the classical definition of intrinsic efficacy of a ligand revolved around a system-independent property that, in essence, was simply the ligand's ability to modulate receptor stimulus. Observations that could not be explained by classical receptor theory were deemed to be caused by experimental artifacts or differences in stimulus-response coupling, and often dismissed. However, there was some data that could not be reconciled with these reasons, (for review see Kenakin, 1995). With the advent of new technologies and more sensitive assay conditions, these anomalies could be more closely examined, and the relatively uncomplicated concept of intrinsic efficacy has become increasingly more complex by the accumulating evidence of a phenomenon known as 'functional selectivity', 'biased agonism', 'ligand-directed trafficking of receptor stimulus' or 'protean agonism' (Kenakin, 1995; Urban et al., 2007). This concept has arisen from evidence that a single GPCR can simultaneously activate more than one signalling pathway, and that the strength of coupling between these pathways is often different depending on the ligand, and in many cases, divergent. Perhaps the simplest model to depict this phenomenon is the three-state model, which describes the interaction between the agonist (A) with the inactive receptor (R), an active conformation of the receptor (R*) and an active conformation of the receptor that is disparate from R* (R**; Figure 1.3A) (Leff et al., 1997). This model can be translated into a cubic ternary complex model that encompasses the agonist (A), G protein (G) and the inactive and active receptor R and R*, respectively. However, in this model, R* describes infinite active states of the receptor (adapted from Weiss et al., 1996). In general, the determination of functional selectivity necessitates the use of multiple and

disparate functional assays and the use of multiple ligands. Thereafter, functional selectivity may be characterised as a change in the rank order of potencies, efficacies or both, between different pathways (Figure 1.4A). In some cases the ligand can activate signalling pathways in a divergent manner, such that it can display positive efficacy for one pathway, and negative efficacy for another (Figure 1.4B); this is also known as protean agonism (Kenakin, 2001). This occasionally enigmatic occurrence has given rise to a paradigm shift in the GPCR field and, indeed, has been shown for many GPCRs (Table 1.2). More importantly, it has led to a nascent approach for drug discovery with regard to pathway-targeting of novel compounds. One of the first studies that provided evidence of a functional selectivity ligand was at the cholecystokinin-1 (CCK_1) receptor (Roettger et al., 1997). This investigation found that the CCK_1 receptor antagonist, D-Tyr-Gly-[(Nle28,31,D-Trp30)cholecystokinin-26–32]-phenethyl ester, (D-Trp-ODE), was able to induce CCK_1 receptor internalisation without eliciting any other agonist responses. It was also revealed that the mode of D-Trp-ODE-stimulated internalisation did not involve receptor phosphorylation, in contrast to the native agonist, CCK, which resulted in CCK_1 receptor phosphorylation and internalisation. It was then suggested that although D-Trp-ODE did not activate G proteins, the induced conformation was conducive to coupling to internalisation machinery independently of phosphorylation events. Since then, there has been more evidence of functionally selective ligands at a vast array of GPCRs, including serotonin, dopamine, and vasopressin receptors (Berg et al., 1998; Cordeaux et al., 2001; Charest et al., 2007). However, the abundance of work performed on the β-adrenoceptors, in particular, has led to profound insights into the molecular basis of functional selectivity. Many clinically used β-adrenoceptor antagonists have been shown to have inverse agonist activity when cAMP accumulation is measured (Chidiac et al., 1994; Azzi et al., 2001),

and some antagonists such as pindolol and atenolol have been demonstrated to possess weak agonist activity for cAMP accumulation (Jasper et al., 1990). It has been further shown that the β-adrenoceptor antagonist, propranolol was able to robustly activate cAMP-binding protein response element-mediated reporter gene transcription and translation, whilst exerting negative efficacy on cAMP accumulation, both of which were β_2-adrenoceptor mediated and able to be antagonised by ICI 118551 (Baker et al., 2003). This sparked much interest in the β-adrenoceptor field, especially given the implications of these findings with regard to clinical efficacy. The divergent efficacies displayed by propranolol were further corroborated with evidence that propranolol could induce β_2- (and β_1-) adrenoceptor-mediated activation of ERK1/2 phosphosphorylation, whilst remaining an inverse agonist for basally activated cAMP accumulation (Galandrin and Bouvier, 2006). This evidence was further tested at the β_1-adrenoceptor by attempting to correlate the functional profile of adrenoceptor ligands with biophysical data obtained by conducting bioluminescence resonance energy transfer (BRET) experiments to measure receptor/G protein interactions. These studies revealed that, indeed, the conformational change induced by propranolol was markedly different to that of the reference agonist, isoproteronol (Galandrin et al., 2008). This biophysical study further confirmed previous work performed to suggest that different ligands potentially induce quite distinct conformations of GPCRs (Ghanouni et al., 2001; Swaminath et al., 2005). The previous examples of functional selectivity have considered ligands that display agonism at one signalling pathway and negative or no efficacy at another. However, functional selectivity can be more subtle, where a ligand may be an agonist for both pathways, but the selectivity of coupling varies. This is where a comparison between two (or more) agonists is required to determine whether any differences in potency seen are due to pathway selectivity or differential strength of

coupling. The serotonin 5-HT_{2C} receptor is pleiotropically coupled and binds ligands that display functional selectivity. The first instance of functional selectivity observed at this receptor was determined by changes in rank order of potencies between agonists that stimulate phosphoinositide accumulation, whilst simultaneously evoking arachidonic acid release (Berg et al., 1998). That study revealed that the agonist (±)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI) was more efficacious at stimulating arachidonic acid release compared to the agonist, quipazine. However, the reverse was true when comparing these agonists in a phosphoinositide accumulation assay. The reversal of agonist efficacy or potency between pathways is a hallmark of functional selectivity, but requires comparison between two or more ligands, as shown in the study by Berg et al. (1998). A similar result has been achieved at the 5-HT_{2C} receptor when comparing Ca^{2+} signalling and ERK1/2 phosphorylation; where DOI was more efficacious at activating intracellular Ca^{2+} mobilisation compared to quipazine, and the converse was seen for ERK1/2 phosphorylation (Werry et al., 2005).

The production of pathway-selective ligands is paramount for the development of more efficacious and targeted drugs. Therefore, the ability to predict GPCR signalling by analysing ligand-induced conformational changes would, no doubt, be of particular use for developing pathway-selective ligands and, indeed, solving this challenge is being attempted with the use of multiple intramolecular biosensors (Zurn et al., 2009). These biosensors can then be used to produce a Förster (or fluorescence) resonance energy transfer (FRET) signal; consequently a FRET profile of ligand can be made and coupled with its pharmacological profile to determine conformational specificity.

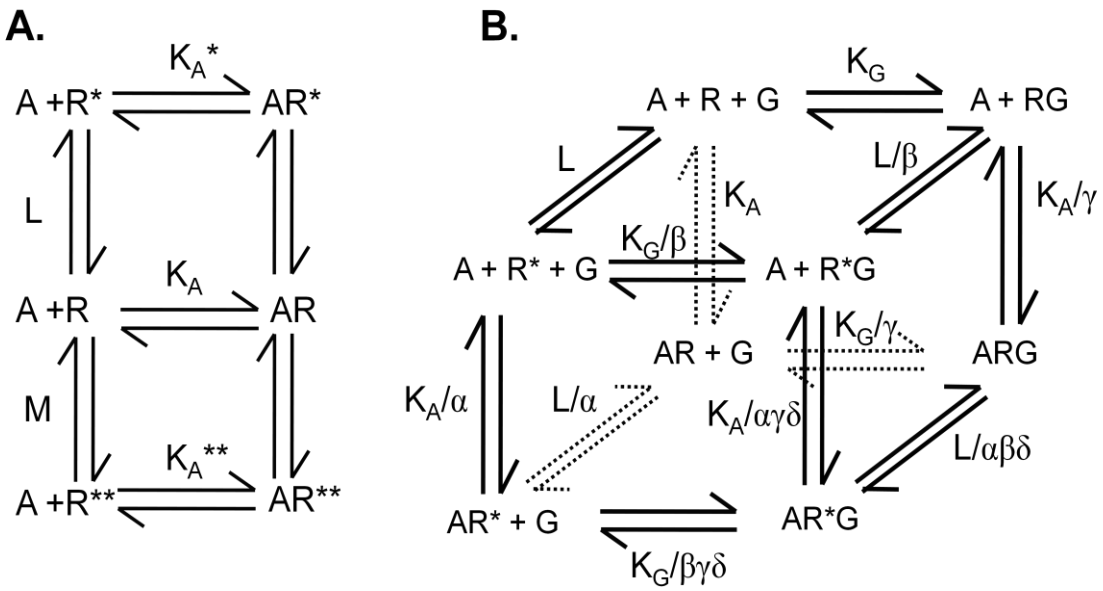

Figure 1.3 Theoretical models depicting the interaction between an agonist and the inactive and multiple active states of a receptor. **A.** The three-state model describes the interaction of agonist (A) with the inactive receptor (R) and two distinct active states (R* and R**), where K_A is the equilibrium affinity constant for each receptor state, and L and M are the isomerisation constants for the R* and R** states, respectively. **B.** The cubic ternary complex model similarly describes the parameters in the three-state model, but further incorporates G protein binding, determined by its affinity of the receptor (K_G). The above parameters are modulated by β, the influence of the activated receptor on G protein binding; γ, the effect of the G protein on ligand binding and δ, the effect of the ternary complex on receptor activation. In this model, R* represents infinite active conformations of the receptor, the abundance of which is determined by the coupling constants.

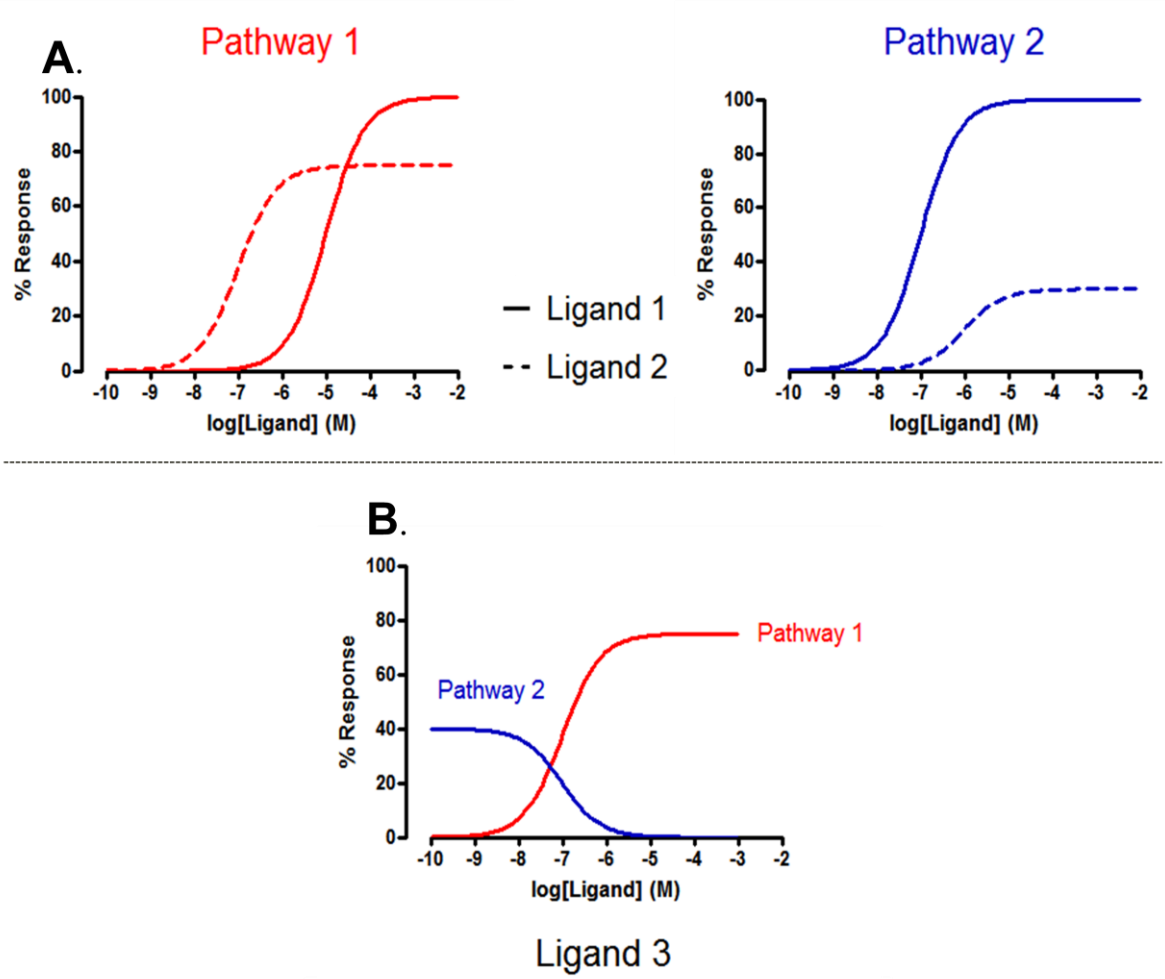

Figure 1.4 Hallmarks of functional selectivity. **A.** Theoretical concentration-response curves of Ligands 1 and 2 when measuring Pathways 1 and 2. These illustrate functional selectivity, denoted by the change in rank order of potency. **B.** Theoretical concentration-curves of Ligand 3-induced activation of Pathways 1 and 2. In this case, functional selectivity is indicated by the divergence of efficacies between the pathways, where Ligand 3 is an agonist with positive efficacy for Pathway 1, and an inverse agonist for Pathway 2.

Table 1.2 Examples of receptors that bind functionally selective ligands. Adapted from Kenakin, (2003a).

Receptors that bind functionally selective ligands and the experimental approach used to determine functional selectivity
Agonist-induced stimulus trafficking
PACAP receptor
Dopamine D_2 receptor
β_2-adrenoceptor
Calcitonin receptor
Bombesin receptor
Cannabinoid receptor
Parathyroid hormone receptor
Differential receptor internalisation
Cholecystokinin receptor
Opioid peptide receptor
Angiotensin II AT_1 receptor
Chemokine CCR5 receptor
Protean agonism (divergent efficacies)
Bradykinin receptor
α_{2A}-adrenoceptor

1.4.2 Allosteric modulation of GPCRs

1.4.2.1 Models defining allosteric interactions

Allosteric (from the Greek meaning 'other site') interactions were first described in the field of enzymology, where indirect interactions between the substrate-binding site and other, topographically distinct, binding sites (Monod et al., 1963). This concept was subsequently extended to ion channels and GPCR. Indeed, the GPCR-G protein interaction is the best example of a GPCR allosteric interaction, since the G protein can modulate the affinity of the receptor for the ligand, and the receptor can modulate the affinity of the G protein for the guanine nucleotide (Christopoulos and Kenakin, 2002). Allosteric modulators can be further defined into subcategories, allosteric enhancer or inhibitor, based on their ability to modulate the stimulus of the orthosteric ligand (May and Christopoulos, 2003). Allosteric modulators may also be agonists independently of orthosteric ligand binding; these are termed allosteric agonists (Langmead and Christopoulos, 2006). Most recently, a new class of ligands has been characterised and are known as bitopic ligands; and they bind to both the orthosteric and allosteric sites (Valant et al., 2008; Valant et al., 2009).

The most parsimonious model for describing an allosteric interaction is the allosteric ternary complex model (ATCM; Figure 1.6A) (Ehlert, 1988; May et al., 2007b). Association of the orthosteric and allosteric ligand is driven by their respective dissociation constants, K_A and K_B. The α value is known as the cooperativity factor, which denotes the magnitude and direction of the allosteric modulation of the affinity of the orthosteric ligand. This interaction is reciprocal, thus it can be described by a single cooperativity factor (Stockton et al., 1983; Lazareno and Birdsall, 1995). If the α value is between 0 and 1, the cooperativity is negative, i.e. it decreases the affinity of the orthosteric ligand for the receptor; if it is equal to 1 the cooperativity is neutral, and

greater than 1 is positive and the modulator will increase the affinity of the orthosteric ligand for the receptor. An extension of the ATCM is the allosteric two state model (ATSM), which incorporates the receptor in its inactive and active state, R*, and how the orthosteric and allosteric ligand, and how the ternary complex influences the distribution of the receptor in the inactive (R) and active (R*) states (Figure 1.6B). The allosteric two-state and ternary complex models are particularly useful for conceptualising effects of allosteric modulators, but are rarely conducive to fitting real experimental data. For data fitting, it is more practical to apply an allosteric 'operational model' to the data, in order to furnish mechanistic and empirical parameter estimates of experimentally-derived allosteric ligand properties (Price et al., 2005; Nawaratne et al., 2008). Parameters for an adaptation of this model are described in section 4.2.9, equation 4.5.

1.4.2.2 Functional selectivity engendered by allosteric modulators

Similarly to orthosteric ligands, allosteric modulators are capable of engendering functional selectivity (Leach et al., 2007). Evidence for this has been shown for the prostaglandin D_2 receptor- (PGD_2R), where phosphoinositide signalling is not affected by the putative allosteric ligands, 1-(4-ethoxyphenyl)-5-methoxy-2-methylindole-3-carboxylic acid and N_{α}-tosyltryptophan, but both are capable of abolishing PGD_2R-mediated arrestin recruitment to the plasma membrane in a G protein-independent mode of action (Mathiesen et al., 2005). Furthermore, there is evidence of functional selectivity mediated by an allosteric ligand at the tachykinin NK_2 receptor, where the allosteric ligand, LPI805, induced a conformation that was favourable to Ca^{2+} signalling, whilst inducing a conformation that was less conducive to cAMP signalling (Maillet et al., 2007).

From the perspective of drug development, targeting an allosteric site with a functionally selective ligand to achieve receptor subtype- and pathway-selectivity, would be a novel approach to reducing side-effects and potentially improving clinical efficacy.

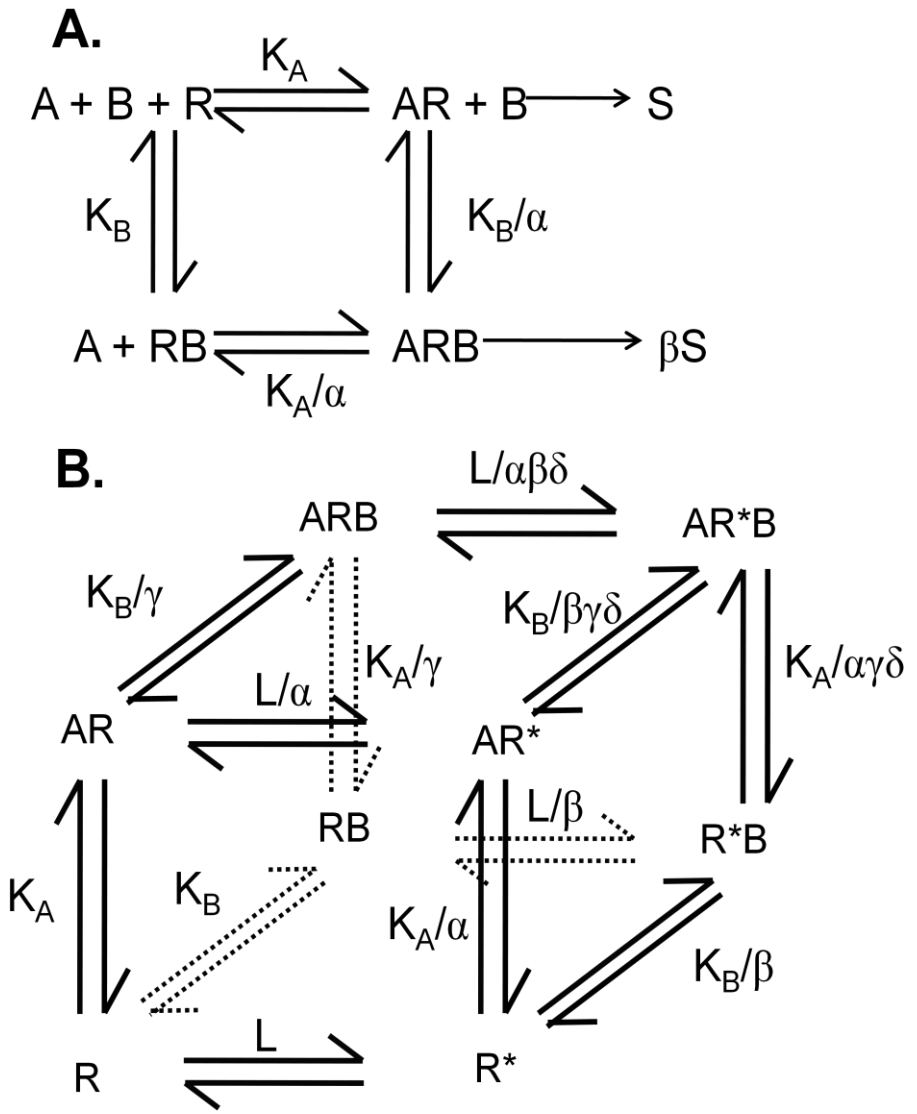

Figure 1.6 Models of receptor, orthosteric and allosteric ligand interactions. **A.** The allosteric ternary complex model (ATCM) describes the binding of the orthosteric ligand (A), allosteric ligand (B) to the receptor (R), where the binding of both is governed by their respective equilibrium dissociation constants (K_A) and (K_B), and the cooperativity factor (α), which denotes the magnitude and direction of each ligand's effect on the other. This model also assumes that AR and ARB complexes impart a stimulus, which for ARB is modulated by β, the scaling factor of efficacy. **B.** The allosteric two state model (ATSM), similarly describes the allosteric ligand's affinity, efficacy and modulation of the orthosteric ligand, but also describes the distribution of these ligands across the inactive (R) and active (R*) states determined by their cooperativity factors in different states. The parameters are as described above, in addition to β, the effect of the orthosteric ligand on receptor isomerisation; γ, the effect of the allosteric ligand on receptor isomerisation and δ, the effect of the ternary complex on receptor isomerisation.

1.5 A novel approach for investigating functional selectivity

The production of functionally selective ligands is a potential approach to targeting not only receptors, but signalling pathways, in an attempt to decrease the side-effect profile of new therapeutics. The most common strategy to screen for functionally selective ligands is to use multiple signalling endpoint assays. However, this is not necessarily conducive to a high-throughput approach and may hinder rapid progress (Butcher, 2005). Some examples of methods for screening for pathway-selective ligands are reporter gene assays (Rodrigues and McLoughlin, 2009), high-content imaging (Heilker, 2006), and recently the use of label-free systems has become popular as a universal screen for agonist stimulated cellular activation (Tran and Ye, 2008; Lee, 2009). These assay formats have their advantages, but they often require further validation once hits have been detected, to ascertain the signalling effectors responsible for the response. However, the use of an assay system that measures coupling of a single G protein subtype to the receptor, and therefore abrogating the need for post-hoc G protein profiling, could be of great value.

Saccharomyces cerevisiae, is a unicellular, eukaryotic, budding yeast, which possesses some striking resemblances to mammalian cells. Biochemists and geneticists alike have found many useful applications for *S. cerevisiae* due to its possession of many mammalian protein homologues. However, the ability to express mammalian GPCRs in yeast has extended its repertoire and proven to be a useful approach for ligand screening and mutagenesis studies (Versele et al., 2001; Dohlman and Slessareva, 2006). *S. cerevisiae* are known to express at least three GPCR types, the Ste2 and Ste3 proteins, **α** or **a** mating factor receptor, respectively, and the Gpr1 glucose-sensing receptor (Kraakman et al., 1999; Versele et al., 2001).

Gpr1 interacts with and activates its cognate G protein, Gpa2, in a similar manner to mammalian Gα subunit activation, and in turn, results in activation of a cAMP-PKA pathway homologue, leading to various cellular effects (Dohlman, 2002).

Activation of Ste2 or Ste3 by their respective mating factors (**α** or **a)**, results in the GDP-GTP exchange at Gpa1 causing the dissociation of the heterotrimeric G protein complex of Gpa1/Ste4/Ste18, Gα, Gβ and Gγ homologues, respectively, and is the beginning of what is known as the pheromone response pathway (Versele et al., 2001). Upon activation of Gpa1 in yeast, the Ste4/Ste18 complex signals to Ste20 and subsequently a MAPK module consisting of a Ste5/Ste11/Ste7/Fus3 complex (Elion et al., 2005; Ishii et al., 2006). Activation of this module results in activation of the G_1 cyclin-dependent kinase inhibitor, Far1 and transcription factors such as Ste12, which leads to cell-cycle arrest and mating behaviours, respectively (Dowell and Brown, 2002). The pheromone response pathway bears a strong resemblance to mammalian GPCR-G protein-MAPK signalling pathways, so it is not surprising that *S. cerevisiae* has been used as a system for reconstituting mammalian GPCRs in many and varied applications. However, the use of *S. cerevisiae* for the detection of mammalian GPCR activation requires a series of modifications to sensitise the agonist response and increase the tractability of the assay (Figure 1.7; Minic et al., 2005b). First, the Sst2 (a Gpa1 RGS protein) is deleted to prolong the length of time that Gpa1 remains in the active, and therefore dissociated state, which allows the Ste4/Ste18 to continue signalling. Second, the Far1 protein is either deleted or disrupted to inhibit cell-cycle arrest, since if the yeast are capable of proliferation there will be a resultant amplification of signal. Third, the Ste12 promoter, Fus1, is modified to be linked to an appropriate reporter gene, such as LacZ, His3, Luc or Can1, which result in transcription of genes for β-galactosidase, imidazoleglycerol-phosphate dehydratase,

luciferase and arginine permease, respectively (Brown et al., 2000; Ishii et al., 2006; Leskinen et al., 2008). Generally, LacZ, His3 or Luc induction is used to identify receptor activation by measuring enzymatic cleavage of non-fluorescent substrates into fluorescent products, growth or luminescence, respectively (Pausch, 1997; Campbell et al., 1999; Schmidt et al., 2003). These reporter genes are commonly used for ligand screening programs for either agonists or antagonists, but have also been used for mutagenic analysis, of GPCRs (Minic et al., 2005b). Conversely, expression of the Can1 gene product with addition of canavanine, a cytotoxic arginine derivative, results in cell death (Li et al., 2007). This approach has shown to be particularly useful for screening loss-of-function mutants of GPCRs, where random mutagenesis is performed by error-prone PCR and upon co-administration of receptor agonist and canavanine, the mutants that remain functional do not survive, whilst non-functional mutants produce colonies; and these non-functional mutations can be determined and further characterised (Li et al., 2007; Scarselli et al., 2007). Screening randomly mutated gene constructs in yeast has proven to be quite useful, with particular regard to ascertaining critical residues involved in the binding of ACh the M_3 muscarinic ACh receptor (Price et al., 1995a; Price et al., 1996). Although there is evidence that mammalian GPCRs are capable of activating Gpa1, a final modification of the yeast system may be necessary (Brown et al., 2000), which is the expression of a chimeric G protein consisting of Gpa1 with the substitution of the five C-terminal amino acids with the corresponding residues from a mammalian Gα protein (Minic et al., 2005b). This approach increases the specificity of coupling of the GPCR to the pheromone response pathway, in addition to potentially furnishing information about G protein selectivity.

The utility of yeast also allowed the de-orphanisation of at least two GPCRs, the UDP-glucose and the Edg-2 (Vzg-1) receptor (Chambers et al., 2000). The orphan GPCR,

KIAA0001 (UDP-glucose receptor) was expressed in yeast and shown to couple to the native Gα protein, Gpa1, when stimulated with UDP-glucose. This was further tested in HEK-293 cells expressing KIAA0001 with $G\alpha_{16}$, a promiscuous Gα protein, where UDP-glucose elicited a release of intracellular Ca^{2+} stores (Dowell and Brown, 2002; Minic et al., 2005b). In the cases where an orphan GPCR does not couple to Gpa1, and the mammalian Gα protein with which the orphan couples is not known, a chimera of Gpa1/$G\alpha_{16}$ has been proposed to be useful in detecting receptor activation (Campbell et al., 1999).

Using the modified pheromone response pathway, many GPCRs have been studied in heterologous *S. cerevisiae* systems, these include the adenosine A_1 receptor (Campbell et al., 1999), M_1, M_3 and M_5 muscarinic acetylcholine receptors (Erlenbach et al., 2001b) and the calcitonin receptor-like receptor co-expressed with receptor activity-modifying proteins 1, 2 and 3 (calcitonin-related gene peptide and adrenomedullin receptor phenotypes; Miret et al., 2002). Interestingly, to increase cell surface expression of M_1, M_3 and M_5 muscarinic ACh receptors in yeast, it was necessary for Erlenbach et al. (2001a) to delete the central region of the intracellular 3rd loop, ΔPro^{231}-Gly^{345}, ΔAla^{274}-Lys^{469} and ΔThr^{237}-Pro^{413}, respectively. The inability of the full-length receptor to express in appreciable quantities at the cell membrane may, in fact, be caused by improper receptor folding and impaired trafficking or inappropriate receptor phosphorylation by yeast kinases, which may trigger internalisation and degradation. Thus, it is important to consider influences such as these when using yeast as a GPCR expression system, in addition to other factors such as differences in glycosylation patterns and ligand permeability through the cell wall.

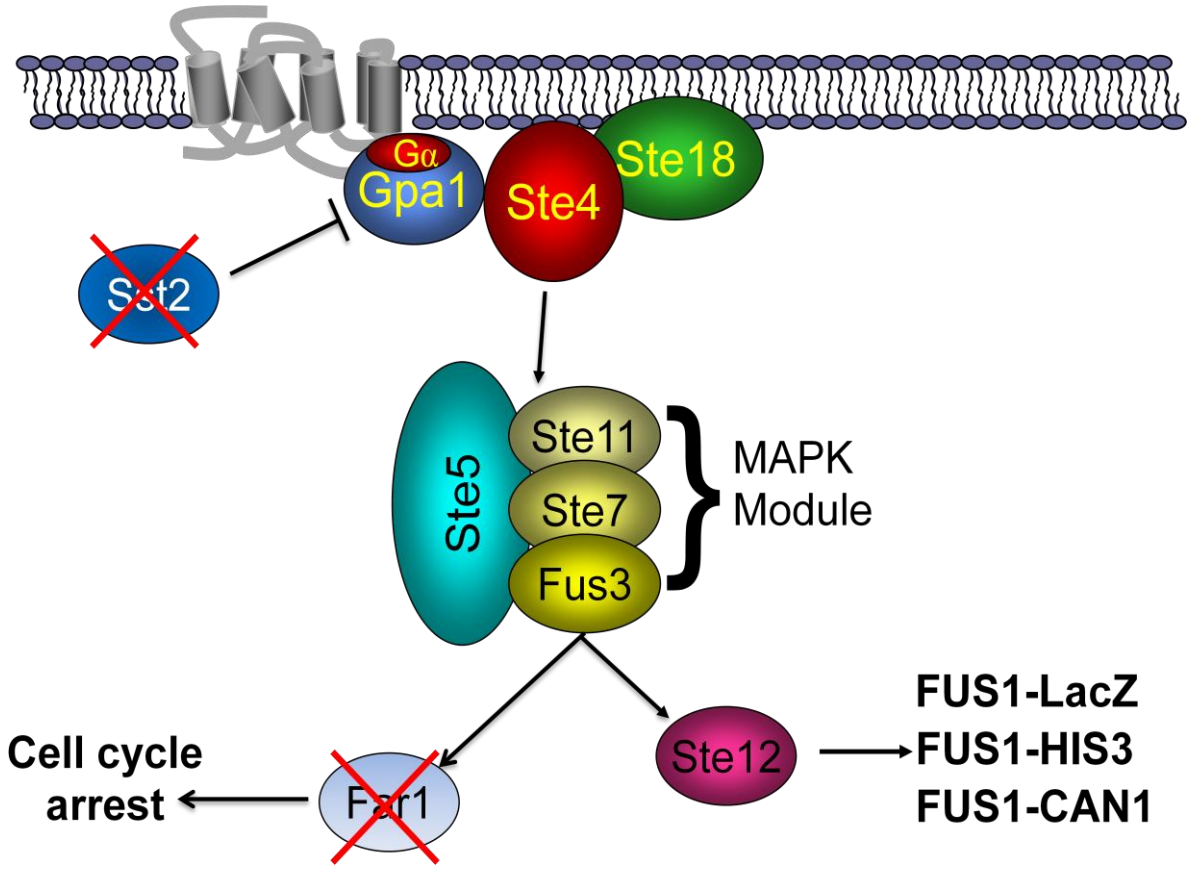

Figure 1.5 Modified S. cerevisiae pheromone response pathway. Pheromone response pathway adapted for functional expression mammalian GPCRs, including the expression of a chimeric Gα protein (Gpa1/Gα), the removal of the RGS protein Sst2 and the cell-cycle mediator Far1. Additionally, the FUS1 promoter is usually linked to either: LacZ, HIS3 or CAN1, (adapted from Dowell and Brown, 2002).

1.6 Scope of thesis

Functionally selective GPCR ligands are highly sought after and potentially offer considerable advantages over non-selective ligands, with regard to clinical efficacy and toxicity. Indeed, many GPCR-ligand combinations have been identified as displaying functional selectivity. However, there remains a hindrance in the ability to detect these signalling consequences, and often this is due to lack of efficient endpoint assays that are conducive to ligand screening, and moreover, the convergence of GPCR signalling on a single endpoint makes it difficult to distinguish upstream effectors. Furthermore, there are other issues such as non-target interacting proteins that may influence the results, by masking or enhancing effects that are not normally present. Functional selectivity is heavily dependent on the cell background, changes in G protein complement and arrangement of signalling effectors will influence the end effect. Hence, using a screen that is not as dependent on cellular background would prove to be invaluable.

This project explores the ability to detect functional selectivity between G proteins using a novel approach in an essentially mammalian-null system. *S. cerevisiae* has been used for many and varied applications. However, no single receptor has yet been systematically investigated across multiple G proteins in this cellular background. Each chapter of this thesis has studied different aspects of functional selectivity, using either the adenosine A_1 receptor or M_3 mAChR as a model.

Chapter 2 investigates the ability of the yeast system to detect functional selectivity between $G\alpha_{i/o}$ family proteins. Since $G\alpha_{i/o}$ proteins are differentially expressed and comparmentalised in different tissues, resulting in altered signalling outcomes. For this reason, a screen that is capable of detecting signalling from each $G\alpha_{i/o}$ protein subtype

independently of each other would be helpful when screening ligands that are targeted toward a single pathway. Hence, Chapter 2 utilises the adenosine A_1 receptor as a model for validating this system with respect to $G\alpha_{i/o}$ signalling, since it predominantly signals though $G\alpha_{i/o}$ subunits. It also explores the ability of the yeast system to estimate affinity values of agonists (R-PIA and VCP-189) and an antagonist (DPCPX), and determine an efficacy profile for high and low efficacy agonists.

The prevalence of ligands that display functional selectivity has increased over the past few years, especially in the β-adrenoceptor field, where many clinically used antagonists display divergent efficacies. Chapter 3 investigates the ability of some clinically relevant muscarinic antagonists to exhibit functional selectivity, by using the yeast system and testing these ligands across various Gα protein subtypes. The chapter uses the M_3 mAChR, to produce a G protein coupling profile for the agonist, carbachol, to estimate affinity values for the antagonist, atropine, and investigate whether some classical muscarinic antagonists display functional selectivity between G proteins.

The targeting of an allosteric site to engender functional selectivity is a nascent approach in drug development, but generally the study of functional properties of allosteric modulators has gone largely unappreciated until recently and, similarly to the study of orthosteric ligand functional selectivity, there are inherent difficulties with dissecting pathway selectivity. Chapter 4 uses the recently described $K^{7.32}E$ mutant of the M_3 mAChR (Iarriccio, 2008), to investigate the capability of the allosteric ligand, brucine, to exhibit functional selectivity across various G protein. Furthermore, the ability of the yeast system to predict allosteric interactions was investigated, which was then confirmed in mammalian cell assays.

CHAPTER 2

DETERMINATION OF ADENOSINE A_1 RECEPTOR AGONIST AND ANTAGONIST PHARMACOLOGY USING *SACCHAROMYCES CEREVISIAE*: IMPLICATIONS FOR LIGAND SCREENING AND FUNCTIONAL SELECTIVITY.

Monash University

Declaration for Thesis Chapter 2

Declaration by candidate

In the case of Chapter 2, the nature and extent of my contribution to the work was the following:

Nature of Contribution	Extent of Contribution (%)
Development of ideas, performed experiments and data analysis, contributed to writing and revision of manuscript	80%

The following co-authors contributed to the work.

Name	Nature of Contribution
Celine Valant	Performed experiments and data analysis
Simon Dowell	Provision of expertise and yeast strains, manuscript preparation
Dalibor Mijaljica	Provision of expertise and facilities, manuscript preparation
Rodney Devenish	Provision of expertise and facilities, manuscript preparation
Peter Scammells	Compound synthesis, manuscript preparation
Patrick Sexton	Development of ideas, manuscript preparation
Arthur Christopoulos	Development of ideas, manuscript preparation

Candidate's signature:..... Date:31/07/09

Declaration by co-authors

The undersigned hereby certify that:
(1) the above declaration correctly reflects the nature and extent of the candidate's contribution to this work, and the nature of the contribution of each of the co-authors;
(2) they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;
(3) they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
(4) there are no other authors of the publication according to these criteria;
(5) potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journal or other publications, and (c) the head of the responsible academic unit; and
(6) the original data are stored at the following location(s) and will be held for at least five years from the date indicated below:

Location: Monash Institute of Pharmaceutical Sciences, Monash University, Parkville

Contributor	Signature	Date
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Rodney Devenish		30/07/'09
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Arthur Christopoulos		31/07/09

2.1 Introduction

Adenosine is a purine nucleoside that is vital in regulating numerous physiological processes, especially in the cardiovascular and central nervous systems (Hasko et al., 2008). Adenosine as a signalling molecule is generated by the catabolism of ATP and adenosine diphosphate (ADP), released from the intracellular space via equilibrative nucleotide transporters during respiration, but local concentrations are largely increased in hypoxic or ischaemic conditions (Linden, 2001). These nucleotides are then sequentially catabolised from ATP and ADP to adenosine monophosphate (AMP) by the ecto-nucleotidase CD39 (ectonucleoside triphosphate diphosphohydrolase 1) and from AMP to adenosine by CD73 (ecto-5′-nucleotidase). Extracellular adenosine is then subject to phosphorylation by adenosine kinase, converting it to AMP, or deamination by adenosine deaminase, which converts adenosine to inosine (Hasko and Cronstein, 2004).

The adenosine receptors are a group of rhodopsin family GPCRs and can be divided into three subcategories, A_1, A_2 and A_3, of which the A_1 and A_3 preferentially couple to $G_{i/o}$ proteins, whereas A_2 receptors prefer to couple to G_s proteins (Linden, 2001). The A_1 receptor is nearly ubiquitously expressed throughout the body, with highest levels in brain, spinal cord, atria and adipose tissue (Baraldi et al., 2000; Kourounakis et al., 2001). The A_1 receptor preferentially couples to $G_{i/o}$ proteins, to inhibit AC activity and consequently decrease intracellular cAMP concentration, activate Kir3 channels, and inactivate Ca_v2 channels in different cell types (Linden, 2001; Benians et al., 2003). A_3 receptors, which also activate $G_{i/o}$ protein, signal with a similar profile to that of the A_1 receptor, are expressed in high concentrations in the testes and mast cells (Fredholm et al., 2001; Hasko et al., 2008). Conversely, the A_2 receptors include the A_{2A} and A_{2B} receptors, which preferentially stimulate G_s, but both receptors can also activate G_{olf}

and $G_{q/11}$ proteins, depending on the cellular background (Fredholm et al., 2001). Despite differences in signalling between the adenosine receptor subtypes, all subtypes are able to signal to pathways that result in ERK1/2 phosphorylation (Schulte and Fredholm, 2003). Interestingly, however, the A_{2A} receptor has been shown to not only activate ERK1/2 signalling pathways, but also inhibit the same pathway when it is first stimulated by other receptors such as the thrombin or nerve growth factor receptors (Hirano et al., 1996; Arslan and Fredholm, 2000). As well as ERK1/2 phosphorylation, there is evidence that the A_{2B} receptor can activate JNK and p38 in human mast cells (Feoktistov et al., 1999), and in untransfected HEK 293 cells the A_{2B}-mediated ERK1/2 phosphorylation appears to be mediated by $G_{q/11}$-PLC signalling (Gao et al., 1999).

In pathophysiological settings, adenosine is commonly regarded as a tissue protective molecule whose actions are mostly mediated by the adenosine A_1 receptor. Activation of the adenosine A_1 receptor remains an attractive therapeutic approach for treating conditions such as ischemia-reperfusion injury, paroxysmal superventricular tachycardia, chronic pain and non-insulin dependent diabetes mellitus (Gao and Jacobson, 2007; Andreadou et al., 2008; Elzein and Zablocki, 2008). For this reason, A_1 receptor-selective compounds have generated much interest as potential novel therapeutic agents.

Traditionally, approaches for selectively targeting the A_1 receptor have exploited differences in the binding properties of this receptor relative to other subtypes, either by focusing on the receptor's orthosteric site or, alternatively, on potential allosteric sites (Gao et al., 2005; Goblyos et al., 2005; Gao and Jacobson, 2007; Aurelio et al., 2009). More recently, there has been a growing appreciation that ligands may be designed that are not only subtype-selective, but also signal pathway-selective. This reflects the recognition that GPCRs adopt multiple active states that can be differentially stabilised

such that only a subset of the entire signalling repertoire associated with a given receptor is activated in a ligand-specific manner – a phenomenon termed "stimulus-trafficking" or "functional selectivity" (Kenakin, 1995; Urban et al., 2007). As with most GPCRs, the A_1 receptor is known to couple promiscuously to multiple signalling pathways (reviewed in Schulte and Fredholm, 2003), and it is thus conceivable that selectively targeting only some of these could potentially lead to a reduction in the side-effect profile associated with indiscriminate activation of the A_1 receptor. However, multiple assay types are generally required to study the promiscuous coupling of GPCRs such that pharmacological parameters (e.g., ligand affinities and relative efficacies) can be obtained. Determination of such information is required to facilitate structure-activity relationships and to understand functional selectivity. Nonetheless, many assays can be influenced by intracellular signalling cross-talk arising from the promiscuous nature of GPCR signalling. Therefore, it would be useful to utilise a single system that could generate the requisite information on selective signalling with minimal contribution from potential confounding influences.

The yeast, *Saccharomyces cerevisiae*, expresses a single type of GPCR that, upon activation, signals to the pheromone response pathway via coupling to a single heterotrimeric G protein (Dowell and Brown, 2002). Importantly, yeast can be adapted to accommodate mammalian GPCR signalling via this one-GPCR-one G protein pathway. Brown et al. (2000) have previously modified this system to allow expression of a human/yeast Gα protein chimera, consisting of five C-terminal amino acids of a given human Gα protein fused with the truncated yeast Gα protein, Gpa1(1-467), (Gpa1/ Gα) (Brown et al., 2000). This modification allows specificity of binding to, potentially, any desired mammalian GPCR, whilst maintaining the capacity to couple to the endogenous yeast Gβγ subunits (Dowell and Brown, 2002), which then signal to a

MAPK pathway to activate reporter gene expression. This yeast signalling assay is thus an attractive system for studying specific pairs of GPCRs and G proteins in the absence of other GPCRs and signal cross-talk.

Despite these properties, the yeast signalling system has not been widely explored to date for quantification of GPCR agonist and antagonist pharmacological properties and G protein coupling profiles. Thus, the aim of the current study was to determine the pharmacological characteristics of A_1 adenosine receptors expressed in various yeast strains together with individual G protein chimeras for each of the main mammalian Gα subunits. Our results suggest that the yeast signalling assay is a robust platform for determining relative efficacies of agonists and affinity values for both agonists and antagonists. The assay is generally predictive of coupling preferences that are also seen in mammalian cell assays. However, there also exists a potential for a lack of sensitivity in the ability of the yeast system to detect functional selectivity between some Gα protein subtypes.

2.2 Methods

2.2.1 Materials

The *Surefire*™ ERK1/2 phosphorylation kit was kindly donated by Dr Michael Crouch (TGR Biosciences, SA, Aust.). All AlphaScreen™ beads and guanosine 5'(γ-thio)triphosphate, [^{35}S], [^{35}S]-GTPγS were purchased from Perkin Elmer, (Boston, MA). 5'-deoxy-N^6-(endo-norborn-2-yl)-5'-(2-fluorophenylthio)adenosine (VCP-189) was synthesized (as Compound 12c) as previously described in Ashton et al. (2008). Flp-In™ Chinese hamster ovary (CHO) cells, Gateway™ plasmids, BP clonase kit, LR clonase kit, hygromycin B, zeocin, Fluo-4-AM, *S. cerevisiae* EasyComp™ transformation kit and fluorescein di(β-D-galactopyranoside) (FDG) were obtained from Invitrogen (Carlsbad, CA). cDNA constructs of the human adenosine A_1 receptor and PTX-insensitive $G\alpha_{i/o}$ proteins were purchased from the Missouri University of Science and Technology cDNA Resource Center (http://cdna.org). Dulbecco's modified Eagle medium (DMEM) and fetal bovine serum (FBS) were purchased from GIBCO (Gaithersburg, MD) and JRH Biosciences (Lenexa, KS), respectively. Metafectene reagent was obtained from Biontex (Martinsried/Planegg, Germany). Ultima gold scintillation cocktail was purchased from Packard Bioscience (Meriden, CT). Bicinchoninic acid protein (BCA) reagents were obtained from Pierce Biotechnology (Rockford, IL) and adenosine deaminase (ADA), derived from calf intestine, was purchased from Roche (Basel, Switzerland). All other reagents were purchased from Sigma Aldrich (St Louis, MO).

2.2.2 Cell culture and transfections

The sequence of the human adenosine A_1 receptor was amplified by PCR and cloned into the Gateway entry vector, pDONR201, using the BP clonase kit according to manufacturer's instructions. The A_1 receptor construct was subsequently transferred in the Gateway destination vector, pEF5/FRT/V5-dest, using the LR clonase kit in accordance with manufacturer's instructions. The construct was then transfected into Flp-In CHO cells using methods described previously (Nawaratne et al., 2008). Flp-In CHO cells stably expressing adenosine A_1 receptors (CHO A_1R) were cultured at 37°C in 5% CO_2 in DMEM supplemented with 5% (v/v) FBS, 16mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and were selected using 400μg mL^{-1} hygromycin B, but maintained using 200μg mL^{-1} hygromycin B. For ERK1/2 phosphorylation experiments requiring transfection of pertussis toxin- (PTX) insensitive G proteins, CHO cells expressing the A_1 receptor were transfected with either pcDNA3.1+ (vector only) or pcDNA3.1+ encoding PTX-insensitive $G\alpha_{i/o}$ family proteins (described in Wise et al. (1997)), in 96-well plates using Metafectene™ reagent according to manufacturer's instructions for CHO cells.

2.2.3 Yeast transformations and signalling assay

Production of *Saccharomyces cerevisiae* strains expressing chimeras of five C-terminal amino acids of human Gα protein with the yeast Gpa1, 1-467, (Gpa1/Gα) has been described previously in Brown et al. (2000). The gene encoding the human adenosine A_1 receptor was cloned into the p426GPD vector using traditional cloning methods. Yeast strains were then transformed with this construct using the *S.cerevisiae* EasyComp™ transformation kit in accordance with manufacturer's instructions. The conditions for the signalling component of the assay were as previously described

(Brown et al., 2000), except that FDG was used as the β-galactosidase substrate rather than chlorophenolred-β-D-galactopyranoside. After appropriate treatment with ligand, the cells were incubated at 30°C for 18-24hrs. Fluorescence was read on an EnVision™ plate reader (Perkin Elmer) at 475nm excitation and 520nm emission.

2.2.4 Membrane preparation

CHO A_1R cells were grown to 90% confluence, harvested and centrifuged at 300 × g for 3 min. The intact cell pellet was suspended in homogenization buffer (20mM HEPES; 10mM ethylenediaminetetraacetic acid **(**EDTA); 0.1mg mL^{-1} saponin, pH 7.7) and further centrifuged (300 × *g*, 3 min). Cells were then resuspended in homogenization buffer and homogenized using a Polytron PT1200 homogenizer for two 10 sec intervals at maximum setting (6), with 30 sec cooling periods on ice between each burst. The homogenate was then treated for 30 min with 1UmL^{-1} ADA. The homogenate was then centrifuged (40,000 × *g*, 1 hr, 4°C). The resulting pellet was resuspended in 5 ml of GTPγS buffer (100mM NaCl; 20mM HEPES; 10mM $MgCl_2$, pH 7.4), and the protein content determined using a BCA assay kit (Pierce Biotechnology) according to the manufacturer's instructions. The homogenate was then divided into 1ml aliquots and either used immediately or stored frozen at −80°C until required.

2.2.5 [^{35}S]GTPγS immunoprecipitation assay

CHO A_1R membranes (20μg per sample) were incubated in GTPγS buffer with ligand (or buffer) and 10μM GDP for 30 min at 30°C, prior to addition of 0.1nM [^{35}S]GTPγS (final concentration) for a further 30 min at 30°C, in a final volume of 500μL. The reaction was terminated by membrane solubilization in GTPγS buffer with 1.25% Igepal

CA630. Samples were then placed on a rotor at 4°C for 30 min, before being incubated with 2μg of appropriate anti-Gα subunit antibody for a further 90 min at 4°C. A slurry of protein A sepharose in assay buffer was added to each sample to achieve a 3% (v/v) final concentration and incubated at 4°C for 1 hr. Samples were centrifuged three times at 60 × g at 4°C, each time washed with 500μL of ice-cold GTPγS buffer with 1.25% Igepal CA630. The final pellet was resuspended and added to 4mL of scintillation cocktail (Ultima gold), and radioactivity was then determined by scintillation counting.

2.2.6 Extracellular signal-regulated kinase 1/2 phosphorylation assays

Initial ERK1/2 phosphorylation time course experiments were performed to determine the time at which ERK1/2 phosphorylation was maximal after stimulation by each agonist. Cells were seeded into transparent 96-well plates at 5×10^4 cells per well and grown overnight or until confluent. Cells were then washed twice with phosphate-buffered saline (PBS) and incubated in serum-free DMEM at 37°C for at least 4 h to allow FBS-stimulated phosphorylated ERK1/2 levels to subside. Prior to stimulation, cells were treated with 1U mL^{-1} ADA for 30 min. Cells were then stimulated with agonist for 5mins and incubated at 37°C in 5% CO_2. For antagonist interaction studies, cells were incubated with 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) for 30 min at 37°C in 5% CO_2, prior to agonist stimulation. Experiments using PTX-insensitive Gα protein subunits were transfected as described above. 24 hr post-transfection, cells were washed with PBS, and cultured overnight in serum-free media containing 100ng mL^{-1} PTX. The assay was then performed as described above. For receptor alkylation experiments, CHO A_1R cells were cultured overnight to approximately 90% confluence in 96-well plates, washed with PBS, and treated for 15 or 30 min (VCP-189 alkylation

assays) with 10μM 8-Cyclopentyl-3-(3-((4(fluorosulfonylbenzoyl)oxy)propyl)-1-propylxanthine (FSCPX), an irreversible A_1 receptor antagonist), or 5μM or 10μM FSCPX for 30mins ((−)-N^6-(2-phenylisopropyl)adenosine (R-PIA) alkylation assays) at 37°C. The cells were extensively washed with PBS, and bathed in serum-free media 4hrs prior to experimentation. The assay was then performed as described above. For all experiments, 3% (v/v) FBS was used as a positive control, and vehicle controls were also performed. The reaction was terminated by removal of drugs and lysis of cells with 100μl of *SureFire* lysis buffer (as provided by the manufacturer). The lysates were agitated for 1 to 2 min and were diluted at a ratio of 4:1 (v/v) lysate/*Surefire* activation buffer in a total volume of 50μL. Under low light conditions a 1:240 (v/v) dilution of AlphaScreen beads: Surefire reaction buffer was prepared and this was mixed with the activated lysate mixture in a ratio of 6:5 (v/v), respectively, in a 384-well opaque Optiplate. Plates were incubated in the dark at 37°C for 1 hr before the fluorescence signal was measured using a Fusion-α plate reader (Perkin Elmer) using standard AlphaScreen settings. Data were normalised to the maximal response elicited by 3% (v/v) FBS at the same time point.

2.2.7 cAMP accumulation assay

CHO A_1R cells were plated into 96-well plates and cultured overnight at 37°C in 5% CO_2. Cells were washed with PBS, and cultured overnight in serum-free media in the absence or presence of 100ng mL^{-1} PTX. Thirty minutes prior to assaying, the culture medium was replaced with phenol red-free DMEM with 0.1% bovine serum albumin (BSA), 1UmL^{-1} ADA and 500μM 3-isobutyl-1-methylxanthine (IBMX) and incubated at 37°C in 5% CO_2. Cells were treated with R-PIA and/or forskolin, and incubated for

30 min at 37°C in 5% CO_2. Media was aspirated and cells were lysed in lysis buffer (dH_2O; 0.3% Tween20; 5mM HEPES; 0.1% BSA). Lysates were transferred to a 384-well plate and mixtures of lysis buffer/donor bead-conjugated anti-cAMP antibody and lysis buffer/biotinylated cAMP/acceptor bead-conjugated streptavidin were added to the lysates according to the PerkinElmer cAMP Alphascreen™ protocol. Plates were incubated in the dark at room temperature overnight before the fluorescence signal was measured using a Fusion-α plate reader (Perkin Elmer) using standard AlphaScreen settings. Data were normalised to the response elicited by 10μM forskolin at the same time point.

2.2.8 Ca^{2+} mobilisation assay

CHO A_1R cells were cultured overnight in 96-well plates at 37°C in 5% CO_2. Cells were washed with PBS, and cultured overnight in serum-free media in the absence or presence of 100ng mL^{-1} PTX. Cells were washed twice in Ca^{2+} assay buffer (150mM NaCl, 2.6mM KCl, 1.2mM $MgCl_2$, 10mM dextrose, 10mM HEPES, 2.2mM $CaCl_2$, 0.5% (w/v) BSA and 4mM probenecid). The final wash replaced with Ca^{2+} assay buffer containing 1μM Fluo-4-AM and incubated for 1 hr at 37°C in 5% CO_2. Cells were washed twice more and replaced with 37°C Ca^{2+} assay buffer containing 1UmL^{-1} ADA. R-PIA was added and fluorescence was measured in a Flexstation™ (Molecular Devices).

2.2.9 Data analysis

Agonist concentration-response curves, in the absence of antagonist, were fitted via nonlinear regression to the following three-parameter logistic function, using Prism 5.02 (GraphPad Software, San Diego, CA):

$$E=basal+\frac{E_{max}-basal}{1+10^{(-pEC_{50}-Log[A])}} \qquad (2.1)$$

where E is response, E_{max} and basal are the top and bottom asymptotes of the curve, respectively, Log[A] is the logarithm of the agonist concentration, and pEC_{50} is the negative logarithm of the agonist concentration that gives a response halfway between E_{max} and basal.

Experiments measuring the interaction between R-PIA and DPCPX, or R-PIA and VCP-189 at the adenosine A_1 receptor were globally fitted to the following logistic equation of agonist-antagonist interaction:

$$E=basal+\frac{(E_{max}-basal)}{1+\left(\frac{10^{-pEC_{50}}\left[1+\left([B]/10^{-pA_2}\right)^{s}\right]}{10^{Log[A]}}\right)} \qquad (2.2)$$

where basal, E_{max}, Log[A] and EC_{50} are as previously described. B is the molar concentration of antagonist. pA_2 is the negative logarithm of the concentration of antagonist that requires a 2-fold increase in the concentration of agonist to achieve an equal response to that in absence of antagonist. The parameter, s, is analogous to the Schild slope factor (Motulsky and Christopoulos, 2004).

To generate agonist affinity and efficacy estimates the following form of an operational model of agonism (Black and Leff, 1983) was applied to the relevant data (see Results):

$$Y=\text{basal}+\frac{E_m\text{-basal}}{1+\left[\frac{10^{\log K_A}+10^{\log[A]}}{10^{\log\tau}+10^{\log[A]}}\right]} \quad (2.3)$$

where $LogK_A$ is the logarithm of the equilibrium dissociation constant of the agonist, E_m is the maximum response of the system and τ is an operational measure of efficacy, equal to the total receptor concentration divided by the concentration of agonist/receptor complexes required to achieve 50% of the maximal system response. All other parameters are as previously described. For application to receptor alkylation experiments, equation 3 was globally fitted to the entire family of curves for a given agonist's responses determined in the absence or after treatment with FSCPX, with all parameters being shared except Log τ. For experiments in yeast comparing the responses of VCP-189 to those of the full agonist, R-PIA, equation 3 was applied to the VCP-189 responses while equation 1 was applied to the R-PIA responses, with the basal parameters shared across datasets and the E_{max} parameter of equation 1 constrained to equal the E_m parameter of equation 3; in this manner, estimates of $LogK_A$ and Logτ could be derived for VCP-189 (Leff et al., 1990; Motulsky and Christopoulos, 2004).

Statistical comparisons between parameters were performed using a one-way ANOVA with a Bonferroni's multiple comparisons or Dunnett's post-test, as appropriate, and a probability (*p*) less than 0.05 was taken to indicate significance.

2.3 Results

2.3.1 Determination of agonist and antagonist pharmacology using S.cerevisiae

Concentration-response curves were constructed to the agonists, R-PIA and VCP-189 in yeast strains expressing chimeras of Gpa1/$G\alpha_q$, Gpa1/$G\alpha_{12}$, Gpa1/$G\alpha_o$, Gpa1/$G\alpha_{i1/2}$, Gpa1/$G\alpha_{i3}$, Gpa1/$G\alpha_s$, or the full-length endogenous yeast Gα protein Gpa1, to test the ability of these compounds to elicit a response when coupled to an individual subtype of G protein. R-PIA produced detectable responses in strains expressing Gpa1/$G\alpha_o$, Gpa1/$G\alpha_{i1/2}$ and Gpa1/$G\alpha_{i3}$. VCP-189 was a partial agonist compared to R-PIA, and only produced significant responses in yeast strains expressing Gpa1/$G\alpha_i$ subunits (Figure 2.1); Table 2.1 lists the estimates of agonist potency derived from these experiments. Subsequent application of an operational model of agonism (equation 2.3) to the data also allowed for the determination of VCP-189 affinity (Log K_A) and relative efficacy (Log τ) at the Gpa1/$G\alpha_{i1/2}$ and Gpa1/$G\alpha_{i3}$ strains, yielding the following values: for Gpa1/$G\alpha_{i1/2}$, $LogK_A$ = -6.40 ± 0.16; Logτ = -0.37 ± 0.07 (τ = 0.42); for Gpa1/$G\alpha_{i3}$, $LogK_A$ = -5.76 ± 0.22; Logτ = -0.61 ± 0.17 (τ = 0.24), n = 3-5. Neither the affinity or relative efficacy values were significantly different from one another between strains ($p > 0.05$).

The lack of response to VCP-189 in the Gpa1/$G\alpha_o$ strain may reflect either a lack of interaction of the agonist with the receptor in that yeast strain, or a lack of signalling efficacy via the Gpa1/$G\alpha_o$ protein. To differentiate between these two possibilities, interaction experiments were performed between R-PIA and VCP-189 at the Gpa1/$G\alpha_o$ strain. As shown in Figure 2.2, increasing concentrations of VCP-189 led to a progressive rightward shift of the R-PIA concentration-response curve in a manner consistent with a competitive interaction, indicating that VCP-189 was indeed able to

interact with the A_1 receptor expressed in this yeast strain. Application of equation 2.2 to the data yielded a $-pA_2$ estimate of -5.98 ± 0.06 (n = 3) for VCP-189. Neither this value, nor the $LogK_A$ values determined from the operational model analysis of the data from the other two yeast strains, were significantly different from one another (p>0.05).

The interaction paradigm utilised above was next extended to investigate the properties of a prototypical A_1 receptor orthosteric antagonist, DPCPX, at each of the Gpa/$G\alpha_o$, Gpa/$G\alpha_{i1/2}$ or Gpa/$G\alpha_{i3}$ strains. Figure 2.3 shows that, in each instance, the antagonist caused parallel rightward shifts of the R-PIA concentration-response curves in a concentration-dependent manner, characterized by the following pA_2 estimates (n = 4): 8.51 ± 0.15, 9.15 ± 0.29 and 8.58 ± 0.32, for Gpa/$G\alpha_o$, Gpa/$G\alpha_{i1/2}$ and Gpa/$G\alpha_{i3}$, respectively. Statistical analysis revealed no significant difference between these values (p>0.05).

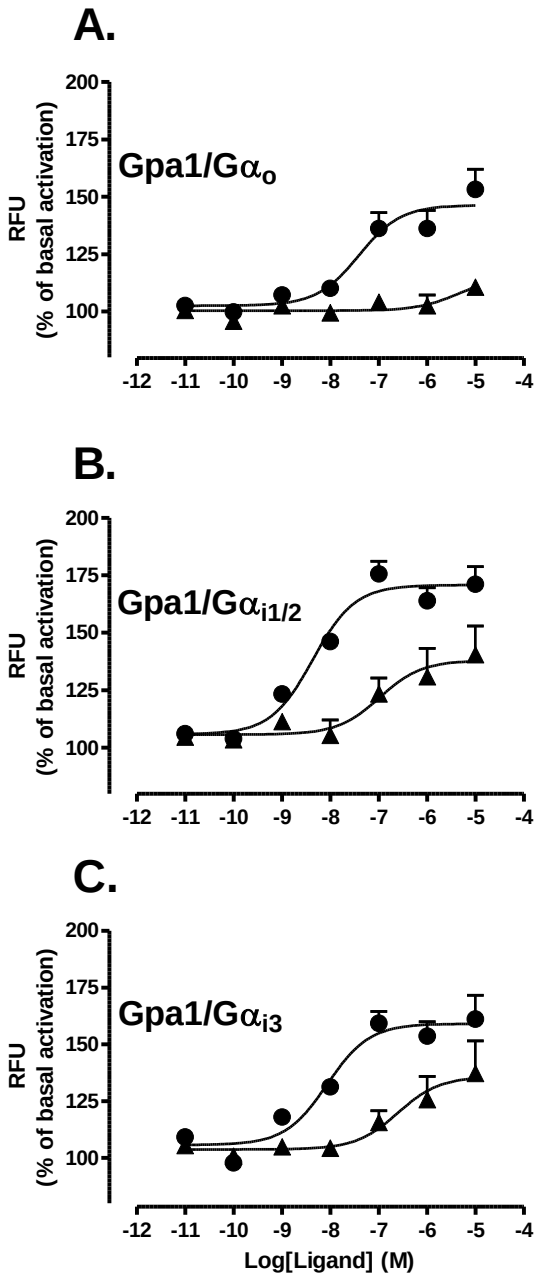

Figure 2.1 Influence of G protein subtype on adenosine A1 receptor agonist concentration-response curves. R-PIA (●) and VCP-189 (▲) concentration-response curves were constructed in yeast strains expressing either: **A.** Gpa1/Gα_o, **B.** Gpa1/G$\alpha_{i1/2}$ or **C.** Gpa1/Gα_{i3}. Each yeast strain was incubated with a range of ligand concentrations for 18-24hrs at 30°C, prior to obtaining fluorescence measurements. Data points represent the mean ± S.E.M. obtained from three to five experiments conducted in duplicate and are normalised to the fluorescence measure in the absence of ligand. Error bars not shown lie within the dimensions of the symbol.

Table 2.1 Potency (pEC50) values generated from concentration-response curves from yeast signalling assays performed in strains expressing the adenosine A1 receptor with either Gpa1/$G\alpha_o$, Gpa1/$G\alpha_{i1/2}$ or Gpa1/$G\alpha_{i3}$. Values represent the mean ± S.E.M. obtained from three to five experiments conducted in duplicate.

Yeast strain	R-PIA	VCP-189
Gpa1/$G\alpha_o$	7.38 ± 0.18	N.D.
Gpa1/$G\alpha_{i1/2}$	8.41 ± 0.17	6.97 ± 0.01
Gpa1/$G\alpha_{i3}$	8.08 ± 0.07	6.29 ± 0.25

(N.D. – Not determined)

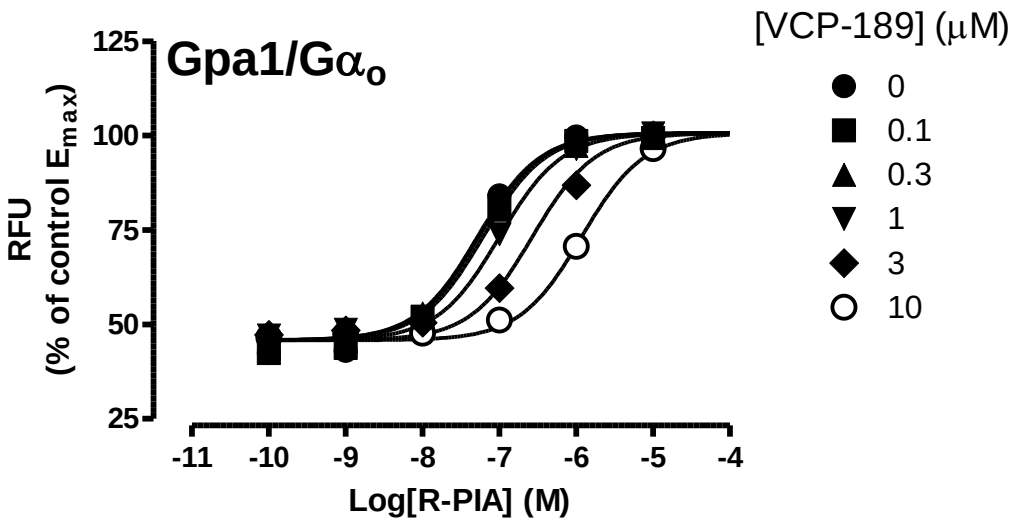

Figure 2.2 Effect of VCP-189 on R-PIA concentration-response curves in yeast strain expressing Gpa1/Gαo. The yeast strain was incubated with a range of R-PIA concentrations in the absence or presence of VCP-189 for 18-24hrs at 30°C, before fluorescence was measured. Data are expressed as a percentage of the maximal response attained by R-PIA in the absence of VCP-189. Data points represent the mean ± S.E.M. obtained from three experiments conducted in duplicate. Error bars not shown lie within the dimensions of the symbol.

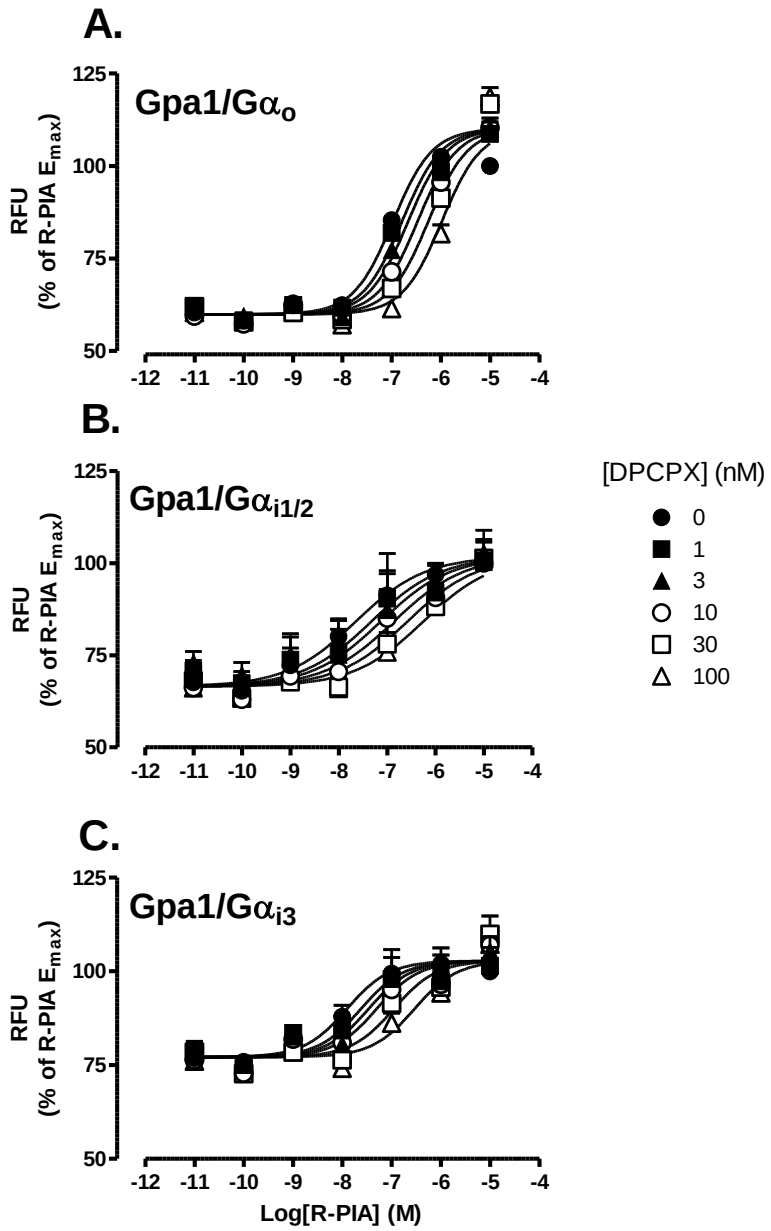

Figure 2.3 Effect of DPCPX on R-PIA concentration-response curves in yeast strains expressing the adenosine A1 receptor and either: **A.** Gpa1/Gα_o**, B.** Gpa1/G$\alpha_{i1/2}$, or **C.** Gpa1/Gα_{i3}. A range of R-PIA concentrations in the absence or presence DPCPX was incubated with the aforementioned yeast strains for 18-24hrs at 30°C, prior to obtaining fluorescence measurements. Data are represented as a percentage of the maximal response elicited by R-PIA in the absence of DPCPX, and each point is the mean ± S.E.M. collected from four experiments. Error bars not shown lie within the dimensions of the symbol.

2.3.2 Validation of $G\alpha_{i/o}$ coupling preference in CHO A_1 cells

The yeast data indicated that the A_1 receptor preferentially couples to members of the $G_{i/o}$ protein family with no appreciable interaction with G_q and G_s proteins. To ascertain whether this finding is relevant to a mammalian cell background, we utilised a CHO cell line stably expressing the human A_1 receptor and monitored intracellular Ca^{2+} mobilisation as a surrogate of either G_q activation and/or $G\beta\gamma$-mediated activation (Selbie and Hill, 1998; Migita et al., 2005; Minelli et al., 2008); inhibition of forskolin-stimulated cAMP accumulation, which indicates $G_{i/o}$ and G_s activation (Levitzki, 1988; Cordeaux et al., 2000); and ERK1/2 phosphorylation, which can result from activation of various G proteins and even non-G protein signalling (Schulte and Fredholm, 2003; Werry et al., 2006). Figure 2.4 shows that the ability of R-PIA to promote ERK1/2 phosphorylation, intracellular Ca^{2+} mobilisation or inhibition of forskolin-stimulated cAMP accumulation was completely abolished by pretreatment with PTX. Furthermore, in the absence of forskolin stimulation with PTX treatment, R-PIA was not able to elicit a cAMP accumulation response, indicating that the A_1 receptor does not couple to G_s proteins in this cell line. Taken together, these results suggest that A_1 receptor-mediated responses in this mammalian cell line are wholly dependent on $G_{i/o}$ activation, as predicted by the findings in the yeast assays.

To confirm that the pharmacology of the antagonist, DPCPX, studied in the yeast cellular background is retained in a mammalian background, we also performed Schild analysis on the ability of DPCPX to inhibit R-PIA-mediated ERK1/2 phosphorylation in the CHO cells. As shown in Figure 2.5, the antagonist produced a concentration-dependent, parallel, dextral shift of the agonist concentration-response curve,

characterised by a pA_2 of 9.11 ± 0.09 (n = 4), which was not significantly different from the values derived in the yeast assays.

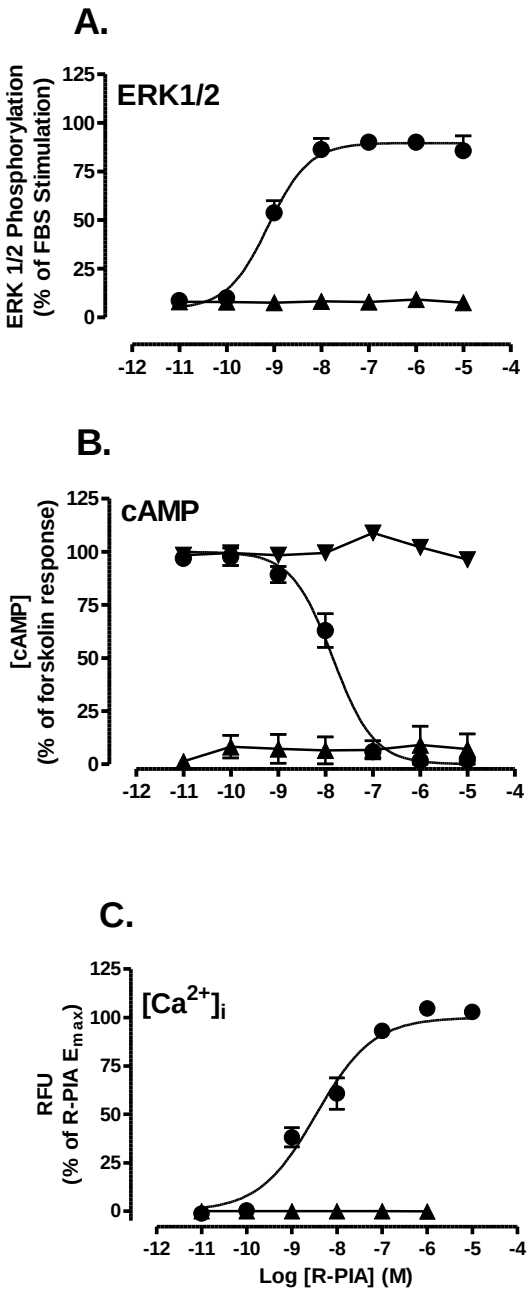

Figure 2.4 Impact of PTX pretreatment on adenosine A1 receptor signalling. R-PIA concentration-response curves for A_1 receptor-mediated ERK1/2 phosphorylation (**A)**; inhibition of forskolin-induced cAMP accumulation (**B**) and intracellular Ca^{2+} mobilisation (**C**). CHO cells expressing the A_1 receptor were serum-starved overnight in the absence (●) or presence (▲) of 100ng mL^{-1} PTX. For Panel **B**, (▼) refers to PTX-treated with no forskolin stimulation. Data points represent the mean ± S.E.M. of three to four experiments performed in duplicate. Error bars not shown lie within the dimensions of the symbol. Data displayed in panels **B** and **C** are from experiments performed by Dr Celine Valant.

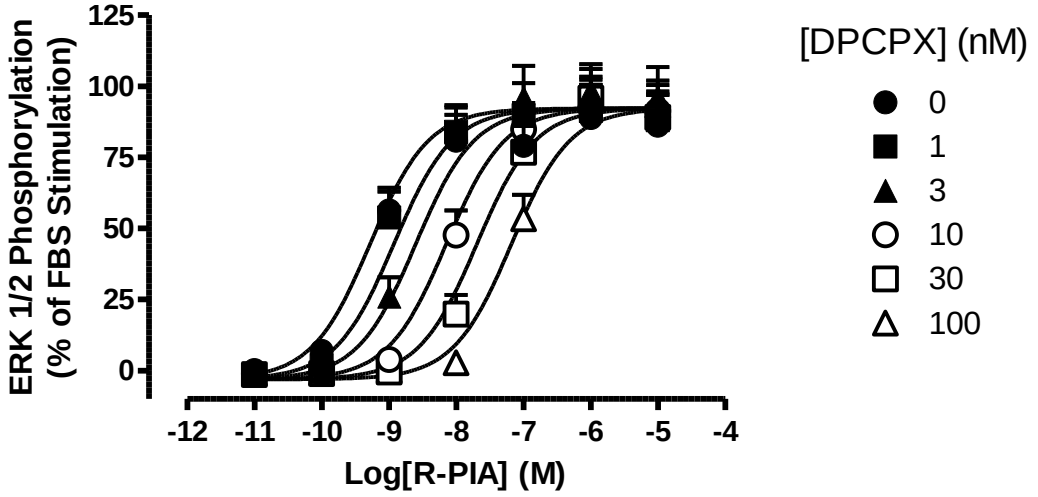

Figure 2.5 Effect of DPCPX on R-PIA-induced ERK1/2 phosphorylation concentration-response curves in the absence or presence of DPCPX performed in CHO cells expressing the adenosine A_1 receptor. CHO cells expressing the adenosine A_1 receptor were pretreated for 30mins with the indicated concentrations of DPCPX prior to a 5 min stimulation of R-PIA at a range of concentrations. Data are expressed as percentage mean of ERK1/2 phosphorylation elicited by 3% FBS ± S.E.M. collected from four experiments performed in duplicate. Error bars not shown lie within the dimensions of the symbol.

2.3.3 Assessment of agonist functional selectivity

The yeast data indicated that VCP-189 had a lower efficacy than R-PIA and a preferential coupling to $G\alpha_i$ proteins relative to $G\alpha_o$ proteins. However, a comparison of the ability of each agonist to mediate ERK1/2 phosphorylation in CHO cells (Figure 2.6) indicated robust and equi-efficacious agonism for both ligands. The differences between these observations in yeast and mammalian cells may reflect differences in stimulus-response coupling between the cellular backgrounds and/or true functional selectivity by VCP-189 for specific Gα subunits that is not detected due to the multi-pathway-convergent nature of ERK1/2 signalling. To first determine the influence of stimulus-response coupling on our observations, we used the irreversible antagonist, FSCPX, to occlude the A_1 receptor's orthosteric site and thus effectively reduce the pool of accessible receptor binding sites that can be activated by each agonist (Figure 2.6). Application of the operational model of agonism (eq. 2.3) to the datasets obtained before and after receptor alkylation yielded the following parameters for R-PIA: Log K_A = -6.96 ± 0.34, Log τ = 1.71 ± 0.34 (τ = 51), whereas for VCP-189 the following values were determined: Log K_A = -5.85 ± 0.21, Log τ = 0.61 ± 0.18 (τ = 4). The differences between the τ values under control conditions (in the absence of alkylation) for the two agonists clearly indicate that VCP-189 is a lower efficacy agonist than R-PIA for ERK1/2 phosphorylation in CHO cells, and that the equivalent maximal agonist responses under the control conditions are thus most likely due to a high degree of stimulus-response coupling and/or receptor expression level in this cellular background compared to the yeast.

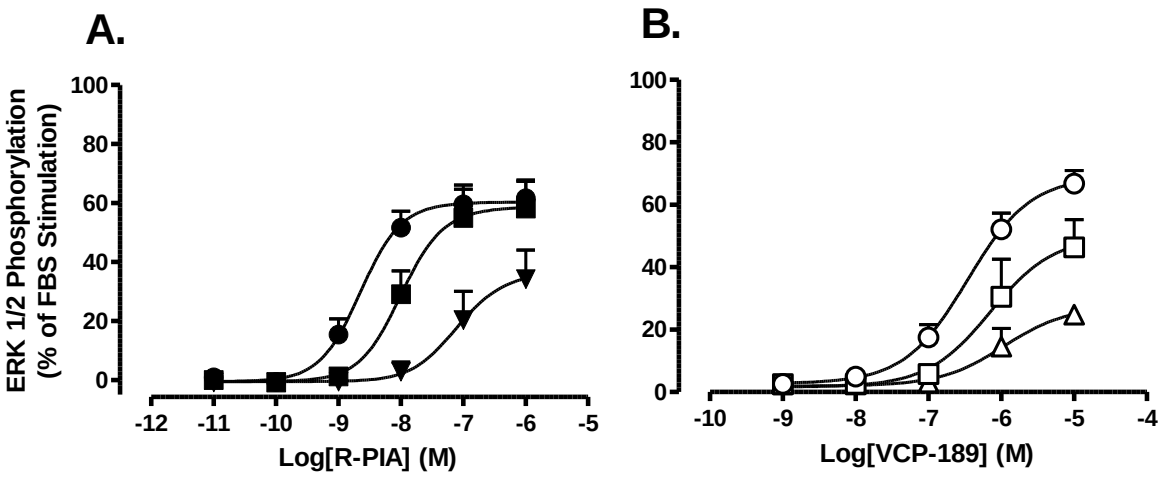

Figure 2.6 Impact of A1 receptor alkylation by FSCPX on R-PIA and VCP-189 ERK1/2 phosphorylation concentration-response curves. **A.** R-PIA-induced ERK1/2 phosphorylation concentration-response curves (5mins incubation) in the absence (●) or presence of 5μM (■) or 10μM (▲) FSCPX pretreatment (30 minutes) in CHO A_1R cells. **B.** VCP-189-induced ERK1/2 phosphorylation concentration-response curves (5 minute incubation) in the absence (○) or presence of 10μM FSCPX pretreatment for 15 (□) or 30 (△) mins. Each data point is expressed as a percentage mean of ERK1/2 phosphorylation elicited by 3% FBS ± S.E.M. obtained from three to five experiments. Error bars not shown lie within the dimensions of the symbol.

Finally, to investigate whether the preferential coupling of VCP-189 to $G\alpha_i$ over $G\alpha_o$ proteins identified in yeast was also indicative of functional selectivity for the former G proteins over the latter, we used two different experimental approaches in CHO cells. The first was to investigate agonist ERK1/2 phosphorylation responses in CHO A_1 cells transfected with cDNA encoding specific PTX-insensitive $G\alpha_{i/o}$ subunits after PTX treatment. These experiments revealed that both R-PIA and VCP-189 were, in fact, able to promote ERK1/2 phosphorylation via either $G\alpha_o$ splice variants ($G\alpha_{oA}$ and $G\alpha_{oB}$), as well as $G\alpha_{i2}$ to varying extents (Figure 2.7). Both ligands had comparable efficacies when coupled to either G_{oA} or G_{i2}, but VCP-189 was a lower efficacy agonist compared to R-PIA when coupled to G_{oB}. The second approach that we utilised was to generate a G protein coupling profile for the A_1 receptor using [^{35}S]-GTPγS immunoprecipitation, which determines activation of native G proteins. Figure 2.8 shows the results from these assays using CHO A_1 cell membranes treated with either buffer (basal), 10μM R-PIA or 10μM VCP-189. The results show that R-PIA and VCP-189 could only significantly alter [^{35}S]-GTPγS binding at $G\alpha_{i3}$ G proteins when compared to basal, in contrast to their effects on $G\alpha_o$ $G\alpha_{i1}$, $G\alpha_{i2}$ and $G\alpha_q$ proteins. However, there did appear to be a trend towards an increase in [^{35}S]-GTPγS binding to $G\alpha_o$ $G\alpha_{i1}$ and $G\alpha_{i2}$ proteins. Collectively, these results suggest that any preferential agonist coupling to G_i proteins over G_o proteins identified in yeast cells are most likely due to differences in strength of coupling rather than functional selectivity due to stimulus-bias.

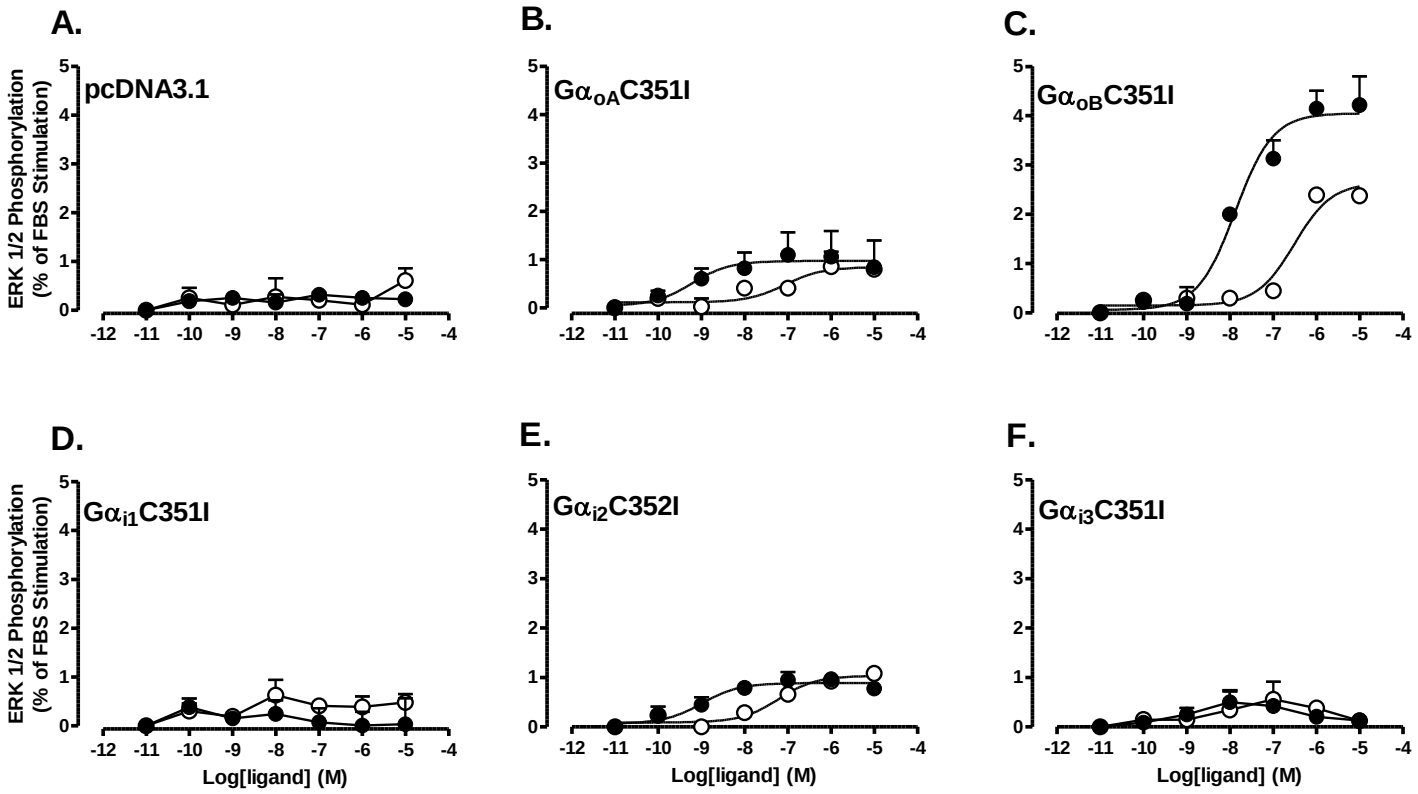

Figure 2.7 Effect of individual PTX-insensitive Gαi/o proteins on R-PIA- (●) and VCP-189- (○) induced ERK1/2 phosphorylation in PTX-pretreated CHO A_1 receptor cells. The cDNA constructs used were: **A.** pcDNA3.1 (vector only); **B.** $G\alpha_{oA}C351I$; **C.** $G\alpha_{oB}C351I$; **D.** $G\alpha_{i1}C351I$; **E.** $G\alpha_{i2}C352I$ and **F.** $G\alpha_{i3}C351I$. cDNA constructs were transfected into CHO cells stably expressing the A_1 receptor 48 hr prior to experimentation. ERK1/2 phosphorylation was determined at the 5 min time point for each concentration of ligand. Each data point is expressed as a percentage mean of ERK1/2 phosphorylation elicited by 3% FBS ± S.E.M. obtained from three experiments. Error bars not shown lie within the dimensions of the symbol.

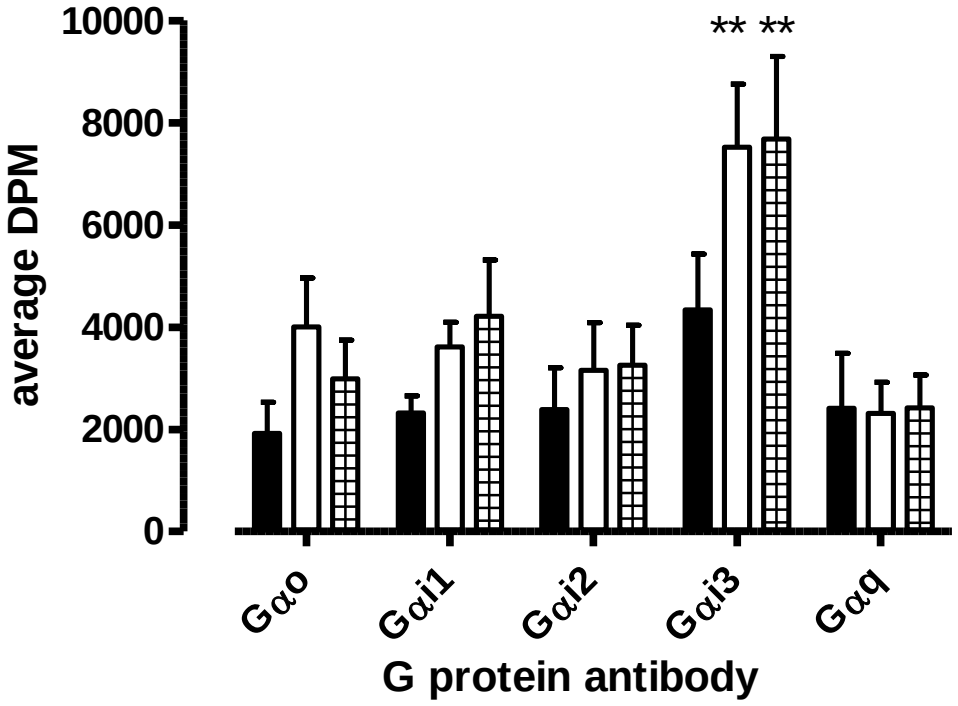

Figure 2.8 Contribution of native mammalian Gαi/o proteins to A1 receptor-mediated [35S]-GTPγS binding using immunoprecipitation. Basal (no ligand) (filled), 10μM R-PIA- (open) and 10μM VCP-189- (thatched) induced [^{35}S]-GTPγS binding at specific $G\alpha_{i/o}$ proteins in CHO A_1R membranes. Each data point is expressed as the mean DPM ± S.E.M. from three to six experiments. ** $P < 0.01$ determined by one-way ANOVA with a Dunnett's post-test for basal $G\alpha_{i3}$ [^{35}S]-GTPγS binding compared to R-PIA- or VCP-189-stimulated [^{35}S]-GTPγS binding.

2.4 Discussion

This is the first study that has used a yeast signalling system to characterize adenosine A_1 receptor ligand affinities, efficacies and G protein coupling profiles. Although there may be issues associated with a potential lack of sensitivity for agonists that are weakly coupled to a particular G protein, the yeast system appears to be a valid platform for determining G protein coupling and generating affinity estimates for A_1 receptor ligands in a manner that is consistent with coupling identified in mammalian cells. This suggests that yeast studies may have applicability to the determination of GPCR ligand-G protein functional selectivity profiles.

There are a number of pharmacological and biochemical approaches to estimating affinities of ligands, the most traditional of which are based on radioligand binding. However, this type of assay does not readily provide any information about events downstream of receptor activation, can be expensive and, for many receptors, is limited by a lack of appropriate radioligands. Another approach to estimate ligand-receptor affinity values is to use functional assays that generate concentration-response data such that appropriate quantitative models can be applied to furnish agonist or antagonist affinity values (Kenakin, 2003b). This approach may also be costly, time-consuming, and/or of insufficient throughput if multiple curves are required. In this regard, the use of a yeast-based assay for determining GPCR ligand pharmacology may prove particularly useful, as it is inexpensive, lacks interacting mammalian proteins and is able to reconstitute many GPCRs with high fidelity (Dowell and Brown, 2002; Minic et al., 2005b). The majority of studies investigating GPCRs in a yeast-based system in the past have exploited the assays for facilitating random mutagenesis studies (Erlenbach et al., 2001a; Schmidt et al., 2003; Armbruster et al., 2007), or as an empirical ligand screening tool with a single pathway endpoint (Bass et al., 1996; Campbell et al., 1999;

Minic et al., 2005a). However, to our knowledge, there has not been a study investigating the ability of the yeast system to ascertain quantitative, system-independent, pharmacological properties of established and novel ligands.

Although an earlier study used an *S. cerevisiae* signalling assay to identify adenosine A_1 receptor antagonists (Campbell et al., 1999), it was restricted to the empirical determination of antagonist potencies rather than a quantification of actual affinity values. In our current study, we have shown that it is possible to apply classic Schild analysis to the interaction between R-PIA and DPCPX in yeast to yield antagonist affinity values that are not significantly different from those obtained in CHO cells. Moreover, these affinity estimates are consistent with values derived for DPCPX in the past (e.g. Townsend-Nicholson and Shine, 1992; Rivkees et al., 1999; de Ligt et al., 2005; Obiefuna et al., 2005), indicating that antagonist pharmacology can be determined in yeast signalling assays and, furthermore, that the G protein subtype present has little bearing on antagonist affinity. In addition to allowing appropriate detection and quantification of antagonist pharmacology, the yeast system also profiled the G protein coupling preferences for A_1 receptor orthosteric agonists, indicating a role only for PTX-sensitive G proteins that was subsequently confirmed in our CHO cell line. These results are consistent with other studies showing that adenosine A_1 receptors are predominantly coupled to $G_{i/o}$ proteins (Freissmuth et al., 1991; Akbar et al., 1994; Jockers et al., 1994), although there is some evidence to suggest that A_1 receptors may couple to G_s (Cordeaux et al., 2000) or G_q proteins (Minelli et al., 2008); this is likely cell background-dependent.

Traditionally, agonist potency estimates derived from functional studies cannot be directly related to agonist affinity estimates for a given GPCR due to the nonlinear nature of stimulus-response coupling in most biological systems (Kenakin, 2003b).

However, a recent study by Yu et al. (2008) has suggested that a general property of *S. cerevisiae* is an apparent lack of signal amplification. This relates to the cell biology of *S. cerevisiae*, which involves a negative-feedback loop in the pheromone response pathway contingent on the phosphorylated Fus3 protein inhibiting Sst2-mediated Ste5 recruitment, resulting in a pronounced decrease in amplification of signal. A consequence of this feedback loop is to "align" the concentration-response relationship with the concentration-occupancy relationship in yeast. Yu et al. (2008) have also shown that deletion of the Sst2 protein provides equivalent results to that of a system where Fus3 is constitutively activated. Given that the yeast system used in our study is Sst2-negative, this suggests that the actual potency values obtained from agonist concentration-response curves should be approximations of agonist affinity for A_1 receptor expressed in our yeast strains. If this is the case, then a number of interesting observations arise from our current results. Although the potency estimates obtained for R-PIA spanned an approx. 10-fold range across the various strains tested, they were generally comparable with the "high-affinity" binding constant derived previously by our laboratory using membrane-based binding assays in CHO cells (May et al., 2005). However, these potency values are not consistent with the Log K_A estimates derived from the receptor alkylation assays of R-PIA-induced ERK1/2 phosphorylation in CHO cells (Figure 2.6); the latter were, instead, consistent with the "low-affinity" binding constant we have previously determined (May et al., 2005). If the hypothesis of a lack of appreciable signal amplification in yeast is valid, then it may be that the high potency of R-PIA as an agonist in yeast, compared to its estimated Log K_A for the A_1 receptor from the operational model analysis in the CHO cells, reflects a pre-formed high affinity state of the A_1 receptor-G protein complex in the intact *S. cerevisiae* cell background that may not be present, or only transiently so, in CHO cells. It is worth noting that a

previous study in HEK-293 cells has suggested that the A_1 receptor can form pre-coupled GPCR-G protein complexes (Nobles et al., 2005).

In addition to allowing for the quantification of parameters describing the drug-receptor interaction, another attractive utility of the yeast assay system is the ability to profile ligands acting unambiguously via a single type of G protein in a common cellular background. In turn, this offers the potential to detect G protein-mediated functional selectivity, which could be manifested as a change in ligand potency or efficacy orders across different yeast strains. However, the subsequent validation of the phenomenon in a mammalian cell line must also consider the impact of differences in stimulus-response coupling and/or receptor expression levels between the cell types on the resultant pharmacology. Indeed, VCP-189 generated the same maximal response as R-PIA when studied as an agonist of ERK1/2 phosphorylation in our CHO cells, but was clearly of lower efficacy than R-PIA in each of the yeast strains tested. The most parsimonious explanation for this difference is that the CHO cell background is characterized by a higher degree of signal-amplification than yeast, such that low efficacy agonists can still generate the maximal cellular response. The results of the receptor alkylation experiments and associated operational model analysis are in agreement with this mechanism; even small degrees of receptor alkylation resulted in a collapse of the VCP-189 concentration-response curve, whereas R-PIA displayed a rightward shift in potency with no collapse of the maximal agonist effect until the extent of receptor alkylation was more pronounced.

Despite the differences in the strength of signal between the two agonists studied, it is still possible that functional selectivity was operative in determining their coupling preferences to various G protein subtypes. Data acquired from the yeast studies indicated that VCP-189 could discriminate between Gpa1/$G\alpha_o$ and Gpa1/$G\alpha_i$ proteins,

and subsequently preferentially activate Gpa1/$G\alpha_i$ proteins, whereas R-PIA did not discriminate. However, the functional data obtained in CHO A_1 cells either transfected with PTX-insensitive $G\alpha_{i/o}$ subunits or following [^{35}S]-GTPγS immunoprecipitation were not in agreement with data collected from the yeast signalling assay, in that VCP-189 also activated $G\alpha_o$ coupling in CHO cells. There are a number of possible reasons for this discrepancy, but the most likely is that, since all Gpa1/Gα chimeras contain only five C-terminal amino acids of the mammalian Gα protein, the sequence in the Gpa1/$G\alpha_o$ chimera is sub-optimal for ensuring sufficient affinity for the A_1 receptor for low efficacy agonists; if an agonist has very low efficacy, it may be undetectable in the yeast system. Thus, we conclude that there is no evidence from these studies of functional selectivity of either agonist in their coupling preferences for $G\alpha_{i/o}$ protein subtypes via the A_1 receptor; the differences in absolute potencies/efficacies noted between cellular backgrounds are most likely due to differences in stimulus-response coupling.

Taken together, our findings indicate that the yeast signalling system is a useful and convenient tool to add to the pharmacological armamentarium, especially in regard to the quantification of agonist and antagonist affinity and relative efficacy estimates. This is likely to apply not only to the A_1 receptor, but to any GPCR that can be expressed in yeast. In theory, the assay can also be used to routinely profile G protein coupling selectivity, although potential issues of sensitivity for detecting low efficacy agonists need to be considered.

CHAPTER 3

NOVEL SIGNALLING PROPERTIES OF 'ANTI-MUSCARINIC' LIGANDS: DETECTION OF FUNCTIONALLY SELECTIVE SIGNALLING AT THE M_3 MUSCARINIC ACETYLCHOLINE RECEPTOR USING *SACCHAROMYCES CEREVISIAE*

3.1 Introduction

The muscarinic acetylcholine receptors (mAChRs) are a prototypical class of rhodopsin family GPCRs, and consist of five subtypes (M_1-M_5) (Caulfield, 1993). The M_1, M_4 and M_5 mAChR subtypes are predominantly expressed in the CNS, whilst M_2 and M_3 mAChRs are expressed widely in the CNS and periphery; the M_3 mAChR performing key roles in various types of smooth muscle (Wess et al., 2007). The activation of $G_{i/o}$ proteins by M_2 and M_4 mAChRs in presynaptic cholinergic and non-cholinergic neurons results in the inhibition of neurotransmission by activation of Kir3 channels and/or inactivation of Ca_v2 channels, thereby inhibiting depolarisation (Hescheler and Schultz, 1994; Ocana et al., 2004; Santafe et al., 2009). M_1 mAChRs also have a role in neurotransmission, but are predominantly expressed postsynaptically, in regions such as the cerebral cortex, hippocampus and striatum (Langmead et al., 2008). Although the M_1 mAChR preferentially couples to $G_{q/11}$, immunoprecipitation studies have shown that the M_1 mAChR also interacts with G_i and $G_{12/13}$ proteins in a cell-specific manner (Luthin et al., 1997). Expression of the M_5 mAChR is nearly exclusively restricted to the brain, in particular the substantia nigra, cerebral cortex, caudate nucleus and putamen, where it exerts its function by coupling to $G_{q/11}$ and, potentially, G_s subunits (Eglen and Nahorski, 2000). The M_3 mAChR is also pleiotropically coupled, with a preference for $G_{q/11}$ activation (Wylie et al., 1999; Rumenapp et al., 2001; Brown et al., 2005; Clark et al., 2007). However, the M_3 mAChR mostly mediates its functions in smooth muscle and endothelial cells of the vasculature, and smooth muscle in the respiratory, gastrointestinal and genitourinary tracts (Eglen and Whiting, 1990; Eglen et al., 1994; Eglen et al., 1996).

One of the major roles of the M_3 mAChR in the airways is regulation of smooth muscle tone, despite it being outnumbered by M_2 mAChRs at an approximate ratio of 4:1

(Gosens et al., 2006). Parasympathetic neurons that innervate the airways are controlled by M_2 mAChR autoreceptors, which modulate ACh neurotransmission (Coulson and Fryer, 2003). Additionally, ACh may be secreted into the airways by inflammatory cells such as both B and T lymphocytes, and epithelial cells (Fujii et al., 1996; Reinheimer et al., 1996; Rinner et al., 1998). This ACh release results in smooth muscle contraction mediated by mAChRs (probably M_3), and is a composition of PLC-IP_3-Ca^{2+} signalling, resulting in Ca^{2+}/calmodulin-mediated activation of myosin light-chain kinase, and a Ca^{2+} sensitising pathway consisting of RhoA-ROCK activation and subsequent inactivation of myosin light-chain phosphatase, producing a synergistic smooth muscle contractile response (Gosens et al., 2006). Given the multiple sources of ACh regulating the airways and the synergistic signalling of the M_3 mAChR to produce a contractile response, it is unsurprising that M_3 mAChR-preferring antagonists, such as tiotropium and iprotropium, have good clinical efficacy for respiratory diseases (Disse et al., 1999).

Despite the widespread utility of M_3-targeting anti-muscarinic drugs, there often remains an issue with subtype selectivity due to the highly conserved nature of the orthosteric binding site (Gregory et al., 2007). Traditionally, ligand selectivity has been ascribed to differential affinities for receptor subtypes, however, there is now accumulating evidence that selectivity can also arises through differential stabilisation of specific receptor states. For example, clinically used β-adrenoceptor 'antagonists', such as propranolol and atenolol, have recently been shown to exhibit both agonist and antagonist/inverse agonist effects depending on which signal pathway was being measured (Baker et al., 2003; Sato et al., 2007; Galandrin et al., 2008). These studies highlight the multi-conformational nature of GPCRs, and that distinct conformations adopted by the ligand/receptor complexes have potential to engender distinct combinations of signalling cascades. As outlined in the previous chapter, the

phenomenon of a ligand selectively activating discrete signalling cascades at the exclusion of others is known as functional selectivity, and there has been a recent paradigm shift toward developing ligands that not only specifically target one type of receptor, but also a distinct pathway that may be implicated in a certain condition. However, this approach adds an extra challenge for drug screening programs.

Given the increasing recent evidence of functional selectivity exhibited by clinically relevant β-adrenoceptors antagonists, it was hypothesised that the M_3 mAChR-targeting anti-muscarinic drugs may also display pathway selectivity. As a proof-of-concept approach, the pharmacology of some classical muscarinic antagonists was re-evaluated at this subtype, using a yeast system as a robust model for detecting ligand-mediated selective G protein signalling. This study has found that the yeast system can not only identify known ligand properties, but also detect novel agonistic properties of anti-muscarinic ligands at a specific G protein-mediated pathway, as well as revealing a role for lipid-rich microdomains as a cell-mediated means of engendering functional selectivity.

3.2 Methods

3.2.1 Materials

The p416GPD $rM_3\Delta i3$ mAChR was a generous gift from Dr Jürgen Wess (NIH, Bethesda, MD). Flp-In™ 3T3 cells were obtained from Invitrogen (Carlsbad, CA). Hoechst 33342 and Alexa™ 568-conjugated phalloidin were purchased from Molecular Probes (Carlsbad, CA). cDNA constructs of the human M_3 mAChR were purchased from the Missouri University of Science and Technology (Missouri S&T), http://cdna.org (Rolla, MO). All other reagents were purchased from Sigma Aldrich (St Louis, MO). Reagents cited in the previous chapter were sourced as per section 2.2.1.

3.2.2 Yeast transformations and signalling assay

Saccharomyces cerevisiae strains expressing chimeras of five C-terminal amino acids of human Gα protein with Gpa1 (1-467) have been previously described in Brown et al. (2000). The yeast strains were further transformed with a p416GPD vector containing the gene encoding the rat M_3 muscarinic ACh receptor with a intracellular 3rd loop deletion ($rM_3\Delta i3$ mAChR), which facilitates enhanced expression in yeast as described in Erlenbach et al. (2001a), using the *S.cerevisiae* EasyComp™ transformation kit in accordance with manufacturer's instructions.

The conditions for the signalling component of the assay were as described in section 2.2.3.

3.2.3 Transfections and cell culture

The cDNA sequence of the human M_3 mAChR was amplified by PCR and cloned, using classical cloning methods, into the Gateway entry vector, pDONR201, using the BP clonase kit according to manufacturer's instructions. The M_3 mAChR construct was subsequently transferred in the Gateway destination vector, pEF5/FRT/V5-dest, using the LR clonase kit in accordance with manufacturer's instructions. The construct was then transfected into Flp-In 3T3 cells using methods described previously (Nawaratne et al., 2008). Flp-In™ 3T3 cells stably expressing the M_3 mAChR (3T3 M_3 mAChR cells) were cultured at 37°C in 5% CO_2 in Dulbecco's modified Eagle medium (DMEM) supplemented with 5% (v/v) FBS, 16mM HEPES and were selected using 200μg mL^{-1} hygromycin B, but maintained using 100μg mL^{-1} hygromycin B.

3.2.4 Ca^{2+} mobilisation assay

3T3 M_3 mAChR cells were cultured overnight in 96-well plates at 37°C in 5% CO_2. Cells were washed twice in Ca^{2+} assay buffer (150mM NaCl, 2.6mM KCl, 1.2mM $MgCl_2$, 10mM dextrose, 10mM HEPES, 2.2mM $CaCl_2$, 0.5% (w/v) BSA and 4mM probenecid). Buffer was then replaced with Ca^{2+} assay buffer with 1μM Fluo-4-AM and incubated for 1 hour at 37°C in 5% CO_2. Cells were washed twice more and replaced with 37°C Ca^{2+} assay buffer, pretreated appropriately with antagonist (if required), agonist was added, and fluorescence was measured in a Flexstation™ (Molecular Devices) at 485 excitation and 520 emission wavelengths.

3.2.5 Cytoskeletal rearrangement assay and image analysis

3T3 M_3 mAChR cells were cultured overnight in 96-well plates at 37°C in 5% CO_2. Samples were serum-starved 4 hr prior to assaying then treated with ligand at appropriate time points (CCh: 2 min, atropine: 15 min, determined by separate time-course assays). Time-course assays were performed by treating cells with serum-free DMEM, 100μM CCh or 100nM atropine for 2, 5, 8, 15 and 30 min. Media was removed and the samples were fixed in 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS) for 10 min, rinsed twice in PBS, and permeabilized in 0.3% (v/v) Tween20 in PBS. Samples were stained in PBS containing 0.2μg mL^{-1} Hoechst 33342 and 2U mL^{-1} Alexa 568-phalloidin, washed twice with PBS and imaged using an IN Cell analyzer 1000™ (GE Healthcare) with 360 excitation, 460 emission (Hoechst 33342); 565 excitation, 620 emission (Alexa 568-phalloidin) filters.

To disrupt lipid-rich microdomains, cells were treated with 5μg mL^{-1} filipin III for 30 min prior to ligand treatment.

The cytoskeletal component of the images was analyzed manually and normalised to the nuclei content per image, which was automatically analyzed using IN Cell Developer™ software.

3.2.6 Data analysis

Individual agonist concentration-response curves, in the absence of antagonist, were fitted via nonlinear regression with equation 2.1 as stated in section 2.2.9, using Prism 5.02 (GraphPad Software, San Diego, CA).

Antagonist affinity estimates were obtained by using the modified Lew/Angus, non-linear regression model (Lew and Angus, 1995). If the data did not satisfy the criteria

of parallel, dextral shift with no depression of E_{max}, the negative logarithm of equi-effective agonist concentrations were utilised for pEC_x in the following model:

$$pEC_x = -\log([B] + 10^{-pA_2}) - \log c \qquad (3.2)$$

where pEC_x is the concentration of agonist that achieves an equi-effective response between basal and E_{max}, [B] is the concentration of agonist, pA_2 is the negative logarithm of the concentration of antagonist that requires a two-fold increase in agonist concentration to achieve an equi-effective response as that in the absence of antagonist, and c is a fitting constant. For a competitive antagonist, the pA_2 value is a measure of the pK_B, ie the equilibrium dissociation constant of the antagonist.

Antagonist potency estimates were analyzed with one-way ANOVA with a Bonferroni's post-test, using Prism 5.02 software (GraphPad, La Jolla, CA). For experiments comparing control and filipin III pretreated atropine concentration-response curves, an extra sum-of-squares F-test was performed to determine whether fitting a single curve to define both sets of data was statistically preferred (p<0.05).

3.3 Results

3.3.1 Characterisation of carbachol and atropine pharmacology in yeast

Concentration-response curves were constructed to carbachol (CCh) in yeast strains expressing the $rM_3\Delta i3$ receptor and chimeras of Gpa1/$G\alpha_q$, Gpa/$G\alpha_{i1/2}$, Gpa1/$G\alpha_{12}$, Gpa1/$G\alpha_s$ or Gpa1, to test its ability to elicit a response when coupled to an individual subtype of G protein. CCh elicited a response in strains expressing chimeras of Gpa1/$G\alpha_q$, Gpa/$G\alpha_{i1/2}$ and Gpa1/$G\alpha_{12}$ with varying potencies (pEC_{50} = 7.15 ± 0.08; 6.67 ± 0.20 and 6.66 ± 0.16, respectively, n = 4), but not Gpa1 or Gpa1/$G\alpha_s$ (Figure 3.1).

To assess whether muscarinic antagonist affinity could be estimated using functional yeast data, interaction studies between CCh and the prototypical, non-selective mAChR antagonist, atropine, were performed in strains expressing Gpa1/$G\alpha_q$, Gpa/$G\alpha_{i1/2}$ or Gpa1/$G\alpha_{12}$. Figure 3.2 shows that, in each case, atropine produced parallel, dextral shifts of the CCh concentration-response curves in a concentration-dependent manner. Application of equation 3.2 to pEC_{20} values yielded the following pA_2 estimates were generated; Gpa1/$G\alpha_q$, (9.16 ± 0.11); Gpa/$G\alpha_{i1/2}$, (9.53 ± 0.17) and Gpa1/$G\alpha_{12}$, (9.19 ± 0.17), n = 4. Statistical analysis using a one-way ANOVA with a Bonferroni's post-test demonstrated no significant difference between these values (p>0.05).

Interestingly, atropine-induced alterations were also observed in the baselines of the interaction studies performed in yeast strains expressing Gpa1/$G\alpha_q$ and Gpa1/$G\alpha_{12}$, suggesting atropine had activity in the absence of CCh. To further probe this phenomenon, concentration-response curves were constructed to atropine in yeast strains expressing chimeras of Gpa1/$G\alpha_q$, Gpa1/$G\alpha_{i1/2}$ and Gpa1/$G\alpha_{12}$ (Figure 3.3). Atropine was an inverse agonist when coupled to Gpa1/$G\alpha_q$, decreasing the basal activity of the system by 25%, with a pIC_{50} value of 9.06 ± 0.51 (n = 4). When coupled

to Gpa1/$G\alpha_{i1/2}$, atropine displayed properties of a neutral antagonist. Surprisingly, however, atropine was a low efficacy *agonist* when coupled to Gpa1/$G\alpha_{12}$ in the yeast system, with a pEC_{50} of 9.06 ± 0.34 and an increase of approximately 15% over basal activation (n = 4). To further probe if these pathway-dependent properties extended to other muscarinic antagonists, concentration-response curves were constructed to N-methylscopolamine (NMS) and pirenzepine in these strains (Figure 3.4). These experiments revealed that both NMS and pirenzepine followed the same pharmacological profile as atropine, with pIC_{50} estimates for inhibition of Gpa1/$G\alpha_q$ activation of 9.14 ± 0.17 (NMS) and 6.60 ± 0.15 (pirenzepine) (n = 3), and pEC_{50} values for Gpa1/$G\alpha_{12}$ signalling of 9.57 ± 0.10 (NMS) and 7.16 ± 0.20 (pirenzepine) (n = 3). These results suggest that the pathway-dependent differential signalling of atropine is a common trait of the other 'anti-muscarinic' ligands.

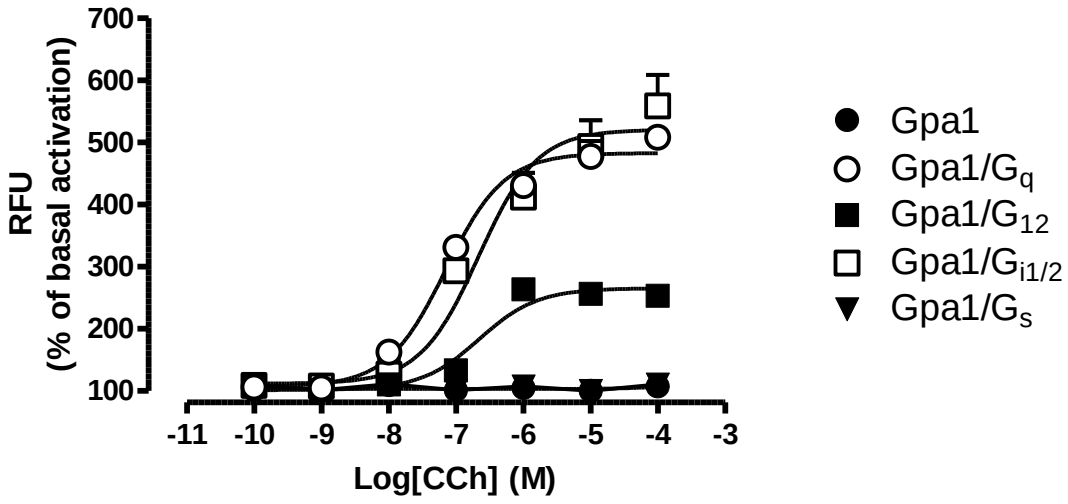

Figure 3.1 CCh concentration-response curves in yeast expressing the rM3Δi3 mAChR and Gpa1/Gαq, Gpa1/Gα12, Gpa1/Gαi1/2 or Gpa1/Gαs. Data points are expressed as mean percentage of basal activation ± S.E.M. obtained from four experiments performed in duplicate. Error bars not shown lie within the dimensions of the symbol.

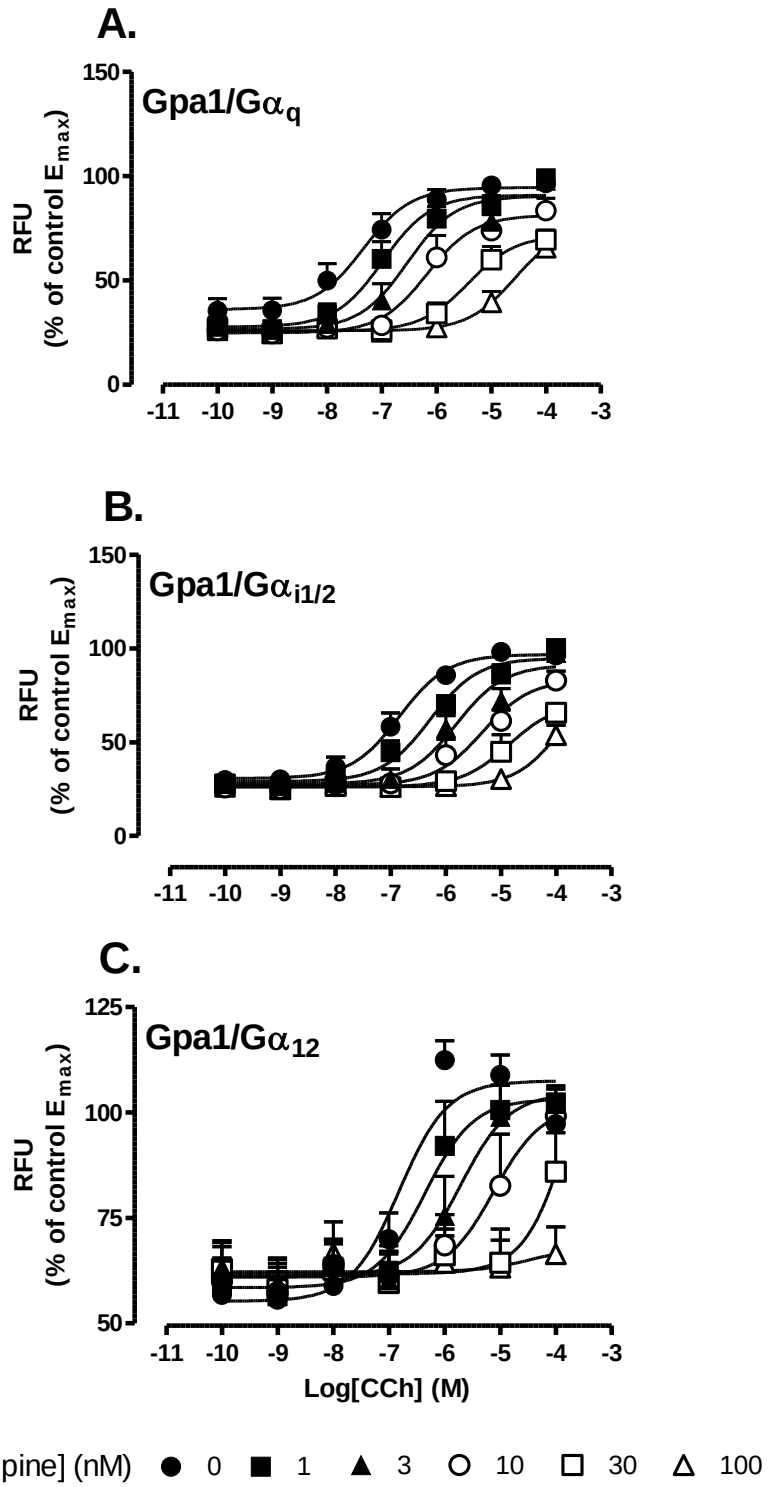

Figure 3.2 Effect of atropine on CCh concentration-response curves in different yeast strains. CCh concentration-response curves, performed in yeast strains expressing the $rM_3\Delta i3$ mAChR and **A.** Gpa1/$G\alpha_q$, **B.**Gpa1/$G\alpha_{i1/2}$ or **C.** Gpa1/$G\alpha_{12}$, in the absence and presence of atropine. Data points are expressed as mean percentage of the CCh E_{max} in the absence of atropine ± S.E.M. obtained from four experiments performed in duplicate. Error bars not shown lie within the dimensions of the symbol.

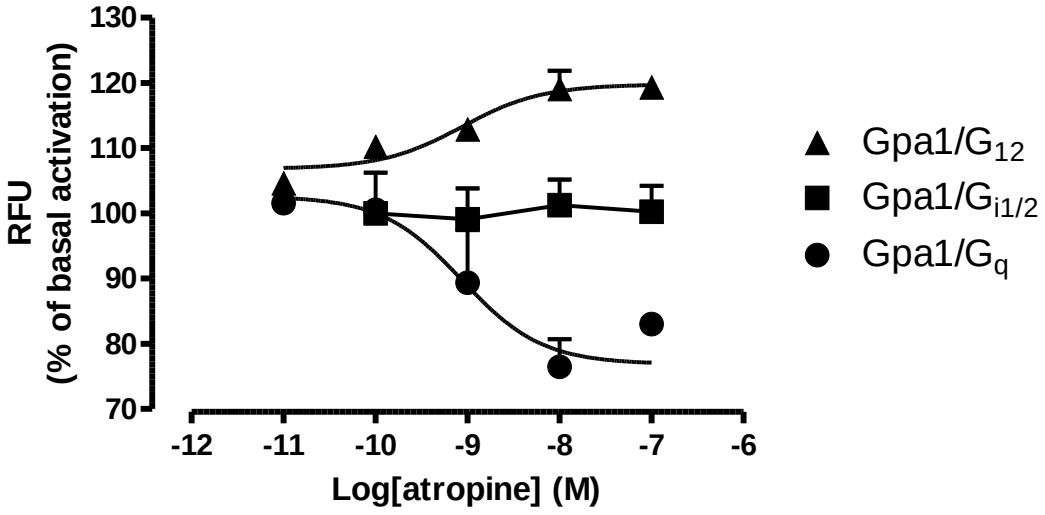

Figure 3.3 Influence of atropine on basal rM3Δi3 mAChR activation in yeast. Atropine concentration-response curves in yeast strains expressing the $rM_3\Delta i3$ mAChR and Gpa1/$G\alpha_q$, Gpa1/$G\alpha_{i1/2}$ or Gpa1/$G\alpha_{12}$. Data is represented as the mean percentage of basal activation ± S.E.M. and is obtained from four experiments performed in duplicate. Error bars not shown lie within the dimensions of the symbol.

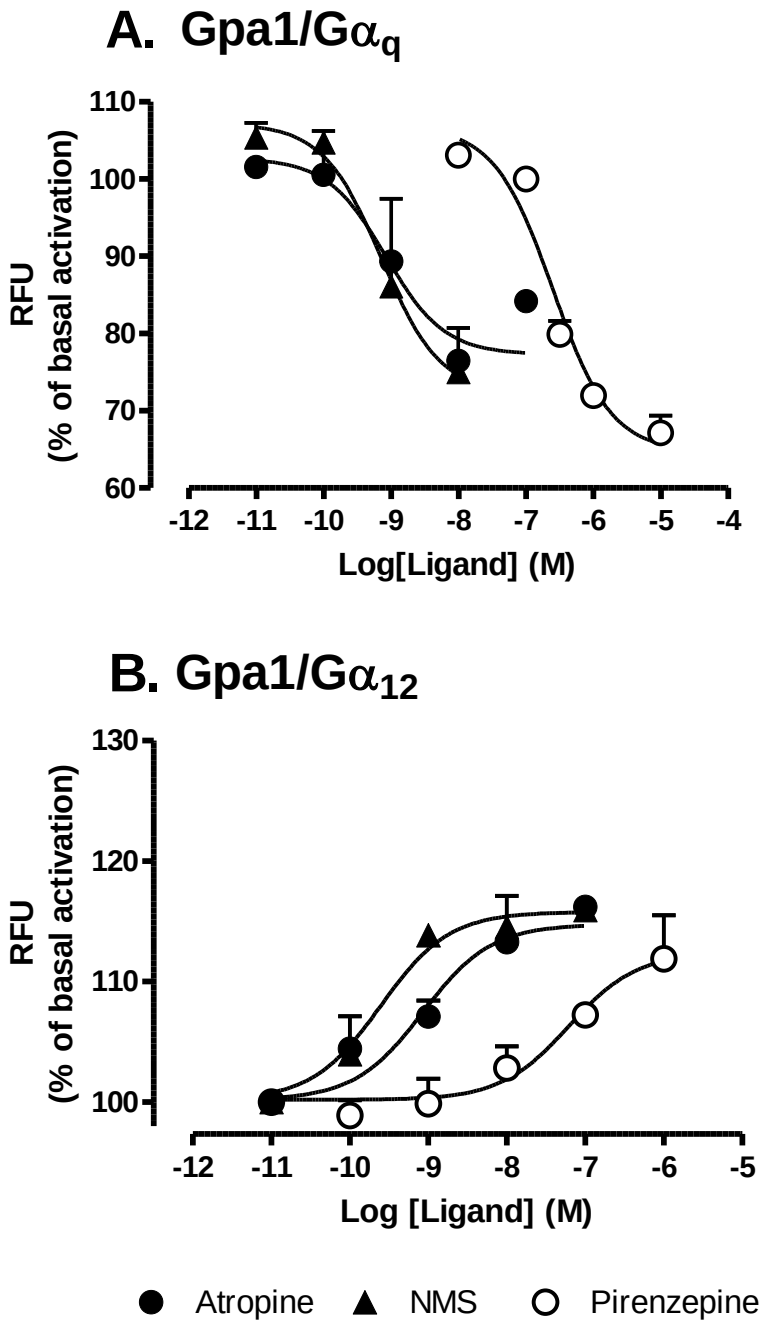

Figure 3.4 Divergent efficacies displayed by prototypical mAChR antagonists in different yeast strains. Atropine, NMS and pirenzepine (solid lines) concentration-response curves in yeast strains expressing, **A.** Gpa1/Gα_q or **B.** Gpa1/Gα_{12}. Data is expressed as mean percentage of basal activation ± S.E.M. collected from three experiments performed in duplicate. Error bars not shown lie within the dimensions of the symbol.

3.3.2 Validation of atropine pharmacology in mammalian cells expressing the human M_3 mAChR

To validate the ability of the yeast system to predict ligand pharmacology in mammalian cells, experiments were performed in 3T3 cells expressing the human M_3 mAChR (3T3 M_3 mAChR cells), using intracellular Ca^{2+} mobilisation and cytoskeletal rearrangement as surrogate assays for $G\alpha_q$ activation and $G\alpha_{12}$ activation, respectively. Atropine was used as a representative ligand for these experiments.

Interaction studies between CCh and atropine were first performed using intracellular Ca^{2+} mobilisation in 3T3 M_3 mAChR cells as a functional output (Figure 3.5A). Atropine pretreatment resulted in a parallel, rightward shift of the CCh concentration-response curve, in a concentration-dependent manner. A potency estimate of atropine was derived by applying equation 3.2 to pEC_{80} values collected from these data, yielding a $pA_2 = 9.15 \pm 0.35$ (n = 6). This value was not statistically different to atropine's affinity value derived in yeast expressing Gpa1/$G\alpha_q$ (p>0.05).

To investigate the coupling of the human M_3 mAChR to G_{12} proteins, assays were performed to determine CCh-induced effects on the membrane ruffling response in 3T3 M_3 mAChR cells (Pertz et al., 2006; Meller et al., 2008); Figure 3.6 shows representative images of membrane ruffling in 3T3 M_3 mAChR cells. A time-course for serum-free DMEM-, CCh- and atropine-mediated membrane ruffling was constructed to determine the time-point of the peak response (Figure 3.6C). CCh-induced membrane ruffling concentration-response curves were then constructed in the absence and presence of atropine in 3T3 M_3 mAChR cells (Figure 3.7A). Because atropine pretreatment resulted in a dextral shift of the CCh curves with a depression in the maximal response, equation 2 was then applied using equi-effective pEC_{10} values to furnish a pA_2 value of 9.65 ± 0.23 (n = 7).

From these Schild analyses, it was apparent that atropine displayed activity that was independent of CCh-mediated receptor activation. In Ca^{2+} mobilisation assays, atropine was an inverse agonist, with a pIC_{50} of 7.98 ± 0.54, (n = 6) (Figure 3.5B). In agreement with the atropine concentration-response curve generated in yeast strains expressing Gpa1/$G\alpha_{12}$, atropine was a low efficacy agonist of the membrane ruffling response in 3T3 M_3 mAChR cells, with a modest amplitude of response and a pEC_{50} of 8.16 ± 0.40 (n = 7) (Figure 3.7B).

Interestingly, the potency values of atropine derived from Gpa1/$G\alpha_q$ and Gpa1/$G\alpha_{12}$ yeast assays, were not consistent with atropine potency values derived in Ca^{2+} mobilisation and membrane ruffling assays. Generally, however, the potency of a low efficacy agonist or inverse agonist should be a good estimation of the agonist's affinity, since as efficacy tends toward zero, the potency tends toward agonist affinity (Kenakin, 2003b). For the G_q-mediated assay CHO cells, the apparent dextral shift in atropine's potency, compared with the Gpa1/$G\alpha_q$ yeast assay, can be ascribed to a large increase in constitutive activity; whereby a higher concentration of atropine is required to surmount said activity, thus reducing its potency (Kenakin, 2003b). However, this does not account for the dextral shift seen in G_{12}-mediated assays in CHO cells. Therefore, this discrepant finding suggests that atropine is binding with a lower affinity in membrane ruffling assays than in Gpa1/$G\alpha_{12}$ yeast assays. Two possible reasons for the discrepancy are differences in receptor concentration between the yeast and mammalian systems or the receptor is restricted to a cellular compartment in a potentially biased conformation. To investigate the latter, CCh and atropine concentration-response curves were constructed in 3T3 M_3 mAChR cells pre- and post-disruption of lipid microdomains through cholesterol sequestration by filipin III (Figure 3.8). These results showed that cholesterol sequestration caused a sinistral shift in the potency of

atropine in membrane ruffling assays from 8.16 ± 0.40 (absence of filipin III) to 9.20 ± 0.54 (presence of filipin III; curves were found to be statistically different, F-test $p<0.05$), whilst having no effect on the CCh concentration-response curve.

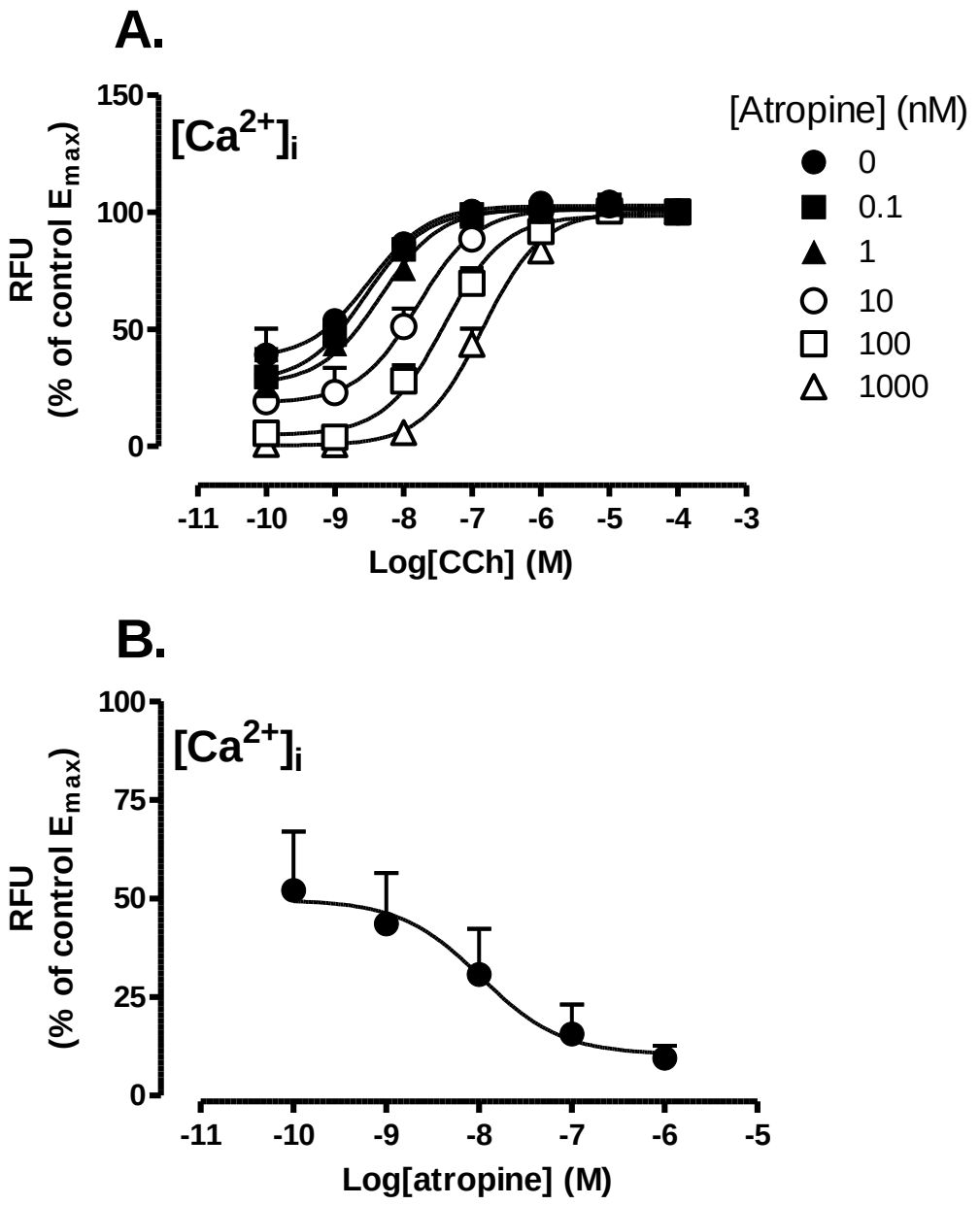

Figure 3.5 The effect of atropine on Ca2+ mobilisation in 3T3 M3 mAChR cells. **A.** Intracellular Ca^{2+} mobilisation concentration-response curves to CCh, performed in 3T3 M_3 cells, in the absence and presence of atropine pretreatment (30 mins). **B.** Intracellular Ca^{2+} signalling concentration-response curve to atropine alone in 3T3 M_3 mAChR cells. Data is represented as mean percentage of E_{max} in the absence of atropine ± S.E.M. collected from six experiments performed in duplicate. Error bars not shown lie within the dimensions of the symbol.

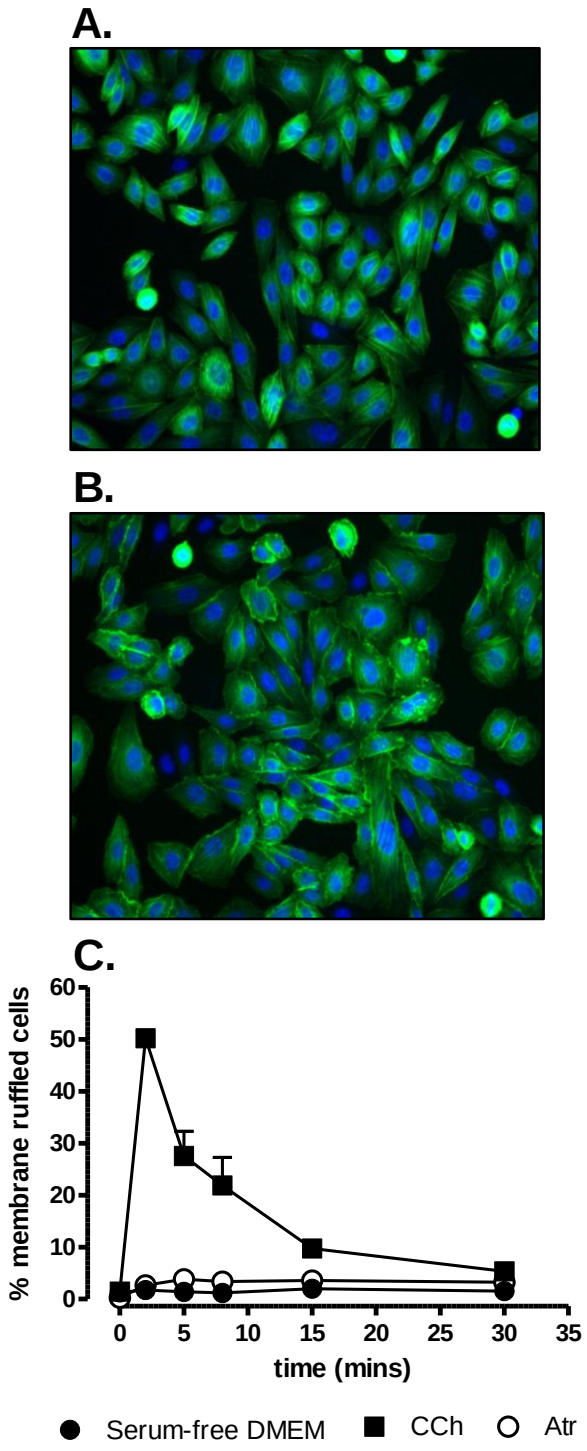

Figure 3.6 CCh induces membrane ruffling in 3T3 M3 mAChR cells. 3T3 M_3 mAChR cells treated for 2 min with **A.** serum-free media or **B.** 100μM CCh, fixed, stained with Hoechst 33342 nuclear dye (blue) and Alexa-568 phalloidin (green) and imaged using a 20X objective on an IN Cell 1000 analyzer**. C.** Time-course of serum-free DMEM-, CCh- and atropine-induced membrane ruffling responses in 3T3 M_3 mAChR cells. Data are expressed as the mean percentage of cells that exhibit membrane ruffling ± S.E.M. obtained from three experiments performed in duplicate. Error bars not shown lie within the dimensions of the symbol.

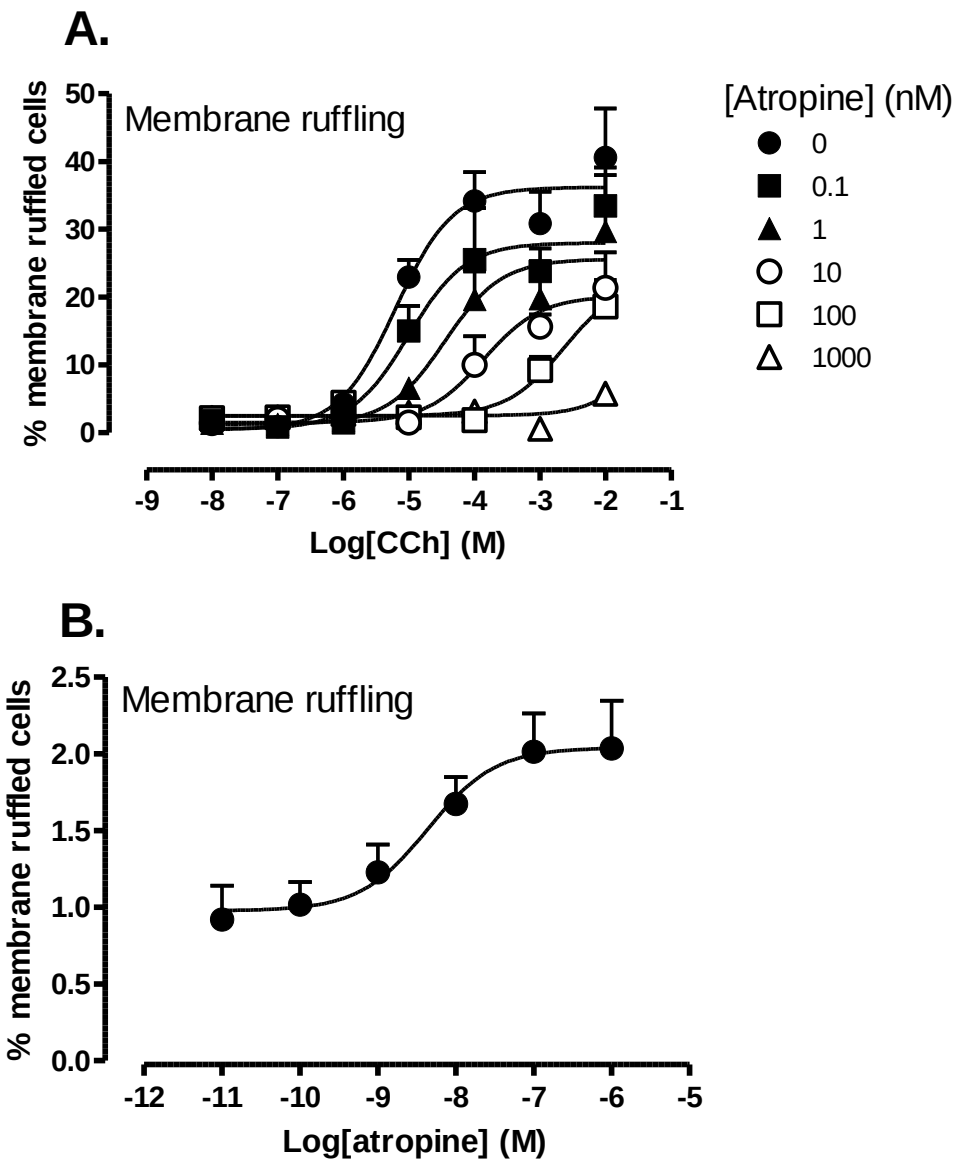

Figure 3.7 Effect of atropine on membrane ruffling response in 3T3 M3 mAChR cells. **A.** Membrane ruffling concentration-response curves to CCh, performed in 3T3 M_3 mAChR cells, in the absence and presence of atropine pretreatment (15 mins). **B.** Membrane ruffling concentration-response curve to atropine alone in 3T3 M_3 mAChR cells. Data are represented as mean percentage of cells exhibiting membrane ruffling in the absence of ligand ± S.E.M. collected from seven experiments performed in duplicate. Error bars not shown lie within the dimensions of the symbol.

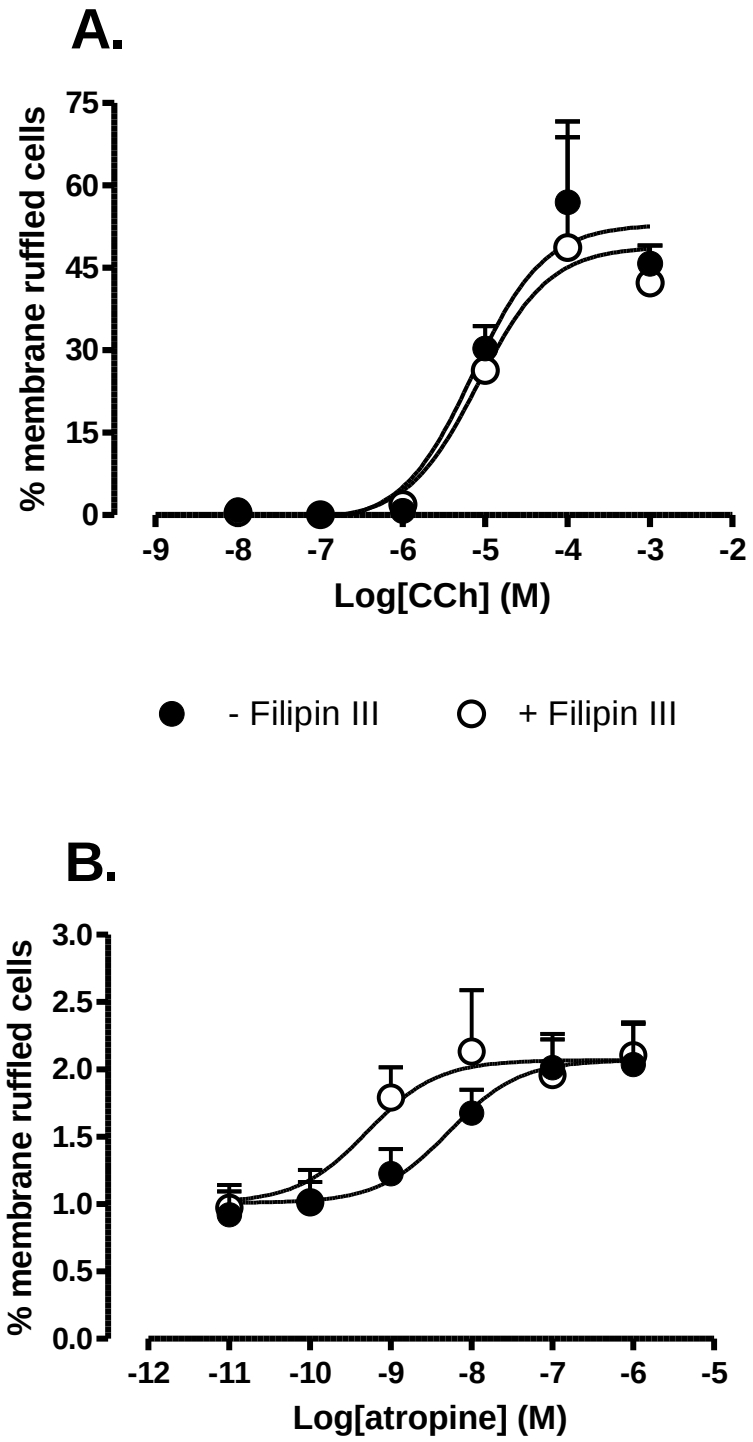

Figure 3.8 Influence of cholesterol sequestration on M3 mAChR-mediated membrane ruffling. CCh- and Atropine-induced membrane ruffling concentration-response curves performed 3T3 M_3 cells in the absence (●) and presence (○) of Filipin III (1µg mL^{-1}). Data are expressed as mean percentage of cells exhibiting membrane ruffling in the absence of ligand ± S.E.M. obtained from three to thirteen experiments performed in duplicate. Error bars not shown lie within the dimensions of the symbol.

3.4 Discussion

This study has identified a hitherto unappreciated signalling bias for ligands classed as prototypical orthosteric muscarinic antagonists, and a highlight potential role for cellular microdomains in engendering functional selectivity through biasing receptor conformations. In addition, these studies further illustrate the utility of the yeast assay in the investigation of functional selectivity across G protein subtypes.

The pleiotropic nature of GPCR coupling presents an often difficult problem in ligand screening, given that a single signalling output may be attributed to multiple G protein subtypes. One of the benefits of the yeast system in this regard is that it can determine specific G protein-coupling in the absence of multiple G protein subtypes. Application of the yeast assay to the G protein-coupling profile of CCh suggested that the $rM_3\Delta i3$ mAChR could couple to G_q, $G_{i1/2}$ and G_{12}, which is consistent with G proteins that have previously been shown to couple to the M_3 mAChR in mammalian cells (Wylie et al., 1999; Rumenapp et al., 2001; Brown et al., 2005; Clark et al., 2007).

In addition to generating a G protein-coupling profile, this study has shown that it is possible to generate antagonist affinity estimates by applying classical Schild analysis to the interaction between CCh and atropine in strains expressing Gpa1/$G\alpha_q$, Gpa1/$G\alpha_{i1/2}$ and Gpa1/$G\alpha_{12}$. The antagonist affinity values yielded from these analyses were not statistically different to those generated from the equivalent experiments performed in 3T3 M_3mAChR cells in Ca^{2+} mobiliation and membrane ruffling assays. Moreover, these estimates are consistent with affinity values derived for atropine at the M_3 mAChR previously (Peralta et al., 1987; Buckley et al., 1989; Smith and Wallis, 1997; Dowling and Charlton, 2006). However, atropine caused a depression of the E_{max} of the CCh concentration-response curve in membrane ruffling assays in 3T3 M_3 mAChR

cells. This result gives the appearance that atropine is not binding in a competitive mode, although, this likely to be a hemi-equilibrium state caused by the slow dissociation rate of atropine and the short time-point of the measured response, resulting in an apparently insurmountable antagonism (Christopoulos et al., 1999).

Atropine, NMS and pirenzepine displayed inverse agonism in yeast strains expressing Gpa1/$G\alpha_q$, as did atropine in Ca^{2+} mobilisation assays in 3T3 M_3 mAChR cells, which is not necessarily a surprising result given that it is well documented that many muscarinic antagonists are, in fact, inverse agonists at the M_3 mAChR (Burstein et al., 1997; Dowling et al., 2006; Thor et al., 2008). However, the pIC_{50} of atropine in Ca^{2+} mobilisation assays in 3T3 M_3 mAChR cells did not approximate the affinity of atropine, in contrast to the pIC_{50} from the atropine concentration-response curve obtained from yeast expressing Gpa1/$G\alpha_q$. This is indicative of a system that exhibits high levels of constitutive activity, hence higher concentrations of inverse agonist are required to surmount this activity, leading to an apparent reduction in inverse agonist potency (Kenakin, 2003b).

Perhaps the most interesting and novel observation from these studies is that atropine, NMS and pirenzepine displayed weak, but detectable agonism when coupled to Gpa1/$G\alpha_{12}$ proteins, in addition to atropine in an assay that is predominately mediated by G_{12} activation (membrane ruffling). In yeast, atropine's potency in the Gpa1/$G\alpha_{12}$ strain approximated the affinity value derived from interaction studies between CCh and atropine, which is expected of a low efficacy agonist since there is no receptor reserve. A similar result was seen for NMS and pirenzepine agonistic potencies in Gpa1/$G\alpha_{12}$ yeast strains when compared to affinity estimates derived in previous studies (Doods et al., 1987; Fisher and Heacock, 1988; Sharif et al., 1995). However, in 3T3 M_3 mAChR cells the potency of atropine as a low efficacy agonist did not approximate its affinity,

and its potency was only aligned with its affinity after lipid-microdomain disruption by cholesterol sequestration. This suggests that the M_3 mAChR is conformationally altered depending on whether it is compartmentalised or not. Indeed, this is consistent with previous studies that have shown the M_3 mAChR exists in lipid compartments in airway smooth muscle cells and detrusor muscle, where disruption of caveolae altered M_3 mAChR function (Lai et al., 2004; Gosens et al., 2007b). Furthermore, it has been shown for other GPCRs that functional selectivity may arise from their differential compartmentalisation with various effectors, such as G_s proteins and adenylate cyclases, or the absence and presence of the $G\alpha_s$ protein's cognate $\beta\gamma$ subunits (Ostrom et al., 2000; Azzi et al., 2001). Together, these studies suggest that functional selectivity may not only be a property of the ligand-receptor complex, but may also be engendered by cell or tissue background.

Functional selectivity has become recognised as a common feature of numerous β-adrenoceptor antagonists, with many clinically used β-adrenoceptor antagonists, such propranolol and carvedilol, exhibiting signalling bias (Baker et al., 2003; Sato et al., 2007; Galandrin et al., 2008). However, in these cases, the signalling bias is between G_s proteins and non-G protein, mostly β-arrestin, signalling. Other examples of functional selectivity between G proteins and β-arrestin exist for the parathyroid hormone (PTH) receptor, where the ligand PTH1-34 activates G_s and β-arrestin, but PTH7-34 exclusively engages β-arrestin (Gesty-Palmer et al., 2006); and for the angiotensin AT_1 receptor, where the agonist, angiotensin II, promotes coupling to G_q and β-arrestin, whereas the angiotensin II analog, $Sar^1Ile^4Ile^4$ angiotensin II solely recruits β-arrestins to signal to mitogen-activated protein kinases (Wei et al., 2003). In addition to this, there is evidence to suggest that some thromoboxane A_2 receptor ligands can preferentially activate G_q over G_{12} signalling, and vice versa (Zhang et al., 2009).

However, to our knowledge, this is the first study to report a muscarinic orthosteric ligand that displays G protein-based protean agonism.

In the context of our results, the inverse agonist properties of atropine at G_q are well-documented (as previously mentioned), but low efficacy agonism at G_{12} is a novel finding from this study. Consequences of signalling through $G\alpha_{12}$ are generally poorly defined compared to other Gα protein subtypes (Worzfeld et al., 2008), however there is evidence that its effectors include Rho guanine nucleotide exchange factors (RhoGEFs) and therefore Rho guanine triphosphatases (RhoGTPases), and RacGTPases (Yuan et al., 2006; Suzuki et al., 2009). The most commonly studied RhoGTPase, RhoA, has two main effectors, ROCK and mDia1 that, together with Rac, play distinct roles in manipulation of the actin cytoskeleton (Fukata et al., 2001; Kurokawa et al., 2005; Pertz et al., 2006). The effects of actin modulation are widespread, from cell morphology changes to alterations in gene transcription by nuclear actin (Miralles and Visa, 2006). Additionally, among the effects of Rho activation is also the upregulation of inflammatory cytokines such as interleukins and chemotactic factors, where inhibition of ROCK has been shown to decrease levels of IL-5, IL-13 and eotaxin in murine airways (Shimamura et al., 2008), interferon γ, IL-2, IL-3 and IL-5 in human asthmatic bronchial lavage fluid (Aihara et al., 2004), and IL-6 and tumor-necrosis factor α in C6 glioma cells (Yamaguchi et al., 2009). Furthermore, ACh has been shown to activate alveolar macrophages via M_3 mAChRs, which results in the release of chemotactic factors (Sato et al., 1998). Interestingly, however, there is evidence to suggest that anti-muscarinics can also cause an increase in inflammatory mediators, where atropine was able to elevate IL-10 levels in mice with lipopolysaccharide-induced inflammation (Fuentes et al., 2008), and enhance major basic protein deposition by eosinophils that was associated with an elevation of IL-5, in airways of antigen-challenged guinea pigs

(Verbout et al., 2007). Moreover, the muscarinic receptor antagonist, tiotropium, which is used to treat chronic obstructive pulmonary disease (COPD), has been shown to cause an increase in IL-6, IL-8 and myeloperoxidase to varying extents, in sputum and serum of patients with COPD (Powrie et al., 2007). Additionally, there has been a reported case of the inflammatory disease, interstitial granulomatous dermatitis, appearing concomitantly with the commencement of darifenacin, an M_3 mAChR-selective antagonist used for the treatment of urinary incontinence, and ceasing at the time of termination of the regime (Mason et al., 2008). Collectively, these studies emphasise how little is actually known about the collateral effects of some therapeutics and how chronic, co-incident activation of GPCR signalling may influence the clinical outcome.

In conclusion, this study has found that atropine displays agonist properties for $G\alpha_{12}$ (and possibly Rho and Rac signalling), a property that has been hitherto unappreciated. Furthermore, the data show that the yeast system is a robust platform to investigate properties of ligands that may remain misunderstood in many conventional systems.

CHAPTER 4

DETECTION OF FUNCTIONALLY SELECTIVE ALLOSTERIC INTERACTIONS AT THE M_3 MUSCARINIC ACETYLCHOLINE RECEPTOR USING *SACCHAROMYCES CEREVISIAE*

4.1 Introduction

The M_3 mAChR is a tractable drug target for conditions such as chronic obstructive pulmonary disease (COPD) and overactive bladder disorder (Wess et al., 2007). However, subtype-selective targeting of mAChRs is often difficult due to their high degree of sequence conservation in the orthosteric binding site (Caulfield, 1993; Gregory et al., 2007). One approach to circumvent this issue is to target an allosteric site on these receptors (Birdsall and Lazareno, 2005).

Although it has been shown that mAChRs possess multiple allosteric ligand binding sites, most studies to date have focused on the so-called 'prototypical' allosteric binding site, which binds neuromuscular-blocking agents, such as gallamine and alcuronium, alkane-*bis*-ammonium compounds, including C_7/3-phth and W84, and alkaloid derivatives, such as brucine (Christopoulos et al., 1998; Gregory et al., 2007).

The prototypical mAChR allosteric site is thought to encompass regions of the 2nd and 3rd extracellular loops, and the top of TMVII (Gnagey et al., 1999; Voigtlander et al., 2003; Avlani et al., 2007; Gregory et al., 2007; May et al., 2007a). Specifically, the amino acid residue at the 7.32 position (Ballesteros-Weinstein (1992) nomenclature) at the junction of TMVII and the extracellular 3rd loop, has been shown to play an important role in the binding and cooperativity of prototypical allosteric ligands (Gnagey et al., 1999; Krejci and Tucek, 2001; Jakubik et al., 2005). At the M_1 mAChR, 7.32 is a glutamic acid, whereas at the M_2 mAChR it is an asparagine, at the M_3 mAChR it is a lysine, at the M_4 mAChR it is an aspartic acid and at the M_5 mAChR it is a valine. It has been proposed that key residues that promote selectivity of prototypical allosteric ligands are in the juxtamembrane region of TMVII (Jakubik et al., 2005). The allosteric ligand, brucine, displays highest affinity for the M_1 mAChR and lowest

affinity at the M_3 and M_5 mAChR subtypes, in addition to displaying positive cooperativity with ACh at the M_1 mAChR, and negative cooperativity at all other subtypes (Lazareno et al., 1998). Indeed, recent studies performed by Iarriccio (2008) provide specific evidence that the substitution of $K^{7.32}$ on the M_3 mAChR with the M_1 mAChR equivalent, $E^{7.32}$, enhances the cooperativity between brucine and ACh in binding and functional studies, compared to the wild-type M_3 mAChR where brucine displayed almost neutral cooperativity. Iarriccio (2008) gave evidence that the interaction of brucine with ACh could increase the potency of ACh up to 10-fold in phosphoinositide hydrolysis assays at the $M_3K^{7.32}E$ mAChR.

Given the fact the $K^{7.32}E$ mutation can engender positive cooperativity between ACh and brucine at the M_3 mAChR, the current study employed the use of the yeast assay in conjunction with brucine at the $M_3K^{7.32}E$ mAChR mutant, as a proof-of-concept approach that the yeast system is capable of detecting allosteric modulation, in addition to investigating G protein functional selectivity of brucine. The pairing of the yeast system with the allosteric potentiation of brucine at the $M_3K^{7.32}E$ mAChR revealed that brucine was a functionally selective allosteric potentiator at this receptor and displayed allosteric agonism when coupled to G_q-mediated pathways. Additionally, the functional selectivity displayed by brucine allowed a G protein 'fingerprint' to be generated, which was then able to ascribe potential G protein candidates for $M_3K^{7.32}E$ mAChR-mediated ERK1/2 signalling in a mammalian cell background.

Together, these results suggest yeast signalling assays may be a practicable platform for predicting pharmacology of allosteric ligands at different G protein subtypes, and as a pharmacological tool with selective ligands to help unravel the complex networks of convergent signalling.

4.2 Methods

4.2.1 Materials

The *Surefire*™ ERK1/2 phosphorylation kit was kindly donated by Dr Michael Crouch (TGR Biosciences, SA, Aust.). The p416GPD $rM_3\Delta i3$ mAChR was a generous gift from Dr Jürgen Wess (NIH, Bethesda, MD). [^{3}H] N-methyl scopolamine, ([^{3}H]-NMS), was purchased from Perkin Elmer, (Boston, MA). Flp-In™ Chinese hamster ovary (CHO) cells were obtained from Invitrogen (Carlsbad, CA). Brucine was purchased from Sigma Aldrich (St Louis, MO). All other reagents were obtained as described in sections 2.2.1 and 3.2.1.

4.2.2 Yeast transformations and signalling assay

Saccharomyces cerevisiae strains were transformed as described in section 3.2.2. The $K^{7.32}E$ mutation was introduced by site-directed mutagenesis using the QuikChange mutagenesis kit (Stratagene) by annealing the following oligo-deoxynucleotide pair into the open vector: 5' GACAGCTGCATACCCGAAACCTATTGGAATC 3' and 5' GATTCCAATAGGTTTCGGGTATGCAGCTGTC 3', the vector containing the mutated gene was then transformed in the same manner as described in section 3.2.2.

The conditions for the signalling component of the assay are as described in section 2.2.3.

4.2.3 Transfections and cell culture

The cDNA sequence of the human M_3 mAChR was amplified by PCR and cloned, using classical cloning methods, into the Gateway entry vector, pDONR201, using the

BP clonase kit according to manufacturer's instructions. The M_3 mAChR construct was subsequently transferred in the Gateway destination vector, pEF5/FRT/V5-dest, using the LR clonase kit in accordance with manufacturer's instructions. The construct was then transfected into Flp-In™ CHO cells using methods described previously (Nawaratne et al., 2008). The same processes were applied to generate a vector containing the gene encoding the human $M_3K^{7.32}E$ mAChR, however prior to the BP clonase reaction, a mutation was introduced using the QuikChange mutagenesis kit (Stratagene) by applying the following oligonucleotides: 5' GTGACAGCTGCATACCCGAGACCTTTTGGAATCTGG 3' and 5' CCAGATTCCAAAAGGTCTCGGGTATGCAGCTGTCAC 3' to the open vector, then following the manufacturer's instructions. Flp-In CHO cells stably expressing the M_3 mAChR (CHO M_3 mAChR cells) or the $M_3K^{7.32}E$ mAChR ($M_3K^{7.32}E$ mAChR), were cultured at 37°C in 5% CO_2 in DMEM supplemented with 5% (v/v) FBS, 16mM HEPES and were selected using 400μg mL^{-1} hygromycin B, but maintained using 200μg mL^{-1} hygromycin B.

4.2.4 Membrane preparation

CHO M_3 mAChR or $M_3K^{7.32}E$ mAChR cells were grown to 90% confluence, harvested and centrifuged at 300 × g for 3 min. The intact cell pellet was suspended in homogenization buffer (20mM HEPES; 10mM EDTA; 0.1mg mL^{-1} saponin, pH 7.7) and further centrifuged (300 × *g*, 3 min). Cells were then resuspended in homogenisation buffer and homogenised using a Polytron PT1200 homogeniser for two 10 sec intervals at maximum setting (6), with 30 sec cooling periods on ice between each burst. The homogenate was then centrifuged (40,000 × *g*, 1 hr, 4°C). The resulting pellet was resuspended in 5 ml of HEPES buffer (100mM NaCl; 20mM HEPES; 10mM

$MgCl_2$, pH 7.4), and the protein content determined using a BCA assay kit (Pierce Biotechnology) according to the manufacturer's instructions, using bovine serum albumin as a standard. The homogenate was then divided into 1 ml aliquots and either used immediately or stored frozen at −80°C until required.

4.2.5 Radioligand binding assays

Saturation and interaction binding assays were performed using 15µg of membrane expressing the M_3 mAChR or $M_3K^{7.32}E$ mAChR receptors. For saturation binding assays, membranes were incubated with the orthosteric antagonist [^{3}H]NMS in HEPES buffer (20mM HEPES, 100mM NaCl, and 10mM MgCl, pH 7.4) at 37°C for 1 hr before termination of the assay by rapid filtration onto GF/B grade filter paper (Whatman, Maidstone, UK) using a Brandel harvester, followed by three 2 mL washes with ice-cold NaCl (0.9%). Nonspecific binding was defined in the presence of 10 µM atropine and radioactivity was determined by liquid scintillation counting. For interaction binding studies, membranes were incubated in HEPES buffer containing 100 µM GppNHp with increasing concentrations of CCh in the absence or presence of brucine (3, 10 or 30µM) and [^{3}H]NMS at a concentration equal to its equilibrium dissociation constant for each receptor (approximately 0.7nM for both receptors) as determined from saturation binding experiments. Determination of non-specific binding and termination of the experiment were as described above.

4.2.6 Ca^{2+} mobilisation assay

CHO M_3 or $M_3K^{7.32}E$ mAChR cells were cultured overnight in 96-well plates at 37°C in 5% CO_2. Cells were washed twice in Ca^{2+} assay buffer (See section 3.2.4 for buffer

composition), then buffer was replaced with Ca^{2+} assay buffer containing 1μM Fluo-4-AM and incubated for 1 hr at 37°C in 5% CO_2. Cells were washed twice more and replaced with 37°C Ca^{2+} assay buffer. Whilst fluorescence was measured, brucine was added for 1 min, carbachol was subsequently added and the response was measured further for 3 min in a Flexstation™ (Molecular Devices) using 485 excitation and 520 emission wavelengths.

4.2.7 Cytoskeletal rearrangement assay and image analysis

CHO M_3 and $M_3K^{7.32}E$ mAChR cells were cultured overnight in 96-well plates at 37°C in 5% CO_2. Samples were serum-starved 4 hr prior to assaying then treated with ligand at appropriate time points (CCh: 2 min, Brucine: 15 min, determined by separate time-course assays). Time-course assays were performed by treating cells with serum-free DMEM, 1mM CCh, 100μM brucine or a combination of 1mM CCh and 100μM brucine for 2, 5, 8, 15 and 30 min. Fixing, staining and analysis of samples were performed as described in section 3.2.5.

4.2.8 Extracellular signal-regulated kinase 1/2 phosphorylation assays

Initial ERK1/2 phosphorylation time-course experiments were performed to determine the time at which ERK1/2 phosphorylation was maximal after stimulation by each agonist at a single concentration, in addition to co-administration of brucine (10μM) with CCh (1μM). Cells were seeded into transparent 96-well plates at 5×10^4 cells per well and grown overnight. Cells were then washed twice with phosphate-buffered saline (PBS) and incubated in serum-free DMEM at 37°C for at least 4 hr. For interaction studies between CCh and brucine, cells were then stimulated with brucine for 1min

prior to CCh stimulation for 5 min and incubated at 37°C in 5% CO_2. For experiments where PTX pretreatment was required, cells were treated with 100ng mL^{-1} PTX in serum-free DMEM overnight. For all experiments, 10% (v/v) FBS was used as a positive control, and vehicle controls were also performed. The reaction was terminated and samples were processed as per section 2.2.6. Data were normalised to the maximal response elicited by 10% (v/v) FBS at the same time point.

4.2.9 Data analysis

All data were analysed using Prism 5.02 (GraphPad Software, San Diego, CA). For radioligand saturation binding data, nonspecific and total binding data were fitted to the following equation.

$$Y = \frac{B_{max} \cdot [\mathrm{A}]}{[\mathrm{A}] + K_{\mathrm{A}}} + \mathrm{NS} \cdot [\mathrm{A}] \quad (4.1)$$

where Y is radioligand binding, B_{max} is the total receptor density, [A] is the radioligand concentration, K_A is the equilibrium dissociation constant of the radioligand, and NS is the fraction of nonspecific radioligand binding.

For interaction binding experiments, the shifts of the competitor versus [^{3}H]NMS competition binding curves obtained in the absence or presence of brucine were fitted to the following allosteric binding model (Christopoulos, 2000):

$$Y = \frac{B_{max} \cdot [\mathrm{A}]}{[\mathrm{A}] + K'_{\mathrm{App}}} \quad (4.2)$$

$$\text{where } K'\mathrm{App} = \frac{K_{\mathrm{A}} \cdot K_{\mathrm{B}}}{\alpha \cdot [\mathrm{B}] + K_{\mathrm{B}}} \cdot \left[1 + \frac{[\mathrm{I}]}{K_{\mathrm{I}}} + \frac{[\mathrm{B}]}{K_{\mathrm{B}}} + \frac{\alpha' \cdot [\mathrm{I}] \cdot [\mathrm{B}]}{K_{\mathrm{I}} \cdot K_{\mathrm{B}}}\right] \quad (4.3)$$

and [I] denotes the concentration of (orthosteric) competitor, [B] denotes the concentration of modulator, K_A, K_B and K_I denote the equilibrium dissociation constants of the radioligand, modulator and competitor, respectively, α denotes the radioligand binding cooperativity factor, which is a measure of the magnitude and direction of the allosteric effect the modulator exerts on the affinity of radioligand and α' defines the binding cooperativity factor between the allosteric ligand and orthosteric competitor. Values of α or α' > 1 denote positive cooperativity, values < 1 (but greater than 0) denote negative cooperativity, values = 1 denote neutral cooperativity, and values approaching zero denote inhibition that is indistinguishable from competitive (orthosteric) antagonism.

Concentration-response data generated from functional assays were fitted to the three-parameter logistic equation 2.1.

Data from functional experiments measuring the interaction between CCh and brucine at the M_3 and $M_3K^{7.32}E$ mAChR were also fitted to the following operational model for the interaction between an agonist and allosteric modulator (Aurelio et al., 2009):

$$E = \text{Basal} + \frac{(E_m - \text{basal}) \cdot ([A] \cdot (K_B + \alpha \cdot \beta \cdot [B]) + \tau_B \cdot [B] \cdot EC_{50})}{([A] \cdot (K_B + \alpha \cdot \beta \cdot [B]) + \tau_B \cdot [B] \cdot EC_{50}) + (EC_{50} \cdot (K_B + [B]))} \qquad (4.4)$$

where E denotes the effect, A denotes the agonist, B denotes the allosteric modulator, α•β denotes a composite cooperativity factor that quantifies the change in affinity and stimulus imparted to the receptor by the agonist as a result of the presence of allosteric modulator, E_m denotes the maximal response of the system, τ_B is the relative efficacy of the allosteric modulator, [A] and K_B are as defined for equation 4.3.

All parametric measures of potency, affinity, operational efficacy, and cooperativity were estimated as logarithms (Christopoulos, 1998). Statistical comparisons between parameters were performed using a Student's *t*-test or one-way analysis of variance (ANOVA) with a Bonferroni's multiple comparison post-test, as appropriate, and $p < 0.05$ taken as indicating significance.

4.3 Results

4.3.1 Effect of brucine on carbachol signalling at the $rM_3\Delta i3$ and $rM_3\Delta i3K^{7.32}E$ mACh receptor in yeast

To ascertain whether allosteric modulation could be detected in yeast signalling assays, interaction studies were performed between CCh and brucine at the $rM_3\Delta i3$ and $rM_3\Delta i3K^{7.32}E$ mAChR in yeast strains expressing Gpa1/$G\alpha_q$, Gpa1/$G\alpha_{12}$ or Gpa1/$G\alpha_{i1/2}$ (Figure 4.1). In all yeast strains expressing the $rM_3\Delta i3$ mAChR, brucine had no effect on the carbachol concentration-response curve. However, in strains expressing the $rM_3\Delta i3K^{7.32}E$ mAChR, brucine exhibited differential effects on carbachol signalling in a G protein-dependent manner. Specifically, brucine displayed agonism in addition to modest potentiation of the carbachol response when coupled to Gpa1/$G\alpha_q$, whereas it displayed no agonism but a robust sinistral shift of the carbachol concentration-response curve when coupled to Gpa1/$G\alpha_{12}$. When the $rM_3\Delta i3K^{7.32}E$ mAChR was coupled to Gpa1/$G\alpha_{i1/2}$ brucine had no effect on carbachol responsiveness, nor did it display agonism. Additionally, from the data in yeast expressing $rM_3\Delta i3K^{7.32}E$ mAChR and Gpa/$G\alpha_q$ or Gpa/$G\alpha_{12}$, brucine affinity (Log K_B), efficacy (Log τ_B) and cooperativity (Log $\alpha\beta$) estimates were derived using equation 4.4 (Table 4.1). Taken together, these data suggest that brucine is able to engender functional selectivity of G protein coupling at the CCh-occupied $rM_3\Delta i3K^{7.32}E$ mAChR.

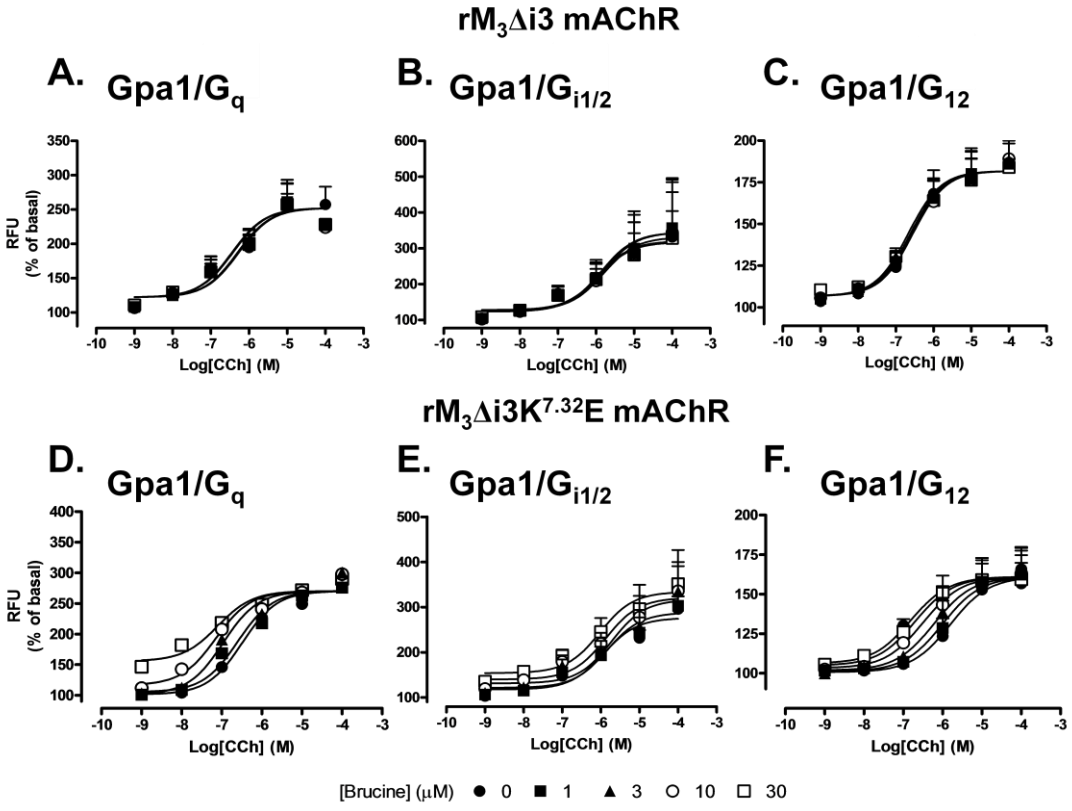

Figure 4.1 Effects of brucine on CCh concentration-response curves in yeast. CCh concentration-response curves were performed in yeast strains expressing the $rM_3\Delta i3$ mAChR and **A.** Gpa1/$G\alpha_q$, **B.** Gpa1/$G\alpha_{i1/2}$ or **C.** Gpa1/$G\alpha_{12}$, or the $rM_3\Delta i3K^{7.32}E$ mAChR and **D.** Gpa1/$G\alpha_q$, **E.** Gpa1/$G\alpha_{i1/2}$ or **F.** Gpa1/$G\alpha_{12}$, in the absence and presence of brucine. Data points are expressed as mean percentage of the basal activity in the absence of brucine ± S.E.M. obtained from three to five experiments performed in duplicate. Error bars not shown lie within the dimensions of the symbol.

Table 4.1 Operational model parameters for the interaction between CCh and brucine at the rM3Δi3K7.32E mAChR in yeast. Data are expressed as the mean ± S.E.M. of three to five separate experiments performed in duplicate.

	Gpa1/Gα_q	**Gpa1/Gα_{12}**
Log K_B[a]	-4.48 ± 0.13	-4.89 ± 0.13
Log τ_B[b]	0.15 ± 0.07 (τ = 1.4)	-0.79 ± 0.17[**] (τ = 0.16)
Log $\alpha\beta$[c]	0.76 ± 0.17 ($\alpha\beta$ = 5.8)	1.06 ± 0.10 ($\alpha\beta$ = 11.5)

[a] – logarithm of the dissociation constant of the allosteric modulator
[b] – logarithm of the operational efficacy of the allosteric modulator
[c] – logarithm of the cooperativity of the allosteric modulator on the potency of the orthosteric agonist
[**] – $p<0.01$ determined by Student's *t*-test (compared to the same parameter in the Gpa1/Gα_q strain)

4.3.2 Differential effects of brucine on carbachol and [^{3}H]-NMS binding at human M_3 and $M_3K^{7.32}E$ mACh receptors in mammalian cell membranes

Radioligand saturation binding studies were performed on CHO cell membranes expressing either the M_3 or $M_3K^{7.32}E$ mAChR, from which B_{max} and [^{3}H]-NMS affinity values were derived using equation 1 (Table 4.2). Interaction binding studies were also performed between [^{3}H]-NMS, CCh and brucine to determine the effect of brucine on the affinity of the radioligand and the non-radiolabelled competitor (Figure 4.2). From these data brucine affinity and cooperativity values were derived using equation 4.2 (Table 4.3). Affinity values for [^{3}H]-NMS, CCh and brucine derived from binding assays were similar across the M_3 and $M_3K^{7.32}E$ mAChRs, suggesting that the mutation had little effect on the orthosteric or allosteric ligand binding pockets. Interestingly, however, the modulatory effect of brucine on orthosteric ligand binding was markedly altered by the $K^{7.32}E$ mutation, since brucine displayed slight negative cooperativity for [^{3}H]-NMS and CCh binding at the M_3 mAChR, but positive cooperativity at the $M_3K^{7.32}E$ mAChR.

Table 4.2 Saturation binding parameters for [3H]-NMS at the M3 or M3K7.32E mAChR in CHO cell membranes. Data are presented as the mean ± S.E.M. of three separate experiments performed in duplicate.

	CHO M_3 mAChR	**CHO $M_3K^{7.32}E$ mAChR**
Log K_D[a]	-9.14 ± 0.05	-9.20 ± 0.04
B_{max} (fmol mg^{-1} protein)[b]	3425 ± 233	2940 ± 338

[a] – logarithm of the equilibrium dissociation constant of the radiolabel
[b] – total number of binding sites, determined by specific binding of the radioligand

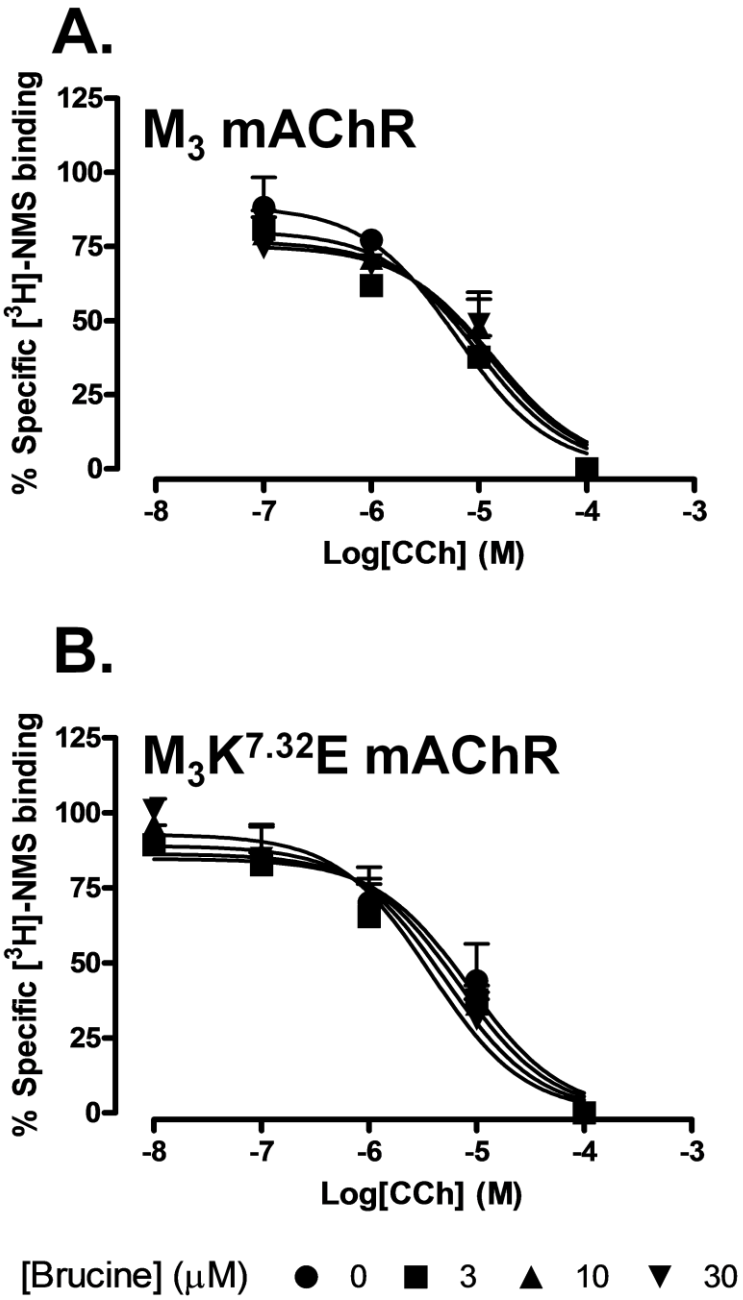

Figure 4.2 The allosteric modulator brucine displays differential cooperativity at the M3 and M3K7.32E mACh receptors. Interaction between [^{3}H]-NMS and carbachol at the **A.** M_3 or **B.** $M_3K^{7.32}E$ mAChR expressed in CHO cell membranes in the absence and presence of brucine. Data points are represented as the mean percentage of specific [^{3}H]-NMS binding in the absence of CCh or brucine ± S.E.M. of three experiments performed in duplicate. Error bars not shown lie within the dimensions of the symbol.

Table 4.3 Allosteric ternary complex model binding parameters for the interaction between [3H]-NMS, CCh and brucine at the M3 and M3K7.32E mAChRs in CHO cell membranes. Data are presented as the mean ± S.E.M. of three separate experiments performed in duplicate. * indicates a statistically significant difference compared to M_3 mAChR, $p<0.05$.

	CHO M_3 mAChR	**CHO $M_3K^{7.32}E$ mAChR**
Log K_B[a]	-5.95 ± 0.37	-5.06 ± 0.15
Log K_I[b]	-5.48 ± 0.03	-5.32 ± 0.04*
Log α[c]	-0.12 ± 0.03 (α = 0.75)	0.10 ± 0.03** (α = 1.25)
Log α'[d]	-0.35 ± 0.26 (α' = 0.45)	0.58 ± 0.04* (α' = 3.80)

[a] – logarithm of the equilibrium dissociation constant of the allosteric modulator
[b] – logarithm of the equilibrium dissociation constant of the orthosteric inhibitor
[c] – logarithm of the cooperativity of the allosteric modulator on the radioligand
[d] – logarithm of the cooperativity of the allosteric modulator on the orthosteric inhibitor
* – $p<0.05$ determined by Student's *t*-test (compared to the same parameter at the CHO M_3 mAChR)
** – $p<0.01$ determined by Student's *t*-test (compared to the same parameter at the CHO M_3 mAChR)

4.3.3 Validation of brucine pharmacology in mammalian cells expressing the human M_3 and $M_3K^{7.32}E$ mACh receptor

Data collected from the yeast assays suggested that brucine had no effect on CCh signalling at the $rM_3\Delta i3$ mAChR, but exhibited agonism in the Gpa/$G\alpha_q$ yeast strain and robust CCh potentiation in the Gpa/$G\alpha_{12}$ strain, at the $rM_3\Delta i3K^{7.32}E$ mAChR. To ascertain whether these data are relevant to a mammalian cell background, experiments were performed in CHO cells expressing either the human M_3 or $M_3K^{7.32}E$ mAChR, using Ca^{2+} mobilisation and membrane ruffling as surrogate assays for G_q and G_{12}, respectively.

Interaction studies were initially performed in CHO cells expressing the M_3 or $M_3K^{7.32}E$ mAChR, with Ca^{2+} mobilisation as a functional endpoint (Figure 4.3). Similarly to what was observed in Gpa1/$G\alpha_q$ yeast strains expressing the $rM_3\Delta i3$ mAChR, brucine had no apparent effect on the CCh concentration-response curve in CHO cells expressing the M_3 mAChR. However, brucine exhibited both agonism and a moderate potentiation of the CCh concentration-response curve in CHO cells expressing the $M_3K^{7.32}E$ mAChR. Application of equation 4.4 to the $M_3K^{7.32}E$ mAChR data yielded the operational model parameter estimates shown in Table 4.4.

Subsequently, time-course studies in CHO M_3 and $M_3K^{7.32}E$ mAChR cells revealed that the CCh-induced membrane ruffling response peaked at 2 min, and that brucine did not alter the time-course profile of CCh nor did brucine exhibit its own agonism in either cell line (Figure 4.4). Carbachol concentration-response curves were then constructed in the absence and presence of brucine in membrane ruffling assays in CHO cells expressing the M_3 or $M_3K^{7.32}E$ mAChR (Figure 4.5). The results were consistent with those found in the yeast signalling assay for Gpa1/$G\alpha_{12}$ coupling, whereby brucine had

no effect on the carbachol concentration-response curves at the $rM_3\Delta i3$ mAChR, but was able to robustly potentiate carbachol-induced signalling at the $rM_3\Delta i3K^{7.32}E$ mAChR, without displaying any allosteric agonism. Table 4.4 shows the Log K_B, Log $\alpha\beta$ and Log τ_B values that were derived by applying equation 4.4 to the $M_3K^{7.32}E$ mAChR data from membrane ruffling assays.

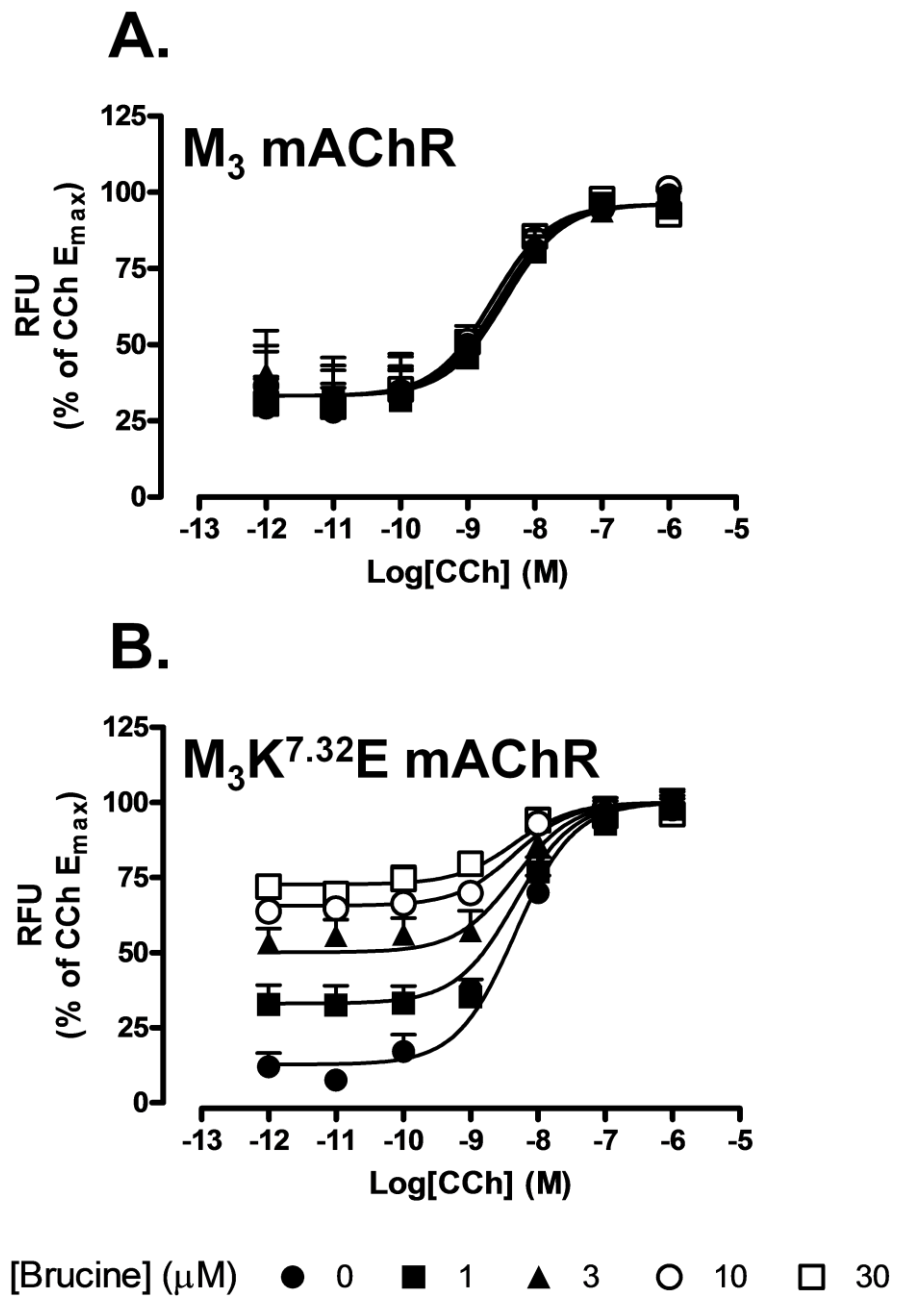

Figure 4.3 Effect of brucine on CCh-induced Ca2+ mobilisation in CHO cells. CCh concentration-response curves, performed in CHO cells expressing the **A.** M_3 the **B.** $M_3K^{7.32}E$ mAChR, in the absence and presence of brucine. Data points are expressed as mean percentage of the maximal CCh-induced Ca^{2+} mobilisation response in the absence of brucine ± S.E.M. obtained from four to six experiments performed in duplicate. Error bars not shown lie within the dimensions of the symbol.

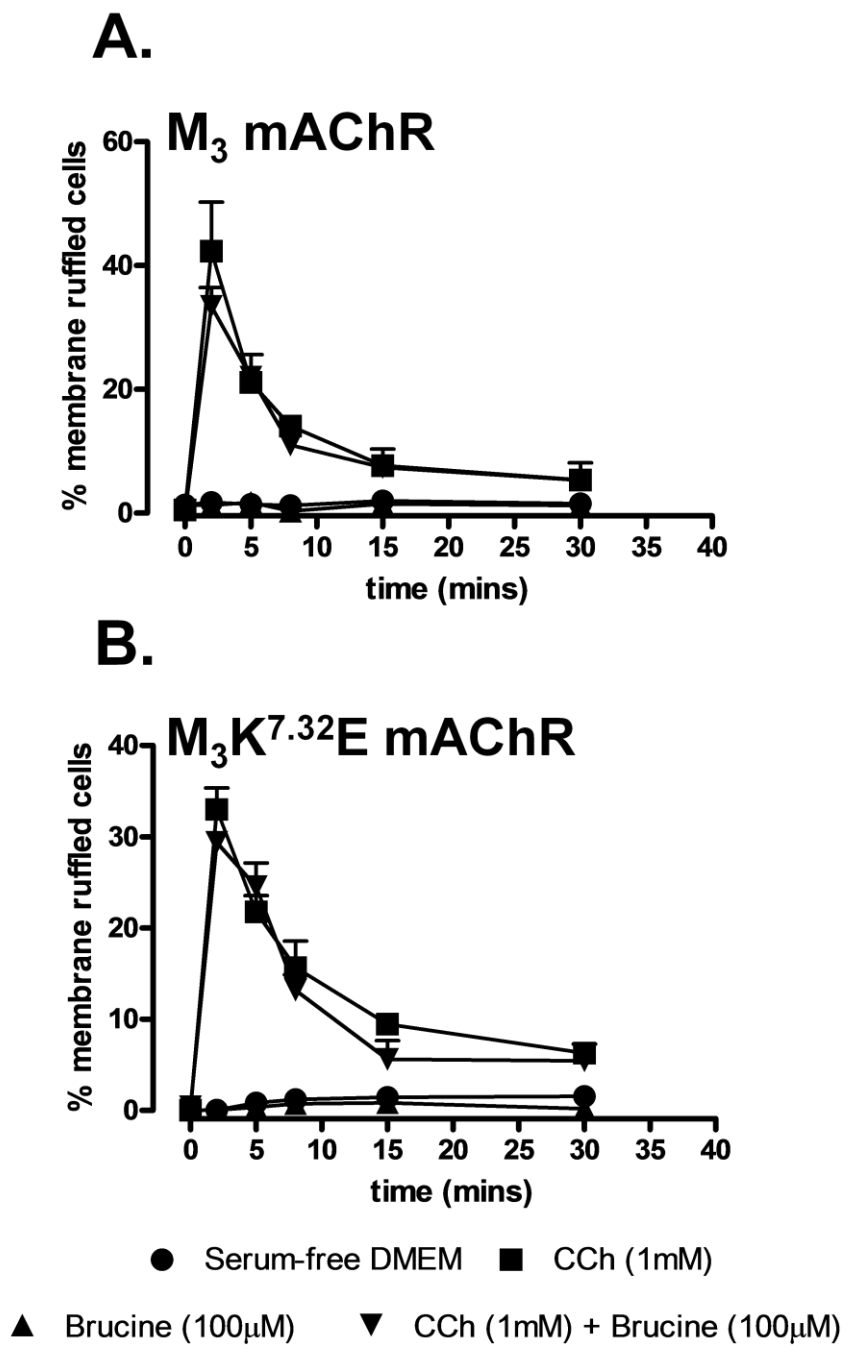

Figure 4.4 Membrane ruffling time-course studies in CHO M3 and M3K7.32E mAChR cells. Stimulation of CHO M_3 and $M_3K^{7.32}E$ mAChR cells with CCh, brucine and co-administered CCh and brucine at different time points. Data are expressed as the mean percentage of cells exhibiting membrane ruffling ± S.E.M., obtained from three experiments performed in duplicate. Error bars not shown lie within the dimensions of the symbol.

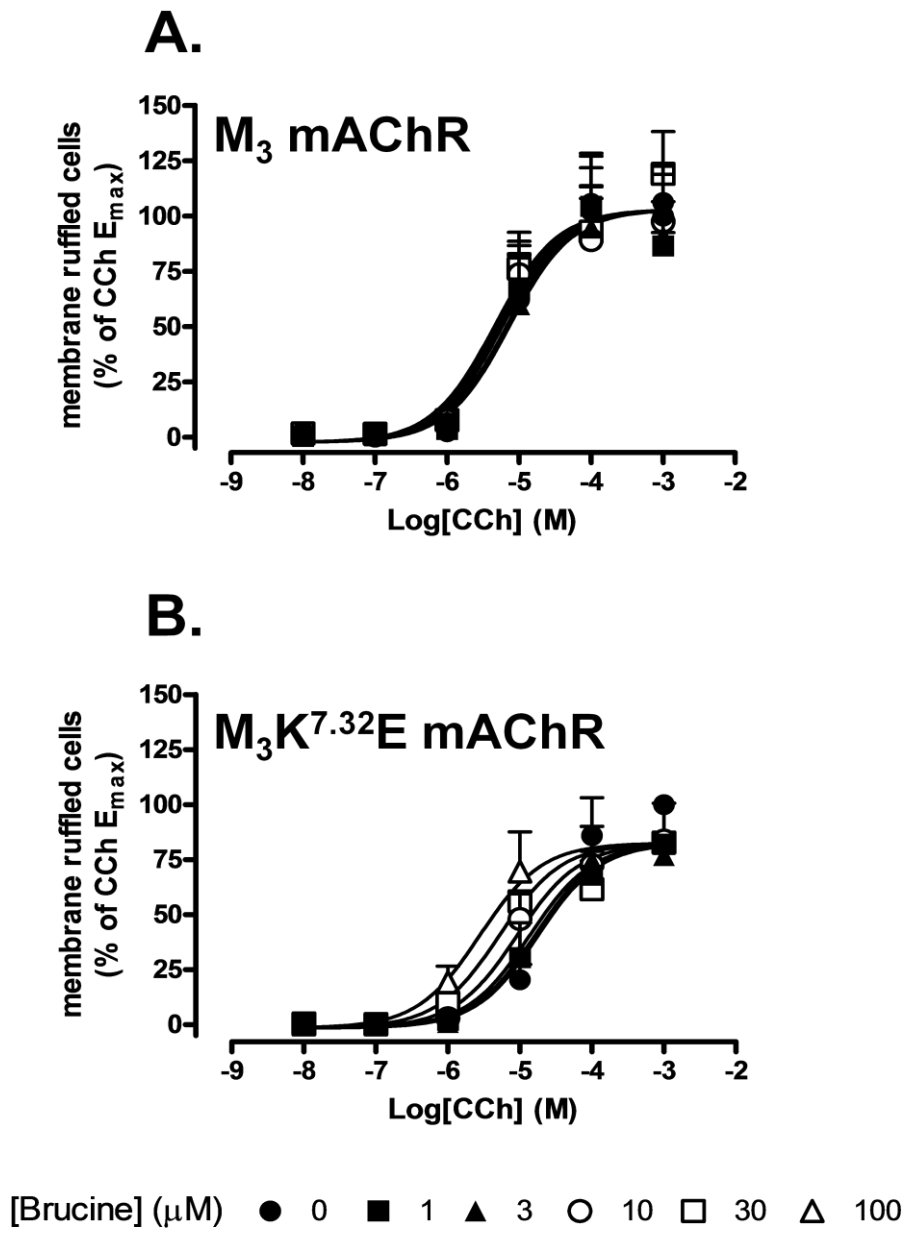

Figure 4.5 Impact of brucine on carbachol-induced membrane ruffling in CHO cells. Carbachol concentration-response curves performed in CHO cells expressing the **A.** M_3 the **B.** $M_3K^{7.32}E$ mAChR, in the absence and presence of brucine. Data points are presented as mean percentage of the maximal CCh-induced membrane ruffling response in the absence of brucine ± S.E.M. obtained from four experiments performed in duplicate. Error bars not shown lie within the dimensions of the symbol.

Table 4.4 Operational model parameters for the interaction between CCh and brucine at the M3K7.32E mAChR. Data are presented as the mean ± S.E.M. of three to six separate experiments performed in duplicate.

	Ca^{2+} mobilisation	Membrane ruffling	ERK1/2 phosphorylation	ERK1/2 phosphorylation (+PTX)
Log K_B[a]	-5.17 ± 0.15	-4.61 ± 0.13	-5.21 ± 0.28	-5.30 ± 0.19
Log τ_B[b]	0.41 ± 0.03** (τ = 2.6)	-1.40 ± 0.48 (τ = 0.04)	-0.66 ± 0.16 (τ = 0.2)	-0.43 ± .16 (τ = 0.4)
Log $\alpha\beta$[c]	0.70 ± 0.17 ($\alpha\beta$ = 5.0)	0.88 ± 0.10 ($\alpha\beta$ = 7.6)	0.84 ± 0.06 ($\alpha\beta$ = 6.9)	0.84 ± 0.07 ($\alpha\beta$ = 6.9)

[a] – logarithm of the equilibrium dissociation constant of the allosteric modulator
[b] – logarithm of the operational efficacy of the allosteric modulator
[c] – logarithm of the cooperativity of the allosteric modulator on the potency of the orthosteric agonist
** – $p<0.01$ determined by one-way ANOVA with Bonferroni's multiple comparisons post-test across Log τ_B values (statistically different to the Log τ_B value derived for membrane ruffling)

4.3.4 Use of yeast-modulator profiling to delineate possible modes of ERK1/2 signalling at the $M_3K^{7.32}E$ muscarinic acetylcholine receptor

In conjunction with generating a G protein profile for receptor-ligand interaction, another utility of the yeast signalling assay could be to aid in the dissection of intracellular G protein mediators of a convergent signalling pathway, such as the phosphorylation of ERK1/2. Specifically, the profile of the effect of brucine at different G protein subtypes may be used to predict which G protein underlies the predominant mode of coupling that leads to $M_3K^{7.32}E$ mAChR-mediated phosphorylation of ERK1/2. Therefore, the effect of brucine on ERK1/2 phosphorylation was investigated. Time-course studies were carried out in CHO M_3 and $M_3K^{7.32}E$ mAChR cells, which demonstrated that maximal CCh-induced ERK1/2 phosphorylation occurred approximately 5 min after stimulation (Figure 4.6). It was also noted that brucine did not display agonism and did not alter the time point of the peak CCh response. Interaction studies between carbachol and brucine at M_3 and $M_3K^{7.32}E$ mACh receptors were then performed to determine the effect of brucine on the potency of CCh at both receptors (Figure 4.7). The results revealed that brucine had little effect on carbachol concentration-response curves from CHO M_3 mAChR cells, whereas brucine potentiated the carbachol response at the $M_3K^{7.32}E$ mAChR in a concentration-dependent manner.

If ERK1/2 phosphorylation was downstream of G protein coupling, then the lack of agonism displayed by brucine suggests an absence of G_q contribution to M_3 mAChR-mediated ERK1/2 phosphorylation. Moreover, the yeast assays also predicted no coupling to $G_{i/o}$ proteins. To confirm the latter, the same ERK1/2 phosphorylation assays were performed in the presence of PTX pretreatment (Figure 4.8). The lack of

effect of PTX on $M_3K^{7.32}E$ mAChR-mediated ERK1/2 phosphorylation suggested that $G_{i/o}$ proteins had no contribution to this pathway, as predicted. Table 4.4 shows operational model parameter estimates derived by applying equation 4.4 to the ERK1/2 phosphorylation data from studies at the $M_3K^{7.32}E$ mAChR in the absence and presence of PTX. Given the absence of contribution by G_q and $G_{i/o}$ proteins, one may speculate that $M_3K^{7.32}E$ mAChR-mediated ERK1/2 phosphorylation is downstream of G_{12} activation and/or G protein-independent effects.

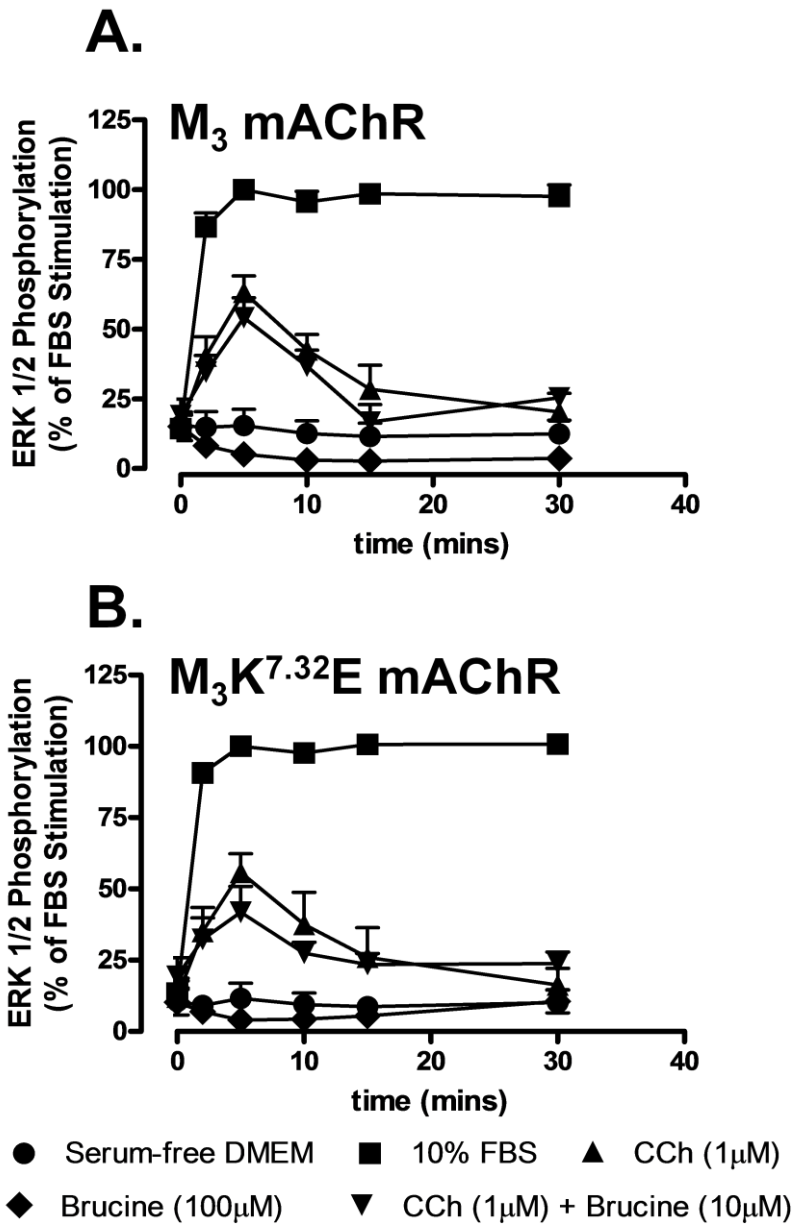

Figure 4.6 ERK1/2 phosphorylation time-course studies in CHO M3 and M3K7.32E mAChR cells. Stimulation of CHO M_3 and $M_3K^{7.32}E$ mAChR cells with single concentrations of FBS, CCh, brucine and co-administered CCh and brucine at different time points. Data are expressed as the mean percentage of the maximal ERK1/2 phosphorylation response elicited by 10% FBS ± S.E.M., collected from three experiments performed in duplicate. Error bars not shown lie within the dimensions of the symbol.

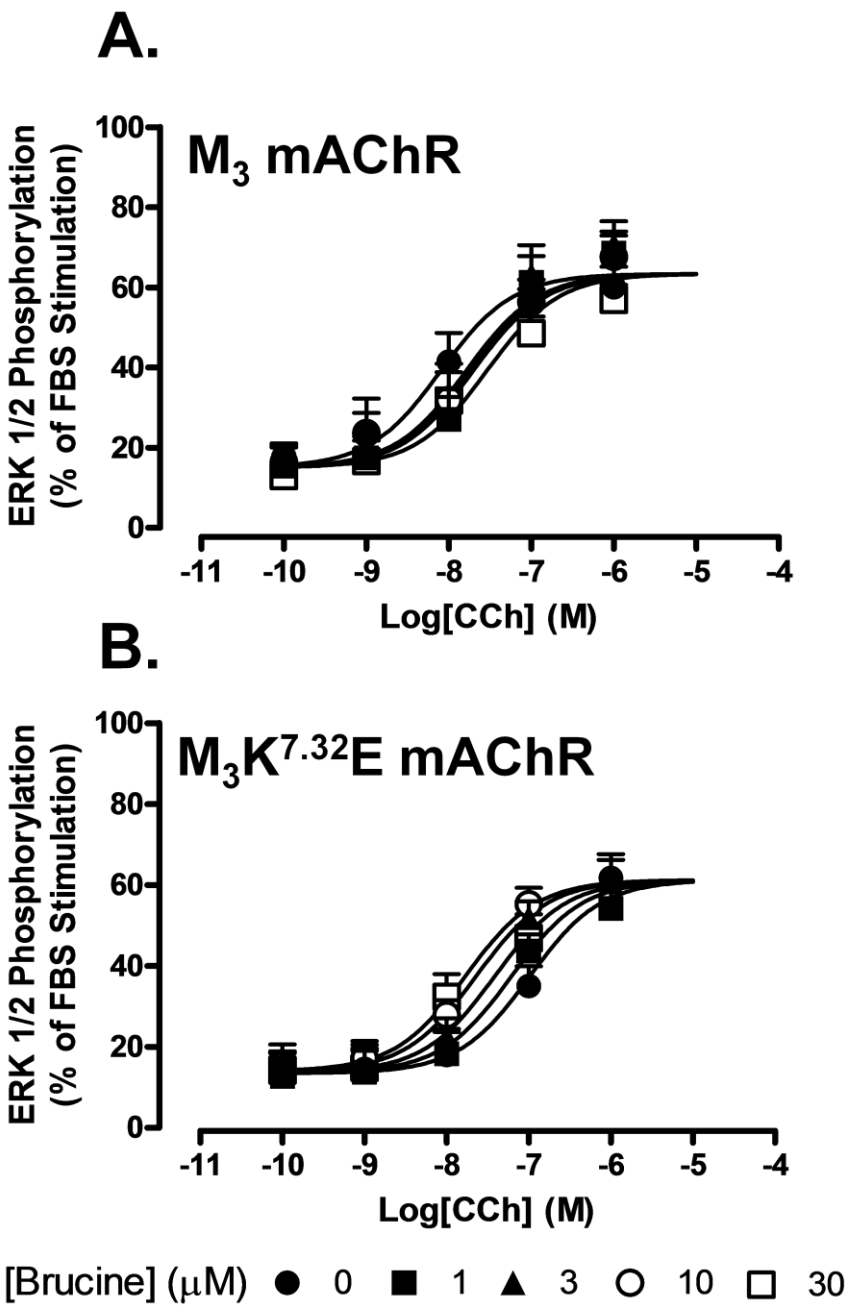

Figure 4.7 Effect of brucine on CCh-induced ERK1/2 phosphorylation in CHO cells. CCh concentration-response curves performed in CHO cells expressing the **A.** M_3 the **B.** $M_3K^{7.32}E$ mAChR, in the absence and presence of brucine. Data points are represented as mean percentage of the peak ERK1/2 phosphorylation response elicited by 10% FBS ± S.E.M. obtained from three experiments performed in duplicate. Error bars not shown lie within the dimensions of the symbol.

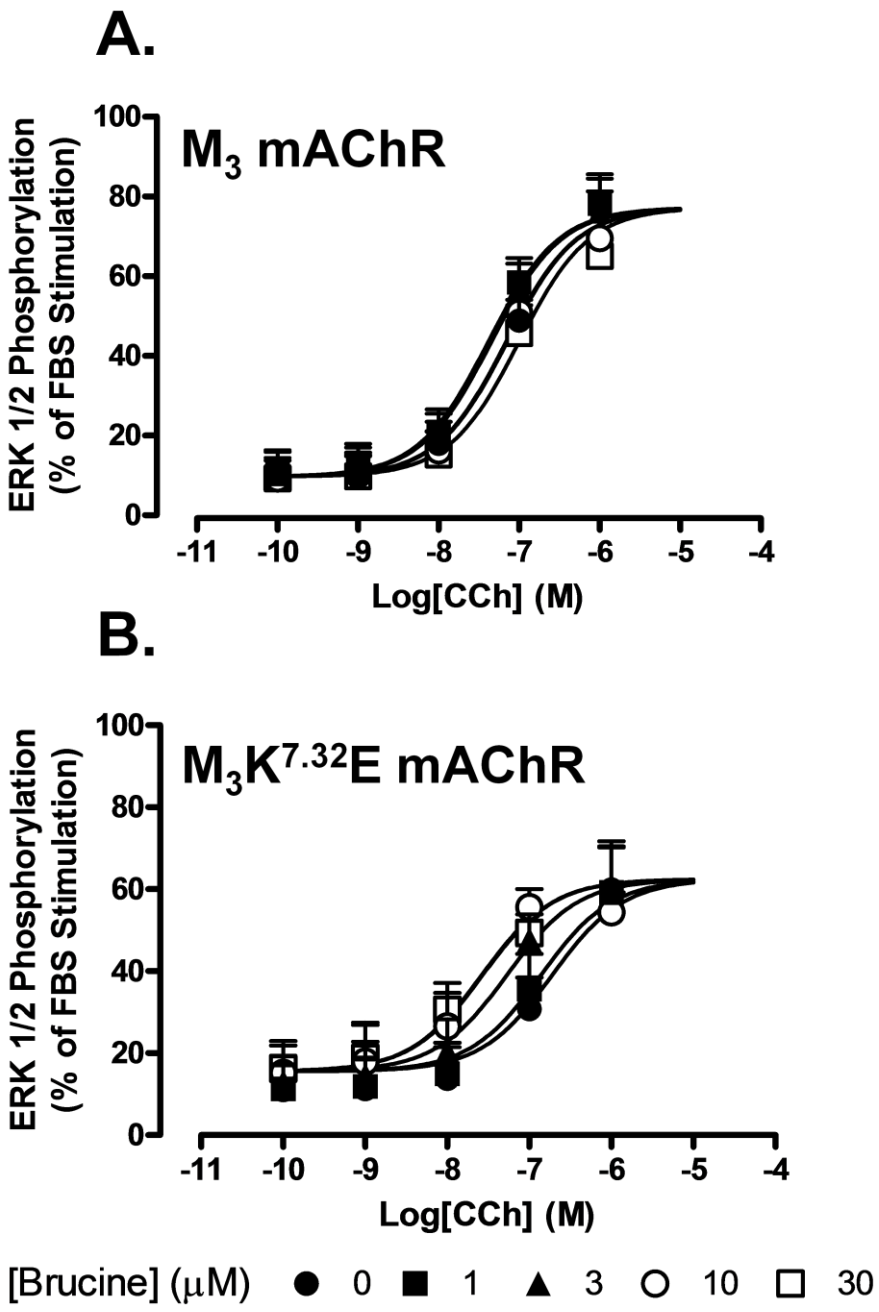

Figure 4.8 Effect of PTX on CCh-induced ERK1/2 phosphorylation in CHO cells. CCh concentration-response curves performed in PTX-treated (100ng mL^{-1} PTX in serum-free DMEM, overnight) CHO cells expressing the **A.** M_3 the **B.** $M_3K^{7.32}E$ mAChR, in the absence and presence of brucine. Data points are represented as mean percentage of the peak ERK1/2 phosphorylation response elicited by 10% FBS ± S.E.M. obtained from three experiments performed in duplicate. Error bars not shown lie within the dimensions of the symbol.

4.4 Discussion

This study is the first to use the yeast system to detect functional selectivity of an allosteric ligand, and to show that brucine is capable of exhibiting pathway selectivity. Furthermore, using the yeast system and the unique properties of brucine at the $K^{7.32}E$ mutant as pharmacological tools, allowed the dissection of the $M_3K^{7.32}E$ mAChR-mediated ERK1/2 phosphorylation pathway in CHO cells.

There have been numerous studies investigating the properties of allosteric ligands that bind to mAChRs (Gregory et al., 2007), with the majority of the studies focusing on ligands that bind to the 'prototypical' allosteric binding site. Indeed, mutagenesis studies have mapped extracellular regions of muscarinic receptors to determine amino acids residues that are pivotal for allosteric binding of the prototypical modulator, gallamine, and associated ligands (Gnagey et al., 1999; Buller et al., 2002; Huang et al., 2005). However, most of these studies focused on the effects of mutagenesis on radioligand binding, or only used a single signalling endpoint to define functional pharmacology (e.g. Jakubik et al. (1996) and Iarriccio (2008)). In contrast, this study investigated the ability of brucine to engender functional selectivity at the $M_3K^{7.32}E$ mAChR, by adopting the use of the yeast signalling assay as a predictive screen in conjunction with a multi-platform approach for system validation.

The results produced in all of the yeast strains expressing the $rM_3\Delta i3$ mAChR showed that brucine had no effect on CCh signalling, which was confirmed in CHO M_3 mAChR cells, whereby the modulator had no effect on the CCh response. This result is consistent with what was found by Iarriccio (2008). The data generated from the yeast signalling assays at the $K^{7.32}E$ mutant, however, suggested that brucine was an agonist with modest enhancement of CCh signalling when coupled to G_q-mediated pathways.

These data are concordant with those generated for CCh-induced Ca^{2+} signalling in CHO $M_3K^{7.32}E$ mAChR cells. In both assay types brucine induced a progressive sinistral shift of the CCh concentration-response curve of approximately 0.5 Log units, as well as brucine displaying its own agonism. The degree of agonism varied between the yeast and CHO cell assays, τ_B = 1.4 and 2.6, respectively, which may be due to a lower receptor concentration in the yeast system compared to CHO $M_3K^{7.32}E$ mAChR cells or decrease stimulus response coupling across the two systems. The agonism displayed by brucine was also observed by Iarriccio (2008) in the $K^{7.32}E$ mutant in a phosphoinositide hydrolysis assay, confirming that brucine is indeed an agonist when coupled to G_q-mediated pathways at this mutant.

Interaction binding assays between CCh and brucine in membranes expressing the M_3 and $M_3K^{7.32}E$ mAChR demonstrated that the $K^{7.32}E$ mutation did not greatly affect the binding of [^{3}H]-NMS, CCh or brucine, but rather enhanced the cooperativity of brucine with the orthosteric ligands. These binding cooperativity profiles are consistent with the recent study by Iarriccio (2008), and the almost neutral cooperativity exhibited by brucine at wild-type M_3 mAChR is consistent with evidence from previous studies at the same receptor (Lazareno et al., 1998; Gharagozloo et al., 1999). However, despite the cooperativities being similar, there is a discrepancy between the affinity estimates of brucine at the unoccupied M_3 mAChR from binding studies (Table 4.3), compared to the values derived by Iarriccio (2008; Log K_B = -3.89) and Lazareno et al. (1998; Log K_B = -3.52); this may be due to different assay conditions.

Interaction studies between CCh and brucine at the $rM_3\Delta i3K^{7.32}E$ in the Gpa1/$G\alpha_{12}$ yeast strain were able to predict the functional profile of brucine in membrane ruffling, in CHO $M_3K^{7.32}E$ mAChR cells, where brucine treatment resulted in a concentration-dependent sinistral shift of the CCh concentration-response curve. Conversely to G_q

signalling, brucine did not display agonism suggesting that brucine is a selective allosteric agonist for G_q-coupling at the mutant receptor; but a selective potentiator for G_{12}-mediated CCh signalling. There was a good accord in the rank order of αβ and τ values derived from yeast assays and their respective CHO cell assay, suggesting that the yeast assay is a reliable surrogate for predicting allosteric ligand pharmacology. However, the Log K_B estimate derived from membrane ruffling assays was lower than the estimates derived from other CHO $M_3K^{7.32}E$ mAChR cell assays. This may be indicative of the $M_3K^{7.32}E$ mAChR being conformationally restricted by lipid compartments, as was the case in the previous chapter for atropine.

As previously mentioned, there are now numerous studies that have investigated mAChR allosteric binding site mutations and allosteric ligand pharmacology, but have not probed the possibilities of functional selectivity. However, given a surge in evidence of functional selectivity induced by orthosteric ligands at various GPCRs (Baker et al., 2003; Sato et al., 2007; Galandrin et al., 2008), some evidence that various allosteric ligands may also induce pathway selective signalling has recently emerged at other GPCRs. For example, prostaglandin D_2 receptor- (PGD_2R) mediated phosphoinositide signalling is not affected by the allosteric ligands, 1-(4-ethoxyphenyl)-5-methoxy-2-methylindole-3-carboxylic acid and N_α-tosyltryptophan, but both abrogate PGD_2R-mediated arrestin recruitment via a non-G protein dependent mechanism (Mathiesen et al., 2005). Likewise, the allosteric ligand LPI805 was able to potentiate NKA-induced Ca^{2+} mobilisation, whilst allosterically inhibiting NKA-induced cAMP accumulation, at the tachykinin NK_2 receptor (Maillet et al., 2007). Thus it should not be surprising that this phenomenon may occur at other pleiotropic receptors, such as the M_3 mAChR.

Another novel utility of the yeast assay in the current study was the use of a functionally selective allosteric modulator as a tool for analysing a convergent signalling pathway,

which allowed a ligand-dependent G protein fingerprint to be ascertained. In yeast, brucine was a selective agonist for Gpa1/$G\alpha_q$ coupling, and the fact that brucine alone did not elicit an ERK1/2 phosphorylation response, suggests a lack of involvement of G_q $M_3K^{7.32}E$ mAChR-mediated ERK1/2 phosphorylation in CHO cells. The pretreatment of CHO $M_3K^{7.32}E$ mAChR cells with PTX did not affect the potency of CCh or cooperativity of brucine, implying a lack of $G_{i/o}$-mediated signalling, which was also predicted by the yeast assay. Taken together, these data suggest that the CCh-stimulated ERK1/2 phosphorylation via the $M_3K^{7.32}E$ mAChR is generated by $G\alpha_{12}$- and/or non-G protein-mediated signalling. Further studies would be required to ascertain the precise components that contribute to the $M_3K^{7.32}E$ mAChR-mediated ERK1/2 phosphorylation response, such as dominant-negative forms of $G\alpha_{12}$ or arrestin 3. Importantly, however, the profile of functional selectivity of brucine at this mutant allows the ascription of a potential G protein candidate of CCh-stimulated ERK1/2 phosphorylation at this receptor.

Together, results from this study suggest that the $K^{7.32}E$ mutation is not necessarily vital for either orthosteric ligand or brucine binding; instead it may be an important region for maintaining the flexibility, and hence possibly activation, of the receptor. Although the finer points of GPCR activation are still largely unknown, there is evidence that some residues in TMVII can form intramolecular interactions with residues in TMIII, to increase the stability of the receptor in an inactive state in the angiotensin II type 1 receptor and opsin, suggesting that TMVII may contribute in part to the activation of the receptor (Groblewski et al., 1997; Rosenbaum et al., 2009). Furthermore, it has been shown through disulphide cross-linking studies that amino acid residues in TMI interact with residues at the bottom of TMVII, and that a large conformational change occurs at the bottom of TMVII upon application of agonist (Wess et al., 2008). There is also

evidence that basic amino acid residues (such as lysine) in membrane proteins 'snorkel' in the lipid, and potentially interact with the charged head-groups in the phospholipid membrane (Mishra et al., 1994). Furthermore, a lysine residue at the juxtamembrane region of a TM helix has been shown to be important for coordinating the helix with the membrane and is also a determinant for the helical tilt (de Planque et al., 1999; Ozdirekcan et al., 2005). Therefore, perhaps, the $K^{7.32}E$ mutation in the M_3 mAChR alters the interaction of TMVII with the plasma membrane and, in turn, increases the propensity of the receptor to be modulated by brucine.

Irrespective of the mode of receptor activation induced by the $K^{7.32}E$ mutation, it is clear that the cooperativity of brucine is increased by the presence this mutation, as previously shown by Iarriccio (2008). Furthermore, the brucine-induced conformational change alters the architecture of the intracellular face of the $M_3K^{7.32}E$ mAChR such that it is conducive to $G\alpha_q$, but not $G\alpha_{12}$ activation in the absence of orthosteric ligands. However, it is also apparent that the same conformation does not promote CCh-induced Ca^{2+} mobilisation, but robustly potentiates CCh-stimulated, $G\alpha_{12}$-mediated signalling. This study has provided evidence that the yeast signalling assay could be a valuable platform for the determination of GPCR ligand-G protein functional selectivity profiles and indeed, the provision of pharmacological parameters such as affinity and relative efficacy estimates.

CHAPTER 5

GENERAL DISCUSSION

GPCRs facilitate many and varied cellular functions that perform vital tasks throughout the body in physiological and pathophysiological settings. Their common architecture yet conformationally diverse character allow them to recognise a vast array of stimuli, in addition to interacting with accessory cellular proteins, such as receptor activity-modifying proteins (RAMPs), and other GPCRs to form oligomeric complexes (Sexton et al., 2006; Milligan, 2007). The ability of GPCRs to promote different phenotypes and their easy accessibility on the plasma membrane make them tractable drug targets for many disease states. However, the inherent nature of signal transduction by GPCRs, once thought to be relatively one-dimensional (one receptor, one linearly-linked signal transduction cascade), has been made increasingly complex by evidence of promiscuous coupling of many GPCRs (Galandrin et al., 2007). This promiscuity creates a potential to 'fine tune' signal transduction by targeting the selected pathways of interest without interfering with collateral functional activity associated with undesired pathways. The conformational malleability of GPCRs paired with pleiotropic coupling thus provides both novel opportunities and great challenges for drug discovery programs to use these receptors as conduits for targeting specific signalling pathways.

The assortment of conformational possibilities displayed by a single GPCR, ranging from inactive to fully active states, is governed by the energy needed to transition into a given state; if a particular conformation is energetically unfavourable, the receptor will remain in a lower energy state, usually the inactive state (Kobilka and Deupi, 2007). However, the formation of a ligand-receptor complex may alter the energy required to transition into a different state, thereby either making it easier or harder to move into a higher energy conformation, as is thought to be the case with agonists and inverse agonists, respectively. The multi-conformational nature of GPCRs is likely the basis for the wide assortment of coupling preferences that mediate receptor signalling. This

concept is further complicated by the array of different G proteins and non-G protein effectors, and the fact that the complement of these effectors can vary widely across different cell types. Moreover, the complexity is further deepened by the alteration in expression of GPCRs, effectors and collateral cellular components in pathophysiological situations. All of these aspects are vital in determining whether a ligand will achieve the desired effect.

The emergence of functional selectivity over the past decade or so has undoubtedly borne consequences for drug discovery in both detrimental and beneficial ways. On the one hand, the recognition of the concept of functional selectivity has given drug discovery programs a new opportunity for targeting various conditions, in a manner that should theoretically yield therapeutics with better selectivity and less side-effects. On the other hand, the difficulty in choosing an appropriate screening platform and selecting which pathways are to be targeted has caused many problems. Drug screens that attempt to discover pathway-selective compounds, by and large, represent a minority, and are in stark contrast to high-throughput screening programs that generally focus on a single signalling output that can be used to target the largest number of GPCRs. In the past this has proven to be a success for generating lead compounds. However, as seen with the β-adrenoceptor antagonists, the collateral but potentially therapeutically-relevant effects of these may remain unnoticed if appropriate studies are not performed. Therefore, proper assay choice is paramount for a successful screen. As mentioned in the Introduction, one of the primary methods for investigating functional selectivity is by using multiple signalling endpoints, which in the case of a high-throughput screen is inefficient and costly. Using newer technologies, such as label-free cell based assays, could be advantageous for identifying compounds that induce a cellular response, but to identify the key effectors in that response would require further

post-hoc analysis of each individual hit compound. However, the use of the yeast assay is a low-cost and tractable platform for studying the effects of ligands on different Gα proteins in isolation. This thesis explores the yeast signalling system as a novel approach to investigating functional selectivity of established and novel ligands and as a means of generating meaningful pharmacological parameters of ligands, such as affinity and efficacy.

In order to generate a ligand-receptor G protein profile in a mammalian system, it is usually necessary to perform experiments in the presence of various signal pathway inhibitors to ascertain the G protein-effector pathway responsible for each outcome. This is the situation where using a mammalian-null system, such as yeast, could be an advantage, as there is no ambiguity in the signalling outcome, there is one receptor type and one G protein type. Chapter 2 assessed the ability of the yeast system to probe for functional selectivity exclusively within $G\alpha_{i/o}$ subtypes and estimate affinity of three adenosine A_1 receptor ligands. The ability to estimate ligand affinity in this system is a major advantage. In addition to estimating DPCPX affinity values using Schild analysis, a further interesting point that arose from this study was the potential for estimating agonist affinity from potency values. This capability is due to the cell biology of *S. cerevisiae*, which involves a negative-feedback system that aligns the concentration-response relationship with the concentration-occupancy relationship to increase the fidelity of the mating response, and therefore 'choose' an appropriate mate based on pheromone concentration (Yu et al., 2008). This intrinsic feature of yeast may be a valuable mechanism for the study of affinity of GPCR ligands without the need for radioligands, and may also be a means of estimating conformation-specific ligand affinities. This may have also been the case for the CCh potency values at $rM_3\Delta i3$ and $rM_3\Delta i3K^{7.32}E$ mAChRs in Chapters 3 and 4.

Results from Chapter 2 also revealed that the yeast system was not sensitive enough to detect A_1 receptor-Gpa1/Gα_o protein interactions in the presence of low efficacy agonists, and that R-PIA and VCP-189 were not functionally selective. This may be indicative of insufficient coupling efficiency of the chimeric G protein to the A_1 receptor in the presence of a low affinity agonist. Therefore, to improve the strength of the interaction between the Gpa1/Gα_o chimera and the adenosine A_1 receptor, the chimera may require more than five amino acids as its recognition sequence. The length of the mammalian component of the chimera is one of the disadvantages of using the yeast system, where there was a compromise between maintaining G protein specificity for the mammalian GPCR and preserving correct function of Gpa1. Thus, depending on the receptor and the chimera, the interaction between the two may not always be strong enough to produce a signal.

To assess the ability of the yeast system to distinguish between G protein subtypes across different families, the M_3 mAChR was used a model of a pleiotropic receptor. Chapter 3 demonstrated that the M_3 mAChR is able to couple to multiple G protein pathways, and that some muscarinic 'antagonists' display true functional selectivity for Gα_q and Gα_{12} coupling. These data were then validated in a mammalian system using atropine as a representative of muscarinic antagonists, which revealed that the expected pharmacology of atropine was biased by the membrane lipid composition, G protein and/or effector complement available to stimulate the Gα_{12}-regulated pathway, providing proof that yeast can detect functional selectivity. The implications of clinically-relevant, functionally selective ligands are far reaching, given that the majority of GPCR-targeting drugs are antagonists and many anti-muscarinics target the M_3 mAChR. Furthermore, it is unknown how many of these ligands display collateral

efficacy, and whether any co-incidental signal pathway activation contributes to their beneficial or detrimental therapeutic effects.

The study in Chapter 3 also assisted in demonstrating that the phenomenon of functional selectivity is not only determined by the character of the ligand-receptor complex, but could be imposed by cellular restrictions. The capacity of the cell to exert functional restrictions on GPCR signalling is further complicated by the localisation of GPCRs into cellular compartments (Patel et al., 2008). The effects of these compartments on function may be exerted in two major modes: functional selectivity imposed by differences in G protein-effector complement, or conformational restriction of the receptor that may be altered by changes in membrane fluidity (Chachisvilis et al., 2006) For example, the adenosine A_{2A} receptor has been shown to couple less efficiently when paired with $\alpha_s\beta_1\gamma_2$, compared to $\alpha_s\beta_4\gamma_2$ when expressed in Sf9 cells (Murphree et al., 2002). This has also been shown to be the case with the M_2 muscarinic acetylcholine receptor (mAChR), where the rate of guanine nucleotide exchange is increased when coupled to $\alpha_o\beta_4\gamma_2$, compared to $\alpha_o\beta_1\gamma_2$ (Hou et al., 2001). The influence of G proteins also extends to γ subunits, for example, the adenosine A_1 receptor -$\alpha_{i2}\beta_1\gamma_2$ or -$\alpha_{i2}\beta_1\gamma_3$ complex had a more rapid rate of guanine nucleotide exchange compared to $\alpha_{i2}\beta_1\gamma_1$ in reconstituted phospholipid vesicles (Figler et al., 1997). Figler et al. (1997) also showed that the A_1 receptor-$\alpha_{i2}\beta_1\gamma_1$ complex is less favourable for agonist binding and is able to increase agonist dissociation kinetics, in comparison to other βγ combinations. These studies emphasise the critical role of distinct combinations of G proteins in ligand binding and signalling. Additionally, based on the studies by Figler et al. (1997), the importance of the βγ subunits in A_1 receptor signalling suggests a role of the non-C-terminal region of the Gα subunit, which in turn would have contributed to the decreased coupling efficiency of Gpa1/$G\alpha_o$, given it lacks all but five C-terminal $G\alpha_o$

amino acids. The change of membrane fluidity also plays a vital role in cell-imposed restriction of GPCR function. Alterations in hippocampal neuron membrane fluidity by cholesterol depletion, have been shown to reduce the affinity of the serotonin 5-HT_{1A} receptor agonist, [^{3}H]-8-hydroxy-2(di-N-propylamino)tetralin, suggesting that a decrease in membrane fluidity forces a receptor conformation that is less favourable than that in the presence of cholesterol (Pucadyil and Chattopadhyay, 2004). A study performed on the oxytocin receptor (OTR) in Madin-Darby canine kidney (MDCK) cells exemplify how differential receptor compartmentalisation can affect receptor signalling (Guzzi et al., 2002). That study provided evidence that the wild-type OTR did not associate with caveolae, and when OTRs were activated, cell proliferation decreased in a concentration-dependent manner. However, when the OTR was fused with caveolin-2, receptor activation resulted in an increase in cell proliferation, suggesting that differential compartmentalisation can achieve quite distinct signalling outcomes. Collectively, findings that demonstrate the ability of a cell to impose restrictions on GPCR function highlight the importance of not only appropriate assay type, but appropriate cell line selection for drug discovery programs.

The ability to modulate GPCRs via an allosteric site is a desirable, yet inherently difficult approach since the allosteric sites often involve regions that are not well characterised compared to the orthosteric site. Regardless, a structure-activity relationship (SAR) -based approach has been applied to the discovery of, for example, many allosteric enhancers of the adenosine A_1 receptor (Aurelio et al., 2009), allosteric agonists of the M_4 mAChR (Nawaratne et al., 2008; Shirey et al., 2008), and even bitopic ligands of the M_2 mAChR such as the oxotremorine-bis(ammonio)alkane compounds (Antony et al., 2009).

The ability of an allosteric ligand to selectively potentiate or inhibit the activity of an orthosteric ligand relies on the nature of the resulting conformational changes in the ligand-receptor complexes. Since allosteric ligands, by their very nature alter the conformation of the receptor in the absence and presence of an orthosteric ligand, they can be exploited in order to target a specific signalling pathway. Similarly to orthosteric ligands, the detection of functionally selective allosteric modulators requires the use of multiple endpoints that are disparate in their effector profile. The utility of the yeast signalling assay could, however, be adapted to high-throughput screening of allosteric ligands across multiple G protein subtypes. Indeed, Chapter 4 investigated the potential of the yeast system to detect allosteric interactions at a $K^{7.32}E$ mutant of the M_3 mAChR, albeit not in a high-throughput fashion. This particular mutation at the M_3 mAChR was chosen because it causes an increase in the cooperativity between ACh and brucine (Iarriccio, 2008), thus providing a suitable model for a proof of concept study for probing functional selectivity of brucine and the sensitivity of the yeast system. In yeast and mammalian cells, brucine exhibited allosteric agonism and robust potentiation of the CCh response when coupled to G_q or G_{12}, respectively. The signalling bias engendered by brucine is an interesting feature of this ligand and, indeed, this has been shown for other allosteric ligands such as LPI805 at the tachykinin NK_2 receptor, where NKA-induced Ca^{2+} mobilisation is potentiated by LPI805, whilst it allosterically inhibited NKA-induced cAMP accumulation (Maillet et al., 2007). Functional selectivity arising from allosteric modulation is not limited to pharmacological agents. It has been shown that an autoantibody against the extracellular Ca^{2+}-sensing receptor (CaSR), from patients with acquired hypocalciuric hypercalcemia, is capable of selectively potentiating Ca^{2+}-stimulated, CaSR-mediated phosphoinositide accumulation, whilst allosterically inhibiting Ca^{2+}-induced, CaSR-mediated ERK1/2

phosphorylation (Makita et al., 2007). The authors postulated that the autoantibody was able to promote signalling pathway-specific conformations of the receptor. Indeed, the differential effects of brucine on the receptor and the CCh-receptor complex suggest distinct conformational states are required for G_q-, $G_{i/o}$- and G_{12}-mediated signalling. From a drug discovery perspective, yeast could not only be used to identify allosteric hit-compounds, but could also be used in tandem with emerging *in silico* methods of drug screening such as virtual ligand screening. By docking ligands with distinct pharmacological profiles (determined in yeast assays) into GPCR homology models, the predicted conformation of the receptor may be used as a guide to investigate the virtual binding properties of novel, potentially pathway-selective pharmacophores. Moreover, it may provide some insight into the conformational changes required for allosteric modulation of a particular receptor, and the alterations in the architecture of the receptor's intracellular face needed to generate a particular signalling response. This approach is not limited to the study of allosteric ligands and could be easily applied to functionally selective orthosteric ligands.

A hitherto unappreciated utility of functionally selective allosteric modulators that was also highlighted in this thesis is the potential 'fingerprinting' of G protein signalling to ascertain the composition of effectors that create a convergent signalling pathway. Many GPCR-mediated signalling pathways, such as Ca^{2+} and MAPK signalling, can be attributed to multiple G protein and non-G protein-mediated events (Werry et al., 2006; Drin and Scarlata, 2007). Thus using pathway-selective allosteric ligands to ascribe potential G protein candidates could serve as an invaluable pharmacological tool for dissecting orthosteric ligand signalling. For example, if an allosteric modulator potentiates the orthosteric ligand-induced $G\alpha_q$ signalling, but allosterically inhibits orthosteric ligand-stimulated $G\alpha_{i/o}$ signalling, and MAPK signalling is also inhibited;

then a candidate for receptor-mediated MAPK signalling is the $G\alpha_{i/o}$ protein. This approach is greatly aided by its use in conjunction with the yeast signalling assay, whereby a preliminary profile of G protein signalling can be made in the presence of allosteric ligand. Using this method, it was possible to show that CCh-mediated ERK1/2 phosphorylation at the $M_3K^{7.32}E$ mAChR was potentially mediated by $G\alpha_{12}$, and not $G\alpha_q$ or $G\alpha_{i/o}$, however, non-G protein-mediated signalling could not be discounted. Indeed, one of the major disadvantages of the yeast system is the inability to detect receptor-non-G protein interactions, which may render it unsuitable for targeting certain signalling pathways.

The yeast assay provides an invaluable platform for which to study GPCR-G protein interactions, in a mammalian-null system. Potentially, it is also suitable for use in a high-throughput setting, as it is tractable and cost-effective. This thesis has provided evidence that the yeast assay, in nearly all cases studied, is an appropriate and valid approach for estimating ligand affinity, relative efficacy and functional selectivity, of both orthosteric and allosteric ligands. Via these means, it was possible to identify hitherto unrecognised properties of some anti-muscarinic ligands and the allosteric muscarinic ligand, brucine. These studies have also given a novel insight into the multidimensional nature of GPCR signalling within and across G protein subtypes, in addition to providing a new approach for screening of functionally selective ligands.

CHAPTER 6

REFERENCES

Abo A, Pick E, Hall A, Totty N, Teahan CG and Segal AW (1991) Activation of the NADPH oxidase involves the small GTP-binding protein p21rac1. *Nature* **353**:668-670.

Abramowitz J and Birnbaumer L (2009) Physiology and pathophysiology of canonical transient receptor potential channels. *Faseb J* **23**:297-328.

Aihara M, Dobashi K, Iizuka K, Nakazawa T and Mori M (2004) Effect of Y-27632 on release of cytokines from peripheral T cells in asthmatic patients and normal subjects. *Int Immunopharmacol* **4**:557-561.

Akbar M, Okajima F, Tomura H, Shimegi S and Kondo Y (1994) A single species of A1 adenosine receptor expressed in Chinese hamster ovary cells not only inhibits cAMP accumulation but also stimulates phospholipase C and arachidonate release. *Mol Pharmacol* **45**:1036-1042.

Amsen EM, Pham N, Pak Y and Rotin D (2006) The guanine nucleotide exchange factor CNrasGEF regulates melanogenesis and cell survival in melanoma cells. *J Biol Chem* **281**:121-128.

Andreadou I, Iliodromitis EK, Koufaki M, Farmakis D, Tsotinis A and Kremastinos DT (2008) Alternative pharmacological interventions that limit myocardial infarction. *Curr Med Chem* **15**:3204-3213.

Antony J, Kellershohn K, Mohr-Andra M, Kebig A, Prilla S, Muth M, Heller E, Disingrini T, Dallanoce C, Bertoni S, Schrobang J, Trankle C, Kostenis E, Christopoulos A, Holtje HD, Barocelli E, De Amici M, Holzgrabe U and Mohr K (2009) Dualsteric GPCR targeting: a novel route to binding and signaling pathway selectivity. *Faseb J* **23**:442-450.

Aoki J, Inoue A, Makide K, Saiki N and Arai H (2007) Structure and function of extracellular phospholipase A1 belonging to the pancreatic lipase gene family. *Biochimie* **89**:197-204.

Armbruster BN, Li X, Pausch MH, Herlitze S and Roth BL (2007) Evolving the lock to fit the key to create a family of G protein-coupled receptors potently activated by an inert ligand. *Proc Natl Acad Sci U S A* **104**:5163-5168.

Arshavsky VY and Pugh EN, Jr. (1998) Lifetime regulation of G protein-effector complex: emerging importance of RGS proteins. *Neuron* **20**:11-14.

Arslan G and Fredholm BB (2000) Stimulatory and inhibitory effects of adenosine A(2A) receptors on nerve growth factor-induced phosphorylation of extracellular regulated kinases 1/2 in PC12 cells. *Neurosci Lett* **292**:183-186.

Ashton TD, Baker SP, Hutchinson SA and Scammells PJ (2008) N6-substituted C5'-modified adenosines as A1 adenosine receptor agonists. *Bioorg Med Chem* **16**:1861-1873.

Aurelio L, Valant C, Flynn BL, Sexton PM, Christopoulos A and Scammells PJ (2009) Allosteric Modulators of the Adenosine A(1) Receptor: Synthesis and Pharmacological Evaluation of 4-Substituted 2-Amino-3-benzoylthiophenes. *J Med Chem*.

Avlani VA, Gregory KJ, Morton CJ, Parker MW, Sexton PM and Christopoulos A (2007) Critical role for the second extracellular loop in the binding of both orthosteric and allosteric G protein-coupled receptor ligands. *J Biol Chem* **282**:25677-25686.

Azzi M, Pineyro G, Pontier S, Parent S, Ansanay H and Bouvier M (2001) Allosteric effects of G protein overexpression on the binding of beta-adrenergic ligands with distinct inverse efficacies. *Mol Pharmacol* **60**:999-1007.

Baillie GS, Scott JD and Houslay MD (2005) Compartmentalisation of phosphodiesterases and protein kinase A: opposites attract. *FEBS Lett* **579**:3264-3270.

Baker JG, Hall IP and Hill SJ (2003) Agonist and inverse agonist actions of beta-blockers at the human beta 2-adrenoceptor provide evidence for agonist-directed signaling. *Mol Pharmacol* **64**:1357-1369.

Balijepalli RC and Kamp TJ (2008) Caveolae, ion channels and cardiac arrhythmias. *Prog Biophys Mol Biol* **98**:149-160.

Ballesteros J, Kitanovic S, Guarnieri F, Davies P, Fromme BJ, Konvicka K, Chi L, Millar RP, Davidson JS, Weinstein H and Sealfon SC (1998) Functional microdomains in G-protein-coupled receptors. The conserved arginine-cage motif in the gonadotropin-releasing hormone receptor. *J Biol Chem* **273**:10445-10453.

Ballesteros JA, Jensen AD, Liapakis G, Rasmussen SG, Shi L, Gether U and Javitch JA (2001) Activation of the beta 2-adrenergic receptor involves disruption of an ionic lock between the cytoplasmic ends of transmembrane segments 3 and 6. *J Biol Chem* **276**:29171-29177.

Ballesteros JA and Weinstein H (1992) Analysis and refinement of criteria for predicting the structure and relative orientations of transmembranal helical domains. *Biophys J* **62**:107-109.

Bansal G, Druey KM and Xie Z (2007) R4 RGS proteins: regulation of G-protein signaling and beyond. *Pharmacol Ther* **116**:473-495.

Baraldi PG, Zaid AN, Lampronti I, Fruttarolo F, Pavani MG, Tabrizi MA, Shryock JC, Leung E and Romagnoli R (2000) Synthesis and biological effects of a new series of 2-amino-3-benzoylthiophenes as allosteric enhancers of A1-adenosine receptor. *Bioorg Med Chem Lett* **10**:1953-1957.

Bass RT, Buckwalter BL, Patel BP, Pausch MH, Price LA, Strnad J and Hadcock JR (1996) Identification and characterization of novel somatostatin antagonists. *Mol Pharmacol* **50**:709-715.

Beaulieu JM, Sotnikova TD, Marion S, Lefkowitz RJ, Gainetdinov RR and Caron MG (2005) An Akt/beta-arrestin 2/PP2A signaling complex mediates dopaminergic neurotransmission and behavior. *Cell* **122**:261-273.

Beazely MA and Watts VJ (2006) Regulatory properties of adenylate cyclases type 5 and 6: A progress report. *Eur J Pharmacol* **535**:1-12.

Bender AT and Beavo JA (2006) Cyclic nucleotide phosphodiesterases: molecular regulation to clinical use. *Pharmacol Rev* **58**:488-520.

Benians A, Leaney JL and Tinker A (2003) Agonist unbinding from receptor dictates the nature of deactivation kinetics of G protein-gated K+ channels. *Proc Natl Acad Sci U S A* **100**:6239-6244.

Benovic JL, Pike LJ, Cerione RA, Staniszewski C, Yoshimasa T, Codina J, Caron MG and Lefkowitz RJ (1985) Phosphorylation of the mammalian beta-adrenergic receptor by cyclic AMP-dependent protein kinase. Regulation of the rate of receptor phosphorylation and dephosphorylation by agonist occupancy and effects on coupling of the receptor to the stimulatory guanine nucleotide regulatory protein. *J Biol Chem* **260**:7094-7101.

Berg KA, Maayani S, Goldfarb J, Scaramellini C, Leff P and Clarke WP (1998) Effector pathway-dependent relative efficacy at serotonin type 2A and 2C receptors: evidence for agonist-directed trafficking of receptor stimulus. *Mol Pharmacol* **54**:94-104.

Berridge MJ and Irvine RF (1989) Inositol phosphates and cell signalling. *Nature* **341**:197-205.

Bhattacharya S, Hall SE and Vaidehi N (2008) Agonist-induced conformational changes in bovine rhodopsin: insight into activation of G-protein-coupled receptors. *J Mol Biol* **382**:539-555.

Birdsall NJ and Lazareno S (2005) Allosterism at muscarinic receptors: ligands and mechanisms. *Mini Rev Med Chem* **5**:523-543.

Birnbaumer L, Zhu X, Jiang M, Boulay G, Peyton M, Vannier B, Brown D, Platano D, Sadeghi H, Stefani E and Birnbaumer M (1996) On the molecular basis and regulation of cellular capacitative calcium entry: roles for Trp proteins. *Proc Natl Acad Sci U S A* **93**:15195-15202.

Black JW and Leff P (1983) Operational models of pharmacological agonism. *Proc R Soc Lond B Biol Sci* **220**:141-162.

Brown AJ, Dyos SL, Whiteway MS, White JH, Watson MA, Marzioch M, Clare JJ, Cousens DJ, Paddon C, Plumpton C, Romanos MA and Dowell SJ (2000) Functional coupling of mammalian receptors to the yeast mating pathway using novel yeast/mammalian G protein alpha-subunit chimeras. *Yeast* **16**:11-22.

Brown JH, Del Re DP and Sussman MA (2006) The Rac and Rho hall of fame: a decade of hypertrophic signaling hits. *Circ Res* **98**:730-742.

Brown SG, Thomas A, Dekker LV, Tinker A and Leaney JL (2005) PKC-delta sensitizes Kir3.1/3.2 channels to changes in membrane phospholipid levels after M3 receptor activation in HEK-293 cells. *Am J Physiol Cell Physiol* **289**:C543-556.

Buckley NJ, Bonner TI, Buckley CM and Brann MR (1989) Antagonist binding properties of five cloned muscarinic receptors expressed in CHO-K1 cells. *Mol Pharmacol* **35**:469-476.

Buday L and Downward J (2008) Many faces of Ras activation. *Biochim Biophys Acta* **1786**:178-187.

Buller S, Zlotos DP, Mohr K and Ellis J (2002) Allosteric site on muscarinic acetylcholine receptors: a single amino acid in transmembrane region 7 is critical to the subtype selectivities of caracurine V derivatives and alkane-bisammonium ligands. *Mol Pharmacol* **61**:160-168.

Bunney TD and Katan M (2006) Phospholipase C epsilon: linking second messengers and small GTPases. *Trends Cell Biol* **16**:640-648.

Burridge K and Wennerberg K (2004) Rho and Rac take center stage. *Cell* **116**:167-179.

Burstein ES, Spalding TA and Brann MR (1997) Pharmacology of muscarinic receptor subtypes constitutively activated by G proteins. *Mol Pharmacol* **51**:312-319.

Butcher EC (2005) Can cell systems biology rescue drug discovery? *Nat Rev Drug Discov* **4**:461-467.

Calaghan S, Kozera L and White E (2008) Compartmentalisation of cAMP-dependent signalling by caveolae in the adult cardiac myocyte. *J Mol Cell Cardiol* **45**:88-92.

Campbell RM, Cartwright C, Chen W, Chen Y, Duzic E, Fu JM, Loveland M, Manning R, McKibben B, Pleiman CM, Silverman L, Trueheart J, Webb DR, Wilkinson V, Witter DJ, Xie X and Castelhano AL (1999) Selective A1-adenosine receptor antagonists identified using yeast Saccharomyces cerevisiae functional assays. *Bioorg Med Chem Lett* **9**:2413-2418.

Catterall WA (2000) Structure and regulation of voltage-gated Ca2+ channels. *Annu Rev Cell Dev Biol* **16**:521-555.

Caulfield MP (1993) Muscarinic receptors--characterization, coupling and function. *Pharmacol Ther* **58**:319-379.

Chachisvilis M, Zhang YL and Frangos JA (2006) G protein-coupled receptors sense fluid shear stress in endothelial cells. *Proc Natl Acad Sci U S A* **103**:15463-15468.

Chakraborti S (2003) Phospholipase A(2) isoforms: a perspective. *Cell Signal* **15**:637-665.

Chambers JK, Macdonald LE, Sarau HM, Ames RS, Freeman K, Foley JJ, Zhu Y, McLaughlin MM, Murdock P, McMillan L, Trill J, Swift A, Aiyar N, Taylor P, Vawter L, Naheed S, Szekeres P, Hervieu G, Scott C, Watson JM, Murphy AJ, Duzic E, Klein C, Bergsma DJ, Wilson S and Livi GP (2000) A G protein-coupled receptor for UDP-glucose. *J Biol Chem* **275**:10767-10771.

Charest PG, Oligny-Longpre G, Bonin H, Azzi M and Bouvier M (2007) The V2 vasopressin receptor stimulates ERK1/2 activity independently of heterotrimeric G protein signalling. *Cell Signal* **19**:32-41.

Chidiac P, Hebert TE, Valiquette M, Dennis M and Bouvier M (1994) Inverse agonist activity of beta-adrenergic antagonists. *Mol Pharmacol* **45**:490-499.

Christopoulos A (1998) Assessing the distribution of parameters in models of ligand-receptor interaction: to log or not to log. *Trends Pharmacol Sci* **19**:351-357.

Christopoulos A (2000) Quantification of Allosteric Interactions at G Protein Coupled Receptors Using Radioligand Binding Assays, in *Current Protocols in Pharmacology* (Enna S ed).

Christopoulos A and Kenakin T (2002) G protein-coupled receptor allosterism and complexing. *Pharmacol Rev* **54**:323-374.

Christopoulos A, Lanzafame A and Mitchelson F (1998) Allosteric interactions at muscarinic cholinoceptors. *Clin Exp Pharmacol Physiol* **25**:185-194.

Christopoulos A, Parsons AM, Lew MJ and El-Fakahany EE (1999) The assessment of antagonist potency under conditions of transient response kinetics. *Eur J Pharmacol* **382**:217-227.

Clark MA, Sethi PR and Lambert NA (2007) Active Galpha(q) subunits and M3 acetylcholine receptors promote distinct modes of association of RGS2 with the plasma membrane. *FEBS Lett* **581**:764-770.

Clerk A, Pham FH, Fuller SJ, Sahai E, Aktories K, Marais R, Marshall C and Sugden PH (2001) Regulation of mitogen-activated protein kinases in cardiac myocytes through the small G protein Rac1. *Mol Cell Biol* **21**:1173-1184.

Cooper DM (2003) Regulation and organization of adenylyl cyclases and cAMP. *Biochem J* **375**:517-529.

Cordeaux Y, Briddon SJ, Megson AE, McDonnell J, Dickenson JM and Hill SJ (2000) Influence of receptor number on functional responses elicited by agonists acting at the human adenosine A(1) receptor: evidence for signaling pathway-dependent changes in agonist potency and relative intrinsic activity. *Mol Pharmacol* **58**:1075-1084.

Cordeaux Y, Nickolls SA, Flood LA, Graber SG and Strange PG (2001) Agonist regulation of D(2) dopamine receptor/G protein interaction. Evidence for agonist selection of G protein subtype. *J Biol Chem* **276**:28667-28675.

Costa T and Cotecchia S (2005) Historical review: Negative efficacy and the constitutive activity of G-protein-coupled receptors. *Trends Pharmacol Sci* **26**:618-624.

Cote JF and Vuori K (2007) GEF what? Dock180 and related proteins help Rac to polarize cells in new ways. *Trends Cell Biol* **17**:383-393.

Coulson FR and Fryer AD (2003) Muscarinic acetylcholine receptors and airway diseases. *Pharmacol Ther* **98**:59-69.

Darby PJ, Kwan CY and Daniel EE (2000) Caveolae from canine airway smooth muscle contain the necessary components for a role in Ca(2+) handling. *Am J Physiol Lung Cell Mol Physiol* **279**:L1226-1235.

de Ligt RA, Rivkees SA, Lorenzen A, Leurs R and AP IJ (2005) A "locked-on," constitutively active mutant of the adenosine A1 receptor. *Eur J Pharmacol* **510**:1-8.

de Planque MR, Kruijtzer JA, Liskamp RM, Marsh D, Greathouse DV, Koeppe RE, 2nd, de Kruijff B and Killian JA (1999) Different membrane anchoring positions of tryptophan and lysine in synthetic transmembrane alpha-helical peptides. *J Biol Chem* **274**:20839-20846.

DeFea KA, Vaughn ZD, O'Bryan EM, Nishijima D, Dery O and Bunnett NW (2000) The proliferative and antiapoptotic effects of substance P are facilitated by formation of a beta -arrestin-dependent scaffolding complex. *Proc Natl Acad Sci U S A* **97**:11086-11091.

Delcourt N, Bockaert J and Marin P (2007) GPCR-jacking: from a new route in RTK signalling to a new concept in GPCR activation. *Trends Pharmacol Sci* **28**:602-607.

DeMali KA and Burridge K (2003) Coupling membrane protrusion and cell adhesion. *J Cell Sci* **116**:2389-2397.

DeWire SM, Ahn S, Lefkowitz RJ and Shenoy SK (2007) Beta-arrestins and cell signaling. *Annu Rev Physiol* **69**:483-510.

Disse B, Speck GA, Rominger KL, Witek TJ, Jr. and Hammer R (1999) Tiotropium (Spiriva): mechanistical considerations and clinical profile in obstructive lung disease. *Life Sci* **64**:457-464.

Dohlman HG (2002) G proteins and pheromone signaling. *Annu Rev Physiol* **64**:129-152.

Dohlman HG and Slessareva JE (2006) Pheromone signaling pathways in yeast. *Sci STKE* **2006**:cm6.

Dolphin AC (2006) A short history of voltage-gated calcium channels. *Br J Pharmacol* **147 Suppl 1**:S56-62.

Doods HN, Mathy MJ, Davidesko D, van Charldorp KJ, de Jonge A and van Zwieten PA (1987) Selectivity of muscarinic antagonists in radioligand and in vivo experiments for the putative M1, M2 and M3 receptors. *J Pharmacol Exp Ther* **242**:257-262.

Doupnik CA (2008) GPCR-Kir channel signaling complexes: defining rules of engagement. *J Recept Signal Transduct Res* **28**:83-91.

Dowell SJ and Brown AJ (2002) Yeast assays for G-protein-coupled receptors. *Receptors Channels* **8**:343-352.

Dowling MR and Charlton SJ (2006) Quantifying the association and dissociation rates of unlabelled antagonists at the muscarinic M3 receptor. *Br J Pharmacol* **148**:927-937.

Dowling MR, Willets JM, Budd DC, Charlton SJ, Nahorski SR and Challiss RA (2006) A single point mutation (N514Y) in the human M3 muscarinic acetylcholine receptor reveals differences in the properties of antagonists: evidence for differential inverse agonism. *J Pharmacol Exp Ther* **317**:1134-1142.

Drin G and Scarlata S (2007) Stimulation of phospholipase Cbeta by membrane interactions, interdomain movement, and G protein binding--how many ways can you activate an enzyme? *Cell Signal* **19**:1383-1392.

Duthey B, Caudron S, Perroy J, Bettler B, Fagni L, Pin JP and Prezeau L (2002) A single subunit (GB2) is required for G-protein activation by the heterodimeric GABA(B) receptor. *J Biol Chem* **277**:3236-3241.

Eglen RM, Hegde SS and Watson N (1996) Muscarinic receptor subtypes and smooth muscle function. *Pharmacol Rev* **48**:531-565.

Eglen RM and Nahorski SR (2000) The muscarinic M(5) receptor: a silent or emerging subtype? *Br J Pharmacol* **130**:13-21.

Eglen RM, Reddy H, Watson N and Challiss RA (1994) Muscarinic acetylcholine receptor subtypes in smooth muscle. *Trends Pharmacol Sci* **15**:114-119.

Eglen RM and Whiting RL (1990) Heterogeneity of vascular muscarinic receptors. *J Auton Pharmacol* **10**:233-245.

Eguchi S, Numaguchi K, Iwasaki H, Matsumoto T, Yamakawa T, Utsunomiya H, Motley ED, Kawakatsu H, Owada KM, Hirata Y, Marumo F and Inagami T (1998) Calcium-dependent epidermal growth factor receptor transactivation mediates the angiotensin II-induced mitogen-activated protein kinase activation in vascular smooth muscle cells. *J Biol Chem* **273**:8890-8896.

Ehlert FJ (1988) Estimation of the affinities of allosteric ligands using radioligand binding and pharmacological null methods. *Mol Pharmacol* **33**:187-194.

Eilers M, Hornak V, Smith SO and Konopka JB (2005) Comparison of class A and D G protein-coupled receptors: common features in structure and activation. *Biochemistry* **44**:8959-8975.

Elion EA, Qi M and Chen W (2005) Signal transduction. Signaling specificity in yeast. *Science* **307**:687-688.

Elzein E and Zablocki J (2008) A1 adenosine receptor agonists and their potential therapeutic applications. *Expert Opin Investig Drugs* **17**:1901-1910.

Erlenbach I, Kostenis E, Schmidt C, Hamdan FF, Pausch MH and Wess J (2001a) Functional expression of M(1), M(3) and M(5) muscarinic acetylcholine receptors in yeast. *J Neurochem* **77**:1327-1337.

Erlenbach I, Kostenis E, Schmidt C, Serradeil-Le Gal C, Raufaste D, Dumont ME, Pausch MH and Wess J (2001b) Single amino acid substitutions and deletions that alter the G protein coupling properties of the V2 vasopressin receptor identified in yeast by receptor random mutagenesis. *J Biol Chem* **276**:29382-29392.

Fain JN, Wallace MA and Wojcikiewicz RJ (1988) Evidence for involvement of guanine nucleotide-binding regulatory proteins in the activation of phospholipases by hormones. *Faseb J* **2**:2569-2574.

Feoktistov I, Goldstein AE and Biaggioni I (1999) Role of p38 mitogen-activated protein kinase and extracellular signal-regulated protein kinase kinase in adenosine A2B receptor-mediated interleukin-8 production in human mast cells. *Mol Pharmacol* **55**:726-734.

Ferguson SS and Caron MG (1998) G protein-coupled receptor adaptation mechanisms. *Semin Cell Dev Biol* **9**:119-127.

Figler RA, Lindorfer MA, Graber SG, Garrison JC and Linden J (1997) Reconstitution of bovine A1 adenosine receptors and G proteins in phospholipid vesicles: betagamma-subunit composition influences guanine nucleotide exchange and agonist binding. *Biochemistry* **36**:16288-16299.

Filardo EJ (2002) Epidermal growth factor receptor (EGFR) transactivation by estrogen via the G-protein-coupled receptor, GPR30: a novel signaling pathway with potential significance for breast cancer. *J Steroid Biochem Mol Biol* **80**:231-238.

Fisher SK and Heacock AM (1988) A putative M3 muscarinic cholinergic receptor of high molecular weight couples to phosphoinositide hydrolysis in human SK-N-SH neuroblastoma cells. *J Neurochem* **50**:984-987.

Foord SM, Bonner TI, Neubig RR, Rosser EM, Pin JP, Davenport AP, Spedding M and Harmar AJ (2005) International Union of Pharmacology. XLVI. G protein-coupled receptor list. *Pharmacol Rev* **57**:279-288.

Frank GD, Mifune M, Inagami T, Ohba M, Sasaki T, Higashiyama S, Dempsey PJ and Eguchi S (2003) Distinct mechanisms of receptor and nonreceptor tyrosine kinase activation by reactive oxygen species in vascular smooth muscle cells: role of metalloprotease and protein kinase C-delta. *Mol Cell Biol* **23**:1581-1589.

Franke TF (2008) PI3K/Akt: getting it right matters. *Oncogene* **27**:6473-6488.

Fredholm BB, AP IJ, Jacobson KA, Klotz KN and Linden J (2001) International Union of Pharmacology. XXV. Nomenclature and classification of adenosine receptors. *Pharmacol Rev* **53**:527-552.

Fredriksson R, Lagerstrom MC, Lundin LG and Schioth HB (2003) The G-protein-coupled receptors in the human genome form five main families. Phylogenetic analysis, paralogon groups, and fingerprints. *Mol Pharmacol* **63**:1256-1272.

Freissmuth M, Schutz W and Linder ME (1991) Interactions of the bovine brain A1-adenosine receptor with recombinant G protein alpha-subunits. Selectivity for rGi alpha-3. *J Biol Chem* **266**:17778-17783.

Fuentes JM, Fulton WB, Nino D, Talamini MA and Maio AD (2008) Atropine treatment modifies LPS-induced inflammatory response and increases survival. *Inflamm Res* **57**:111-117.

Fujii T, Onohara N, Maruyama Y, Tanabe S, Kobayashi H, Fukutomi M, Nagamatsu Y, Nishihara N, Inoue R, Sumimoto H, Shibasaki F, Nagao T, Nishida M and Kurose H (2005) Galpha12/13-mediated production of reactive oxygen species is critical for angiotensin receptor-induced NFAT activation in cardiac fibroblasts. *J Biol Chem* **280**:23041-23047.

Fujii T, Tsuchiya T, Yamada S, Fujimoto K, Suzuki T, Kasahara T and Kawashima K (1996) Localization and synthesis of acetylcholine in human leukemic T cell lines. *J Neurosci Res* **44**:66-72.

Fukata Y, Amano M and Kaibuchi K (2001) Rho-Rho-kinase pathway in smooth muscle contraction and cytoskeletal reorganization of non-muscle cells. *Trends Pharmacol Sci* **22**:32-39.

Galandrin S and Bouvier M (2006) Distinct signaling profiles of beta1 and beta2 adrenergic receptor ligands toward adenylyl cyclase and mitogen-activated protein kinase reveals the pluridimensionality of efficacy. *Mol Pharmacol* **70**:1575-1584.

Galandrin S, Oligny-Longpre G, Bonin H, Ogawa K, Gales C and Bouvier M (2008) Conformational rearrangements and signaling cascades involved in ligand-biased mitogen-activated protein kinase signaling through the beta1-adrenergic receptor. *Mol Pharmacol* **74**:162-172.

Galandrin S, Oligny-Longpre G and Bouvier M (2007) The evasive nature of drug efficacy: implications for drug discovery. *Trends Pharmacol Sci* **28**:423-430.

Galvez T, Duthey B, Kniazeff J, Blahos J, Rovelli G, Bettler B, Prezeau L and Pin JP (2001) Allosteric interactions between GB1 and GB2 subunits are required for optimal GABA(B) receptor function. *Embo J* **20**:2152-2159.

Gao Z, Chen T, Weber MJ and Linden J (1999) A2B adenosine and P2Y2 receptors stimulate mitogen-activated protein kinase in human embryonic kidney-293

cells. cross-talk between cyclic AMP and protein kinase c pathways. *J Biol Chem* **274**:5972-5980.

Gao ZG and Jacobson KA (2007) Emerging adenosine receptor agonists. *Expert Opin Emerg Drugs* **12**:479-492.

Gao ZG, Kim SK, Ijzerman AP and Jacobson KA (2005) Allosteric modulation of the adenosine family of receptors. *Mini Rev Med Chem* **5**:545-553.

Gesty-Palmer D, Chen M, Reiter E, Ahn S, Nelson CD, Wang S, Eckhardt AE, Cowan CL, Spurney RF, Luttrell LM and Lefkowitz RJ (2006) Distinct beta-arrestin- and G protein-dependent pathways for parathyroid hormone receptor-stimulated ERK1/2 activation. *J Biol Chem* **281**:10856-10864.

Ghanouni P, Gryczynski Z, Steenhuis JJ, Lee TW, Farrens DL, Lakowicz JR and Kobilka BK (2001) Functionally different agonists induce distinct conformations in the G protein coupling domain of the beta 2 adrenergic receptor. *J Biol Chem* **276**:24433-24436.

Gharagozloo P, Lazareno S, Popham A and Birdsall NJ (1999) Allosteric interactions of quaternary strychnine and brucine derivatives with muscarinic acetylcholine receptors. *J Med Chem* **42**:438-445.

Ghosh S and Bell RM (1997) Regulation of Raf-1 kinase by interaction with the lipid second messenger, phosphatidic acid. *Biochem Soc Trans* **25**:561-565.

Giehl K (2005) Oncogenic Ras in tumour progression and metastasis. *Biol Chem* **386**:193-205.

Gnagey AL, Seidenberg M and Ellis J (1999) Site-directed mutagenesis reveals two epitopes involved in the subtype selectivity of the allosteric interactions of gallamine at muscarinic acetylcholine receptors. *Mol Pharmacol* **56**:1245-1253.

Goblyos A, Santiago SN, Pietra D, Mulder-Krieger T, von Frijtag Drabbe Kunzel J, Brussee J and Ijzerman AP (2005) Synthesis and biological evaluation of 2-aminothiazoles and their amide derivatives on human adenosine receptors. Lack of effect of 2-aminothiazoles as allosteric enhancers. *Bioorg Med Chem* **13**:2079-2087.

Gomez-Cambronero J and Keire P (1998) Phospholipase D: a novel major player in signal transduction. *Cell Signal* **10**:387-397.

Gosens R, Dueck G, Gerthoffer WT, Unruh H, Zaagsma J, Meurs H and Halayko AJ (2007a) p42/p44 MAP kinase activation is localized to caveolae-free membrane domains in airway smooth muscle. *Am J Physiol Lung Cell Mol Physiol* **292**:L1163-1172.

Gosens R, Stelmack GL, Dueck G, Mutawe MM, Hinton M, McNeill KD, Paulson A, Dakshinamurti S, Gerthoffer WT, Thliveris JA, Unruh H, Zaagsma J and Halayko AJ (2007b) Caveolae facilitate muscarinic receptor-mediated intracellular Ca2+ mobilization and contraction in airway smooth muscle. *Am J Physiol Lung Cell Mol Physiol* **293**:L1406-1418.

Gosens R, Zaagsma J, Meurs H and Halayko AJ (2006) Muscarinic receptor signaling in the pathophysiology of asthma and COPD. *Respir Res* **7**:73.

Gregory KJ, Sexton PM and Christopoulos A (2007) Allosteric modulation of muscarinic acetylcholine receptors. *Curr Neuropharmacol* **5**:157-167.

Groblewski T, Maigret B, Larguier R, Lombard C, Bonnafous JC and Marie J (1997) Mutation of Asn111 in the third transmembrane domain of the AT1A angiotensin II receptor induces its constitutive activation. *J Biol Chem* **272**:1822-1826.

Gu S, Cifelli C, Wang S and Heximer SP (2009) RGS proteins: identifying new GAPs in the understanding of blood pressure regulation and cardiovascular function. *Clin Sci (Lond)* **116**:391-399.

Guzzi F, Zanchetta D, Cassoni P, Guzzi V, Francolini M, Parenti M and Chini B (2002) Localization of the human oxytocin receptor in caveolin-1 enriched domains turns the receptor-mediated inhibition of cell growth into a proliferative response. *Oncogene* **21**:1658-1667.

Hamm HE (1998) The many faces of G protein signaling. *J Biol Chem* **273**:669-672.

Hansen MR, Krabbe S, Ankorina-Stark I and Novak I (2009) Purinergic receptors stimulate Na+/Ca2+ exchange in pancreatic duct cells: possible role of proteins handling and transporting Ca2+. *Cell Physiol Biochem* **23**:387-396.

Hanyaloglu AC and von Zastrow M (2008) Regulation of GPCRs by endocytic membrane trafficking and its potential implications. *Annu Rev Pharmacol Toxicol* **48**:537-568.

Hasko G and Cronstein BN (2004) Adenosine: an endogenous regulator of innate immunity. *Trends Immunol* **25**:33-39.

Hasko G, Linden J, Cronstein B and Pacher P (2008) Adenosine receptors: therapeutic aspects for inflammatory and immune diseases. *Nat Rev Drug Discov* **7**:759-770.

Heilker R (2006) High content screening to monitor G protein-coupled receptor internalisation. *Ernst Schering Found Symp Proc*:229-247.

Hepler JR (1999) Emerging roles for RGS proteins in cell signalling. *Trends Pharmacol Sci* **20**:376-382.

Herlitze S, Garcia DE, Mackie K, Hille B, Scheuer T and Catterall WA (1996) Modulation of Ca2+ channels by G-protein beta gamma subunits. *Nature* **380**:258-262.

Herroeder S, Reichardt P, Sassmann A, Zimmermann B, Jaeneke D, Hoeckner J, Hollmann MW, Fischer KD, Vogt S, Grosse R, Hogg N, Gunzer M, Offermanns S and Wettschureck N (2009) Guanine nucleotide-binding proteins of the G12 family shape immune functions by controlling CD4+ T cell adhesiveness and motility. *Immunity* **30**:708-720.

Hescheler J and Schultz G (1994) Heterotrimeric G proteins involved in the modulation of voltage-dependent calcium channels of neuroendocrine cells. *Ann N Y Acad Sci* **733**:306-312.

Hilgemann DW, Feng S and Nasuhoglu C (2001) The complex and intriguing lives of PIP2 with ion channels and transporters. *Sci STKE* **2001**:RE19.

Hirano D, Aoki Y, Ogasawara H, Kodama H, Waga I, Sakanaka C, Shimizu T and Nakamura M (1996) Functional coupling of adenosine A2a receptor to inhibition of the mitogen-activated protein kinase cascade in Chinese hamster ovary cells. *Biochem J* **316 (Pt 1)**:81-86.

Hoare SR (2005) Mechanisms of peptide and nonpeptide ligand binding to Class B G-protein-coupled receptors. *Drug Discov Today* **10**:417-427.

Hong-Geller E and Cerione RA (2000) Cdc42 and Rac stimulate exocytosis of secretory granules by activating the IP(3)/calcium pathway in RBL-2H3 mast cells. *J Cell Biol* **148**:481-494.

Horinouchi T, Miyake Y, Nishiya T, Nishimoto A, Morishima S, Muramatsu I and Miwa S (2008) Functional role of Na+/H+ exchanger in Ca2+ influx mediated via human endothelin type A receptor stably expressed in Chinese hamster ovary cells. *J Pharmacol Sci* **107**:456-459.

Horinouchi T, Nishimoto A, Nishiya T, Lu L, Kajita E and Miwa S (2007) Endothelin-1 decreases [Ca2+]i via Na+/Ca2+ exchanger in CHO cells stably expressing endothelin ETA receptor. *Eur J Pharmacol* **566**:28-33.

Hou Y, Chang V, Capper AB, Taussig R and Gautam N (2001) G Protein beta subunit types differentially interact with a muscarinic receptor but not adenylyl cyclase type II or phospholipase C-beta 2/3. *J Biol Chem* **276**:19982-19988.

Houslay MD and Milligan G (1997) Tailoring cAMP-signalling responses through isoform multiplicity. *Trends Biochem Sci* **22**:217-224.

Huang XP, Prilla S, Mohr K and Ellis J (2005) Critical amino acid residues of the common allosteric site on the M2 muscarinic acetylcholine receptor: more similarities than differences between the structurally divergent agents gallamine and bis(ammonio)alkane-type hexamethylene-bis-[dimethyl-(3-phthalimidopropyl)ammonium]dibromide. *Mol Pharmacol* **68**:769-778.

Huovila AP, Turner AJ, Pelto-Huikko M, Karkkainen I and Ortiz RM (2005) Shedding light on ADAM metalloproteinases. *Trends Biochem Sci* **30**:413-422.

Hur EM and Kim KT (2002) G protein-coupled receptor signalling and cross-talk: achieving rapidity and specificity. *Cell Signal* **14**:397-405.

Hwang JI, Heo K, Shin KJ, Kim E, Yun C, Ryu SH, Shin HS and Suh PG (2000) Regulation of phospholipase C-beta 3 activity by Na+/H+ exchanger regulatory factor 2. *J Biol Chem* **275**:16632-16637.

Iarriccio L (2008) Allosteric interactions at the M_3 muscarinic acetylcholine receptor, in *Chemical Engineering and Physical Biochemistry*, Polytechnic University and National Institute of Medical Research.

Ifuku M, Farber K, Okuno Y, Yamakawa Y, Miyamoto T, Nolte C, Merrino VF, Kita S, Iwamoto T, Komuro I, Wang B, Cheung G, Ishikawa E, Ooboshi H, Bader M, Wada K, Kettenmann H and Noda M (2007) Bradykinin-induced microglial migration mediated by B1-bradykinin receptors depends on Ca2+ influx via reverse-mode activity of the Na+/Ca2+ exchanger. *J Neurosci* **27**:13065-13073.

Ishii J, Matsumura S, Kimura S, Tatematsu K, Kuroda S, Fukuda H and Kondo A (2006) Quantitative and dynamic analyses of G protein-coupled receptor signaling in yeast using Fus1, enhanced green fluorescence protein (EGFP), and His3 fusion protein. *Biotechnol Prog* **22**:954-960.

Jaakola VP, Griffith MT, Hanson MA, Cherezov V, Chien EY, Lane JR, Ijzerman AP and Stevens RC (2008) The 2.6 angstrom crystal structure of a human A2A adenosine receptor bound to an antagonist. *Science* **322**:1211-1217.

Jacobowitz O, Chen J, Premont RT and Iyengar R (1993) Stimulation of specific types of Gs-stimulated adenylyl cyclases by phorbol ester treatment. *J Biol Chem* **268**:3829-3832.

Jacobowitz O and Iyengar R (1994) Phorbol ester-induced stimulation and phosphorylation of adenylyl cyclase 2. *Proc Natl Acad Sci U S A* **91**:10630-10634.

Jakubik J, Bacakova L, Lisa V, el-Fakahany EE and Tucek S (1996) Activation of muscarinic acetylcholine receptors via their allosteric binding sites. *Proc Natl Acad Sci U S A* **93**:8705-8709.

Jakubik J, Krejci A and Dolezal V (2005) Asparagine, valine, and threonine in the third extracellular loop of muscarinic receptor have essential roles in the positive cooperativity of strychnine-like allosteric modulators. *J Pharmacol Exp Ther* **313**:688-696.

Janowski E, Day R, Kraev A, Roder JC, Cleemann L and Morad M (2009) {beta}-Adrenergic regulation of a novel isoform of NCX: sequence and expression of

shark heart NCX in human kidney cells. *Am J Physiol Heart Circ Physiol* **296**:H1994-2006.

Jasper JR, Michel MC and Insel PA (1990) Amplification of cyclic AMP generation reveals agonistic effects of certain beta-adrenergic antagonists. *Mol Pharmacol* **37**:44-49.

Jensen AA, Hansen JL, Sheikh SP and Brauner-Osborne H (2002) Probing intermolecular protein-protein interactions in the calcium-sensing receptor homodimer using bioluminescence resonance energy transfer (BRET). *Eur J Biochem* **269**:5076-5087.

Jockers R, Linder ME, Hohenegger M, Nanoff C, Bertin B, Strosberg AD, Marullo S and Freissmuth M (1994) Species difference in the G protein selectivity of the human and bovine A1-adenosine receptor. *J Biol Chem* **269**:32077-32084.

Jones GA and Carpenter G (1993) The regulation of phospholipase C-gamma 1 by phosphatidic acid. Assessment of kinetic parameters. *J Biol Chem* **268**:20845-20850.

Kamenetsky M, Middelhaufe S, Bank EM, Levin LR, Buck J and Steegborn C (2006) Molecular details of cAMP generation in mammalian cells: a tale of two systems. *J Mol Biol* **362**:623-639.

Katan M (1998) Families of phosphoinositide-specific phospholipase C: structure and function. *Biochim Biophys Acta* **1436**:5-17.

Kelley GG, Reks SE, Ondrako JM and Smrcka AV (2001) Phospholipase C(epsilon): a novel Ras effector. *Embo J* **20**:743-754.

Kelly E, Bailey CP and Henderson G (2008) Agonist-selective mechanisms of GPCR desensitization. *Br J Pharmacol* **153 Suppl 1**:S379-388.

Kenakin T (1995) Agonist-receptor efficacy. II. Agonist trafficking of receptor signals. *Trends Pharmacol Sci* **16**:232-238.

Kenakin T (2001) Inverse, protean, and ligand-selective agonism: matters of receptor conformation. *Faseb J* **15**:598-611.

Kenakin T (2003a) Ligand-selective receptor conformations revisited: the promise and the problem. *Trends Pharmacol Sci* **24**:346-354.

Kenakin TP (2003b) *A pharmacology primer : theory, application and methods*. Academic, San Diego, Calif. London.

Kobilka B (2009) Structure and dynamics of the human β2-adrenoceptor, in *British Pharmacological Society - Cell Signalling*, Leicester, UK.

Kobilka BK and Deupi X (2007) Conformational complexity of G-protein-coupled receptors. *Trends Pharmacol Sci* **28**:397-406.

Kohout TA and Lefkowitz RJ (2003) Regulation of G protein-coupled receptor kinases and arrestins during receptor desensitization. *Mol Pharmacol* **63**:9-18.

Kourounakis A, Visser C, de Groote M and AP IJ (2001) Differential effects of the allosteric enhancer (2-amino-4,5-dimethyl-trienyl)[3-trifluoromethyl) phenyl]methanone (PD81,723) on agonist and antagonist binding and function at the human wild-type and a mutant (T277A) adenosine A1 receptor. *Biochem Pharmacol* **61**:137-144.

Kraakman L, Lemaire K, Ma P, Teunissen AW, Donaton MC, Van Dijck P, Winderickx J, de Winde JH and Thevelein JM (1999) A Saccharomyces cerevisiae G-protein coupled receptor, Gpr1, is specifically required for glucose activation of the cAMP pathway during the transition to growth on glucose. *Mol Microbiol* **32**:1002-1012.

Krasnoperov V, Lu Y, Buryanovsky L, Neubert TA, Ichtchenko K and Petrenko AG (2002) Post-translational proteolytic processing of the calcium-independent

receptor of alpha-latrotoxin (CIRL), a natural chimera of the cell adhesion protein and the G protein-coupled receptor. Role of the G protein-coupled receptor proteolysis site (GPS) motif. *J Biol Chem* **277**:46518-46526.

Krejci A and Tucek S (2001) Changes of cooperativity between N-methylscopolamine and allosteric modulators alcuronium and gallamine induced by mutations of external loops of muscarinic M(3) receptors. *Mol Pharmacol* **60**:761-767.

Kristiansen K (2004) Molecular mechanisms of ligand binding, signaling, and regulation within the superfamily of G-protein-coupled receptors: molecular modeling and mutagenesis approaches to receptor structure and function. *Pharmacol Ther* **103**:21-80.

Krupinski J, Coussen F, Bakalyar HA, Tang WJ, Feinstein PG, Orth K, Slaughter C, Reed RR and Gilman AG (1989) Adenylyl cyclase amino acid sequence: possible channel- or transporter-like structure. *Science* **244**:1558-1564.

Kunishima N, Shimada Y, Tsuji Y, Sato T, Yamamoto M, Kumasaka T, Nakanishi S, Jingami H and Morikawa K (2000) Structural basis of glutamate recognition by a dimeric metabotropic glutamate receptor. *Nature* **407**:971-977.

Kurokawa K, Nakamura T, Aoki K and Matsuda M (2005) Mechanism and role of localized activation of Rho-family GTPases in growth factor-stimulated fibroblasts and neuronal cells. *Biochem Soc Trans* **33**:631-634.

Lagerstrom MC and Schioth HB (2008) Structural diversity of G protein-coupled receptors and significance for drug discovery. *Nat Rev Drug Discov* **7**:339-357.

Lai HH, Boone TB, Yang G, Smith CP, Kiss S, Thompson TC and Somogyi GT (2004) Loss of caveolin-1 expression is associated with disruption of muscarinic cholinergic activities in the urinary bladder. *Neurochem Int* **45**:1185-1193.

Landry Y, Niederhoffer N, Sick E and Gies JP (2006) Heptahelical and other G-protein-coupled receptors (GPCRs) signaling. *Curr Med Chem* **13**:51-63.

Langmead CJ and Christopoulos A (2006) Allosteric agonists of 7TM receptors: expanding the pharmacological toolbox. *Trends Pharmacol Sci* **27**:475-481.

Langmead CJ, Watson J and Reavill C (2008) Muscarinic acetylcholine receptors as CNS drug targets. *Pharmacol Ther* **117**:232-243.

Lazareno S and Birdsall NJ (1995) Detection, quantitation, and verification of allosteric interactions of agents with labeled and unlabeled ligands at G protein-coupled receptors: interactions of strychnine and acetylcholine at muscarinic receptors. *Mol Pharmacol* **48**:362-378.

Lazareno S, Gharagozloo P, Kuonen D, Popham A and Birdsall NJ (1998) Subtype-selective positive cooperative interactions between brucine analogues and acetylcholine at muscarinic receptors: radioligand binding studies. *Mol Pharmacol* **53**:573-589.

Leach K, Sexton PM and Christopoulos A (2007) Allosteric GPCR modulators: taking advantage of permissive receptor pharmacology. *Trends Pharmacol Sci* **28**:382-389.

Lee PH (2009) Label-free optical biosensor: A tool for G protein-coupled receptors pharmacology profiling and inverse agonists identification. *J Recept Signal Transduct Res*.

Leff P, Prentice DJ, Giles H, Martin GR and Wood J (1990) Estimation of agonist affinity and efficacy by direct, operational model-fitting. *J Pharmacol Methods* **23**:225-237.

Leff P, Scaramellini C, Law C and McKechnie K (1997) A three-state receptor model of agonist action. *Trends Pharmacol Sci* **18**:355-362.

Lefkimmiatis K, Srikanthan M, Maiellaro I, Moyer MP, Curci S and Hofer AM (2009) Store-operated cyclic AMP signalling mediated by STIM1. *Nat Cell Biol* **11**:433-442.

Lefkowitz RJ (1998) G protein-coupled receptors. III. New roles for receptor kinases and beta-arrestins in receptor signaling and desensitization. *J Biol Chem* **273**:18677-18680.

Leskinen P, Hilscherova K, Sidlova T, Kiviranta H, Pessala P, Salo S, Verta M and Virta M (2008) Detecting AhR ligands in sediments using bioluminescent reporter yeast. *Biosens Bioelectron* **23**:1850-1855.

Levitzki A (1988) From epinephrine to cyclic AMP. *Science* **241**:800-806.

Lew MJ and Angus JA (1995) Analysis of competitive agonist-antagonist interactions by nonlinear regression. *Trends Pharmacol Sci* **16**:328-337.

Li B, Scarselli M, Knudsen CD, Kim SK, Jacobson KA, McMillin SM and Wess J (2007) Rapid identification of functionally critical amino acids in a G protein-coupled receptor. *Nat Methods* **4**:169-174.

Li J, Yen C, Liaw D, Podsypanina K, Bose S, Wang SI, Puc J, Miliaresis C, Rodgers L, McCombie R, Bigner SH, Giovanella BC, Ittmann M, Tycko B, Hibshoosh H, Wigler MH and Parsons R (1997) PTEN, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer. *Science* **275**:1943-1947.

Li S, Okamoto T, Chun M, Sargiacomo M, Casanova JE, Hansen SH, Nishimoto I and Lisanti MP (1995) Evidence for a regulated interaction between heterotrimeric G proteins and caveolin. *J Biol Chem* **270**:15693-15701.

Liao Y, Erxleben C, Abramowitz J, Flockerzi V, Zhu MX, Armstrong DL and Birnbaumer L (2008) Functional interactions among Orai1, TRPCs, and STIM1 suggest a STIM-regulated heteromeric Orai/TRPC model for SOCE/Icrac channels. *Proc Natl Acad Sci U S A* **105**:2895-2900.

Liggett SB, Freedman NJ, Schwinn DA and Lefkowitz RJ (1993) Structural basis for receptor subtype-specific regulation revealed by a chimeric beta 3/beta 2-adrenergic receptor. *Proc Natl Acad Sci U S A* **90**:3665-3669.

Linden J (2001) Molecular approach to adenosine receptors: receptor-mediated mechanisms of tissue protection. *Annu Rev Pharmacol Toxicol* **41**:775-787.

Lohse MJ, Benovic JL, Codina J, Caron MG and Lefkowitz RJ (1990) beta-Arrestin: a protein that regulates beta-adrenergic receptor function. *Science* **248**:1547-1550.

Lopez-Ilasaca M, Crespo P, Pellici PG, Gutkind JS and Wetzker R (1997) Linkage of G protein-coupled receptors to the MAPK signaling pathway through PI 3-kinase gamma. *Science* **275**:394-397.

Luthin GR, Wang P, Zhou H, Dhanasekaran D and Ruggieri MR (1997) Role of m1 receptor-G protein coupling in cell proliferation in the prostate. *Life Sci* **60**:963-968.

Luttrell LM, Ferguson SS, Daaka Y, Miller WE, Maudsley S, Della Rocca GJ, Lin F, Kawakatsu H, Owada K, Luttrell DK, Caron MG and Lefkowitz RJ (1999) Beta-arrestin-dependent formation of beta2 adrenergic receptor-Src protein kinase complexes. *Science* **283**:655-661.

Luttrell LM, Hawes BE, van Biesen T, Luttrell DK, Lansing TJ and Lefkowitz RJ (1996) Role of c-Src tyrosine kinase in G protein-coupled receptor- and Gbetagamma subunit-mediated activation of mitogen-activated protein kinases. *J Biol Chem* **271**:19443-19450.

Luttrell LM, Roudabush FL, Choy EW, Miller WE, Field ME, Pierce KL and Lefkowitz RJ (2001) Activation and targeting of extracellular signal-regulated kinases by beta-arrestin scaffolds. *Proc Natl Acad Sci U S A* **98**:2449-2454.

Lutz S, Freichel-Blomquist A, Yang Y, Rumenapp U, Jakobs KH, Schmidt M and Wieland T (2005) The guanine nucleotide exchange factor p63RhoGEF, a specific link between Gq/11-coupled receptor signaling and RhoA. *J Biol Chem* **280**:11134-11139.

Maehama T and Dixon JE (1998) The tumor suppressor, PTEN/MMAC1, dephosphorylates the lipid second messenger, phosphatidylinositol 3,4,5-trisphosphate. *J Biol Chem* **273**:13375-13378.

Mahaut-Smith MP, Martinez-Pinna J and Gurung IS (2008) A role for membrane potential in regulating GPCRs? *Trends Pharmacol Sci* **29**:421-429.

Maillet EL, Pellegrini N, Valant C, Bucher B, Hibert M, Bourguignon JJ and Galzi JL (2007) A novel, conformation-specific allosteric inhibitor of the tachykinin NK2 receptor (NK2R) with functionally selective properties. *Faseb J* **21**:2124-2134.

Makita N, Sato J, Manaka K, Shoji Y, Oishi A, Hashimoto M, Fujita T and Iiri T (2007) An acquired hypocalciuric hypercalcemia autoantibody induces allosteric transition among active human Ca-sensing receptor conformations. *Proc Natl Acad Sci U S A* **104**:5443-5448.

Marchese A, Paing MM, Temple BR and Trejo J (2008) G protein-coupled receptor sorting to endosomes and lysosomes. *Annu Rev Pharmacol Toxicol* **48**:601-629.

Mark MD and Herlitze S (2000) G-protein mediated gating of inward-rectifier K+ channels. *Eur J Biochem* **267**:5830-5836.

Mark MD, Wittemann S and Herlitze S (2000) G protein modulation of recombinant P/Q-type calcium channels by regulators of G protein signalling proteins. *J Physiol* **528 Pt 1**:65-77.

Mason HR, Swanson JK, Ho J and Patton TJ (2008) Interstitial granulomatous dermatitis associated with darifenacin. *J Drugs Dermatol* **7**:895-897.

Mathiesen JM, Ulven T, Martini L, Gerlach LO, Heinemann A and Kostenis E (2005) Identification of indole derivatives exclusively interfering with a G protein-independent signaling pathway of the prostaglandin D2 receptor CRTH2. *Mol Pharmacol* **68**:393-402.

May LT, Avlani VA, Langmead CJ, Herdon HJ, Wood MD, Sexton PM and Christopoulos A (2007a) Structure-function studies of allosteric agonism at M2 muscarinic acetylcholine receptors. *Mol Pharmacol* **72**:463-476.

May LT and Christopoulos A (2003) Allosteric modulators of G-protein-coupled receptors. *Curr Opin Pharmacol* **3**:551-556.

May LT, Leach K, Sexton PM and Christopoulos A (2007b) Allosteric modulation of G protein-coupled receptors. *Annu Rev Pharmacol Toxicol* **47**:1-51.

May LT, Sexton PM and Christopoulos A (2005) Effects of urea pretreatment on the binding properties of adenosine A1 receptors. *Br J Pharmacol* **146**:1119-1129.

Meller J, Vidali L and Schwartz MA (2008) Endogenous RhoG is dispensable for integrin-mediated cell spreading but contributes to Rac-independent migration. *J Cell Sci* **121**:1981-1989.

Migita K, Lu L, Zhao Y, Honda K, Iwamoto T, Kita S and Katsuragi T (2005) Adenosine induces ATP release via an inositol 1,4,5-trisphosphate signaling pathway in MDCK cells. *Biochem Biophys Res Commun* **328**:1211-1215.

Miller LJ, Dong M, Harikumar KG and Gao F (2007) Structural basis of natural ligand binding and activation of the Class II G-protein-coupled secretin receptor. *Biochem Soc Trans* **35**:709-712.

Milligan G (2007) G protein-coupled receptor dimerisation: molecular basis and relevance to function. *Biochim Biophys Acta* **1768**:825-835.

Minelli A, Bellezza I, Collodel G and Fredholm BB (2008) Promiscuous coupling and involvement of protein kinase C and extracellular signal-regulated kinase 1/2 in the adenosine A1 receptor signalling in mammalian spermatozoa. *Biochem Pharmacol* **75**:931-941.

Mineo C, James GL, Smart EJ and Anderson RG (1996) Localization of epidermal growth factor-stimulated Ras/Raf-1 interaction to caveolae membrane. *J Biol Chem* **271**:11930-11935.

Minic J, Persuy MA, Godel E, Aioun J, Connerton I, Salesse R and Pajot-Augy E (2005a) Functional expression of olfactory receptors in yeast and development of a bioassay for odorant screening. *Febs J* **272**:524-537.

Minic J, Sautel M, Salesse R and Pajot-Augy E (2005b) Yeast system as a screening tool for pharmacological assessment of g protein coupled receptors. *Curr Med Chem* **12**:961-969.

Miralles F and Visa N (2006) Actin in transcription and transcription regulation. *Curr Opin Cell Biol* **18**:261-266.

Miret JJ, Rakhilina L, Silverman L and Oehlen B (2002) Functional expression of heteromeric calcitonin gene-related peptide and adrenomedullin receptors in yeast. *J Biol Chem* **277**:6881-6887.

Mishra VK, Palgunachari MN, Segrest JP and Anantharamaiah GM (1994) Interactions of synthetic peptide analogs of the class A amphipathic helix with lipids. Evidence for the snorkel hypothesis. *J Biol Chem* **269**:7185-7191.

Monod J, Changeux JP and Jacob F (1963) Allosteric proteins and cellular control systems. *J Mol Biol* **6**:306-329.

Motulsky HJ and Christopoulos A (2004) *Fitting models to biological data using linear and nonlinear regression. A practical guide to curve fitting. New York, Oxford University Press.*

Murphree LJ, Marshall MA, Rieger JM, MacDonald TL and Linden J (2002) Human A(2A) adenosine receptors: high-affinity agonist binding to receptor-G protein complexes containing Gbeta(4). *Mol Pharmacol* **61**:455-462.

Narumiya S, Tanji M and Ishizaki T (2009) Rho signaling, ROCK and mDia1, in transformation, metastasis and invasion. *Cancer Metastasis Rev* **28**:65-76.

Nawaratne V, Leach K, Suratman N, Loiacono RE, Felder CC, Armbruster BN, Roth BL, Sexton PM and Christopoulos A (2008) New insights into the function of M4 muscarinic acetylcholine receptors gained using a novel allosteric modulator and a DREADD (designer receptor exclusively activated by a designer drug). *Mol Pharmacol* **74**:1119-1131.

Neubig RR (1994) Membrane organization in G-protein mechanisms. *Faseb J* **8**:939-946.

Neubig RR and Siderovski DP (2002) Regulators of G-protein signalling as new central nervous system drug targets. *Nat Rev Drug Discov* **1**:187-197.

Nobles M, Benians A and Tinker A (2005) Heterotrimeric G proteins precouple with G protein-coupled receptors in living cells. *Proc Natl Acad Sci U S A* **102**:18706-18711.

Noda M, Yasuda-Fukazawa C, Moriishi K, Kato T, Okuda T, Kurokawa K and Takuwa Y (1995) Involvement of rho in GTP gamma S-induced enhancement of phosphorylation of 20 kDa myosin light chain in vascular smooth muscle cells: inhibition of phosphatase activity. *FEBS Lett* **367**:246-250.

Nordstrom KJ, Lagerstrom MC, Waller LM, Fredriksson R and Schioth HB (2009) The Secretin GPCRs descended from the family of Adhesion GPCRs. *Mol Biol Evol* **26**:71-84.

Norman JC, Price LS, Ridley AJ and Koffer A (1996) The small GTP-binding proteins, Rac and Rho, regulate cytoskeletal organization and exocytosis in mast cells by parallel pathways. *Mol Biol Cell* **7**:1429-1442.

Obiefuna PC, Batra VK, Nadeem A, Borron P, Wilson CN and Mustafa SJ (2005) A novel A1 adenosine receptor antagonist, L-97-1 [3-[2-(4-aminophenyl)-ethyl]-8-benzyl-7-{2-ethyl-(2-hydroxy-ethyl)-amino]- ethyl}-1-propyl-3,7-dihydro-purine-2,6-dione], reduces allergic responses to house dust mite in an allergic rabbit model of asthma. *J Pharmacol Exp Ther* **315**:329-336.

Ocana M, Cendan CM, Cobos EJ, Entrena JM and Baeyens JM (2004) Potassium channels and pain: present realities and future opportunities. *Eur J Pharmacol* **500**:203-219.

Ohtsu H, Dempsey PJ and Eguchi S (2006) ADAMs as mediators of EGF receptor transactivation by G protein-coupled receptors. *Am J Physiol Cell Physiol* **291**:C1-10.

Okamoto T, Schlegel A, Scherer PE and Lisanti MP (1998) Caveolins, a family of scaffolding proteins for organizing "preassembled signaling complexes" at the plasma membrane. *J Biol Chem* **273**:5419-5422.

Onohara N, Nishida M, Inoue R, Kobayashi H, Sumimoto H, Sato Y, Mori Y, Nagao T and Kurose H (2006) TRPC3 and TRPC6 are essential for angiotensin II-induced cardiac hypertrophy. *Embo J* **25**:5305-5316.

Ostrom RS, Post SR and Insel PA (2000) Stoichiometry and compartmentation in G protein-coupled receptor signaling: implications for therapeutic interventions involving G(s). *J Pharmacol Exp Ther* **294**:407-412.

Ozdirekcan S, Rijkers DT, Liskamp RM and Killian JA (2005) Influence of flanking residues on tilt and rotation angles of transmembrane peptides in lipid bilayers. A solid-state 2H NMR study. *Biochemistry* **44**:1004-1012.

Palczewski K, Kumasaka T, Hori T, Behnke CA, Motoshima H, Fox BA, Le Trong I, Teller DC, Okada T, Stenkamp RE, Yamamoto M and Miyano M (2000) Crystal structure of rhodopsin: A G protein-coupled receptor. *Science* **289**:739-745.

Patel HH, Murray F and Insel PA (2008) Caveolae as organizers of pharmacologically relevant signal transduction molecules. *Annu Rev Pharmacol Toxicol* **48**:359-391.

Patel M, McInnes IB and Graham G (2009) Atypical chemokine receptors in inflammatory disease. *Curr Mol Med* **9**:86-93.

Pausch MH (1997) G-protein-coupled receptors in Saccharomyces cerevisiae: high-throughput screening assays for drug discovery. *Trends Biotechnol* **15**:487-494.

Peleg S, Varon D, Ivanina T, Dessauer CW and Dascal N (2002) G(alpha)(i) controls the gating of the G protein-activated K(+) channel, GIRK. *Neuron* **33**:87-99.

Peralta EG, Ashkenazi A, Winslow JW, Smith DH, Ramachandran J and Capon DJ (1987) Distinct primary structures, ligand-binding properties and tissue-specific expression of four human muscarinic acetylcholine receptors. *Embo J* **6**:3923-3929.

Pertz O, Hodgson L, Klemke RL and Hahn KM (2006) Spatiotemporal dynamics of RhoA activity in migrating cells. *Nature* **440**:1069-1072.

Pin JP and Acher F (2002) The metabotropic glutamate receptors: structure, activation mechanism and pharmacology. *Curr Drug Targets CNS Neurol Disord* **1**:297-317.

Pin JP, Kniazeff J, Binet V, Liu J, Maurel D, Galvez T, Duthey B, Havlickova M, Blahos J, Prezeau L and Rondard P (2004a) Activation mechanism of the heterodimeric GABA(B) receptor. *Biochem Pharmacol* **68**:1565-1572.

Pin JP, Kniazeff J, Goudet C, Bessis AS, Liu J, Galvez T, Acher F, Rondard P and Prezeau L (2004b) The activation mechanism of class-C G-protein coupled receptors. *Biol Cell* **96**:335-342.

Pitcher JA, Freedman NJ and Lefkowitz RJ (1998) G protein-coupled receptor kinases. *Annu Rev Biochem* **67**:653-692.

Powrie DJ, Wilkinson TM, Donaldson GC, Jones P, Scrine K, Viel K, Kesten S and Wedzicha JA (2007) Effect of tiotropium on sputum and serum inflammatory markers and exacerbations in COPD. *Eur Respir J* **30**:472-478.

Price LA, Kajkowski EM, Hadcock JR, Ozenberger BA and Pausch MH (1995a) Functional coupling of a mammalian somatostatin receptor to the yeast pheromone response pathway. *Mol Cell Biol* **15**:6188-6195.

Price LA, Strnad J, Pausch MH and Hadcock JR (1996) Pharmacological characterization of the rat A2a adenosine receptor functionally coupled to the yeast pheromone response pathway. *Mol Pharmacol* **50**:829-837.

Price LS, Norman JC, Ridley AJ and Koffer A (1995b) The small GTPases Rac and Rho as regulators of secretion in mast cells. *Curr Biol* **5**:68-73.

Price MR, Baillie GL, Thomas A, Stevenson LA, Easson M, Goodwin R, McLean A, McIntosh L, Goodwin G, Walker G, Westwood P, Marrs J, Thomson F, Cowley P, Christopoulos A, Pertwee RG and Ross RA (2005) Allosteric modulation of the cannabinoid CB1 receptor. *Mol Pharmacol* **68**:1484-1495.

Pucadyil TJ and Chattopadhyay A (2004) Cholesterol modulates ligand binding and G-protein coupling to serotonin(1A) receptors from bovine hippocampus. *Biochim Biophys Acta* **1663**:188-200.

Rasmussen SG, Choi HJ, Rosenbaum DM, Kobilka TS, Thian FS, Edwards PC, Burghammer M, Ratnala VR, Sanishvili R, Fischetti RF, Schertler GF, Weis WI and Kobilka BK (2007) Crystal structure of the human beta2 adrenergic G-protein-coupled receptor. *Nature* **450**:383-387.

Reinheimer T, Bernedo P, Klapproth H, Oelert H, Zeiske B, Racke K and Wessler I (1996) Acetylcholine in isolated airways of rat, guinea pig, and human: species differences in role of airway mucosa. *Am J Physiol* **270**:L722-728.

Reiter E and Lefkowitz RJ (2006) GRKs and beta-arrestins: roles in receptor silencing, trafficking and signaling. *Trends Endocrinol Metab* **17**:159-165.

Ren XR, Reiter E, Ahn S, Kim J, Chen W and Lefkowitz RJ (2005) Different G protein-coupled receptor kinases govern G protein and beta-arrestin-mediated signaling of V2 vasopressin receptor. *Proc Natl Acad Sci U S A* **102**:1448-1453.

Rhee SG (2001) Regulation of phosphoinositide-specific phospholipase C. *Annu Rev Biochem* **70**:281-312.

Ribas C, Penela P, Murga C, Salcedo A, Garcia-Hoz C, Jurado-Pueyo M, Aymerich I and Mayor F, Jr. (2007) The G protein-coupled receptor kinase (GRK) interactome: role of GRKs in GPCR regulation and signaling. *Biochim Biophys Acta* **1768**:913-922.

Ridley AJ (2006) Rho GTPases and actin dynamics in membrane protrusions and vesicle trafficking. *Trends Cell Biol* **16**:522-529.

Ridley AJ and Hall A (1992) The small GTP-binding protein rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors. *Cell* **70**:389-399.

Ridley AJ, Paterson HF, Johnston CL, Diekmann D and Hall A (1992) The small GTP-binding protein rac regulates growth factor-induced membrane ruffling. *Cell* **70**:401-410.

Rinner I, Kawashima K and Schauenstein K (1998) Rat lymphocytes produce and secrete acetylcholine in dependence of differentiation and activation. *J Neuroimmunol* **81**:31-37.

Rivkees SA, Barbhaiya H and AP IJ (1999) Identification of the adenine binding site of the human A1 adenosine receptor. *J Biol Chem* **274**:3617-3621.

Rodrigues DJ and McLoughlin D (2009) Using reporter gene technologies to detect changes in cAMP as a result of GPCR activation. *Methods Mol Biol* **552**:319-328.

Roettger BF, Ghanekar D, Rao R, Toledo C, Yingling J, Pinon D and Miller LJ (1997) Antagonist-stimulated internalization of the G protein-coupled cholecystokinin receptor. *Mol Pharmacol* **51**:357-362.

Rosenbaum DM, Rasmussen SG and Kobilka BK (2009) The structure and function of G-protein-coupled receptors. *Nature* **459**:356-363.

Rossman KL, Der CJ and Sondek J (2005) GEF means go: turning on RHO GTPases with guanine nucleotide-exchange factors. *Nat Rev Mol Cell Biol* **6**:167-180.

Rumenapp U, Asmus M, Schablowski H, Woznicki M, Han L, Jakobs KH, Fahimi-Vahid M, Michalek C, Wieland T and Schmidt M (2001) The M3 muscarinic acetylcholine receptor expressed in HEK-293 cells signals to phospholipase D via G12 but not Gq-type G proteins: regulators of G proteins as tools to dissect pertussis toxin-resistant G proteins in receptor-effector coupling. *J Biol Chem* **276**:2474-2479.

Salhia B, Rutten F, Nakada M, Beaudry C, Berens M, Kwan A and Rutka JT (2005) Inhibition of Rho-kinase affects astrocytoma morphology, motility, and invasion through activation of Rac1. *Cancer Res* **65**:8792-8800.

Salom D, Lodowski DT, Stenkamp RE, Le Trong I, Golczak M, Jastrzebska B, Harris T, Ballesteros JA and Palczewski K (2006) Crystal structure of a photoactivated deprotonated intermediate of rhodopsin. *Proc Natl Acad Sci U S A* **103**:16123-16128.

Sands WA and Palmer TM (2008) Regulating gene transcription in response to cyclic AMP elevation. *Cell Signal* **20**:460-466.

Santafe MM, Garcia N, Lanuza MA, Tomas M, Besalduch N and Tomas J (2009) Presynaptic muscarinic receptors, calcium channels, and protein kinase C modulate the functional disconnection of weak inputs at polyinnervated neonatal neuromuscular synapses. *J Neurosci Res* **87**:1195-1206.

Sargiacomo M, Sudol M, Tang Z and Lisanti MP (1993) Signal transducing molecules and glycosyl-phosphatidylinositol-linked proteins form a caveolin-rich insoluble complex in MDCK cells. *J Cell Biol* **122**:789-807.

Sato E, Koyama S, Okubo Y, Kubo K and Sekiguchi M (1998) Acetylcholine stimulates alveolar macrophages to release inflammatory cell chemotactic activity. *Am J Physiol* **274**:L970-979.

Sato M, Horinouchi T, Hutchinson DS, Evans BA and Summers RJ (2007) Ligand-directed signaling at the beta3-adrenoceptor produced by 3-(2-Ethylphenoxy)-1-[(1,S)-1,2,3,4-tetrahydronapth-1-ylamino]-2S-2-propan ol oxalate (SR59230A) relative to receptor agonists. *Mol Pharmacol* **72**:1359-1368.

Scarselli M, Li B, Kim SK and Wess J (2007) Multiple residues in the second extracellular loop are critical for M3 muscarinic acetylcholine receptor activation. *J Biol Chem* **282**:7385-7396.

Scheer A, Fanelli F, Costa T, De Benedetti PG and Cotecchia S (1996) Constitutively active mutants of the alpha 1B-adrenergic receptor: role of highly conserved polar amino acids in receptor activation. *Embo J* **15**:3566-3578.

Schmidt C, Li B, Bloodworth L, Erlenbach I, Zeng FY and Wess J (2003) Random mutagenesis of the M3 muscarinic acetylcholine receptor expressed in yeast. Identification of point mutations that "silence" a constitutively active mutant M3 receptor and greatly impair receptor/G protein coupling. *J Biol Chem* **278**:30248-30260.

Schulte G and Bryja V (2007) The Frizzled family of unconventional G-protein-coupled receptors. *Trends Pharmacol Sci* **28**:518-525.

Schulte G and Fredholm BB (2003) Signalling from adenosine receptors to mitogen-activated protein kinases. *Cell Signal* **15**:813-827.

Selbie LA and Hill SJ (1998) G protein-coupled-receptor cross-talk: the fine-tuning of multiple receptor-signalling pathways. *Trends Pharmacol Sci* **19**:87-93.

Sexton PM, Morfis M, Tilakaratne N, Hay DL, Udawela M, Christopoulos G and Christopoulos A (2006) Complexing receptor pharmacology: modulation of family B G protein-coupled receptor function by RAMPs. *Ann N Y Acad Sci* **1070**:90-104.

Sharif NA, To ZP, Wong KH, Delmendo RE, Whiting RL and Eglen RM (1995) M3 muscarinic receptors on murine HSDM1C1 cells: further functional, regulatory, and receptor binding studies. *Neurochem Res* **20**:61-68.

Shenoy SK, Drake MT, Nelson CD, Houtz DA, Xiao K, Madabushi S, Reiter E, Premont RT, Lichtarge O and Lefkowitz RJ (2006) beta-arrestin-dependent, G protein-independent ERK1/2 activation by the beta2 adrenergic receptor. *J Biol Chem* **281**:1261-1273.

Shi L, Liapakis G, Xu R, Guarnieri F, Ballesteros JA and Javitch JA (2002) Beta2 adrenergic receptor activation. Modulation of the proline kink in transmembrane 6 by a rotamer toggle switch. *J Biol Chem* **277**:40989-40996.

Shimamura T, Hiraki K, Takahashi N, Hori T, Ago H, Masuda K, Takio K, Ishiguro M and Miyano M (2008) Crystal structure of squid rhodopsin with intracellularly extended cytoplasmic region. *J Biol Chem* **283**:17753-17756.

Shirey JK, Xiang Z, Orton D, Brady AE, Johnson KA, Williams R, Ayala JE, Rodriguez AL, Wess J, Weaver D, Niswender CM and Conn PJ (2008) An allosteric potentiator of M4 mAChR modulates hippocampal synaptic transmission. *Nat Chem Biol* **4**:42-50.

Siderovski DP and Willard FS (2005) The GAPs, GEFs, and GDIs of heterotrimeric G-protein alpha subunits. *Int J Biol Sci* **1**:51-66.

Smith CM and Wallis RM (1997) Characterisation of [3H]-darifenacin as a novel radioligand for the study of muscarinic M3 receptors. *J Recept Signal Transduct Res* **17**:177-184.

Stewart AJ, Morgan K, Farquharson C and Millar RP (2007) Phospholipase C-eta enzymes as putative protein kinase C and Ca2+ signalling components in neuronal and neuroendocrine tissues. *Neuroendocrinology* **86**:243-248.

Stockton JM, Birdsall NJ, Burgen AS and Hulme EC (1983) Modification of the binding properties of muscarinic receptors by gallamine. *Mol Pharmacol* **23**:551-557.

Suh PG, Hwang JI, Ryu SH, Donowitz M and Kim JH (2001) The roles of PDZ-containing proteins in PLC-beta-mediated signaling. *Biochem Biophys Res Commun* **288**:1-7.

Suh PG, Park JI, Manzoli L, Cocco L, Peak JC, Katan M, Fukami K, Kataoka T, Yun S and Ryu SH (2008) Multiple roles of phosphoinositide-specific phospholipase C isozymes. *BMB Rep* **41**:415-434.

Sunahara RK, Dessauer CW and Gilman AG (1996) Complexity and diversity of mammalian adenylyl cyclases. *Annu Rev Pharmacol Toxicol* **36**:461-480.

Sunahara RK and Taussig R (2002) Isoforms of mammalian adenylyl cyclase: multiplicities of signaling. *Mol Interv* **2**:168-184.

Suzuki N, Hajicek N and Kozasa T (2009) Regulation and physiological functions of G12/13-mediated signaling pathways. *Neurosignals* **17**:55-70.

Swaminath G, Deupi X, Lee TW, Zhu W, Thian FS, Kobilka TS and Kobilka B (2005) Probing the beta2 adrenoceptor binding site with catechol reveals differences in binding and activation by agonists and partial agonists. *J Biol Chem* **280**:22165-22171.

Swann K, Saunders CM, Rogers NT and Lai FA (2006) PLCzeta(zeta): a sperm protein that triggers Ca2+ oscillations and egg activation in mammals. *Semin Cell Dev Biol* **17**:264-273.

Tang WJ and Gilman AG (1991) Type-specific regulation of adenylyl cyclase by G protein beta gamma subunits. *Science* **254**:1500-1503.

Thor D, Schulz A, Hermsdorf T and Schoneberg T (2008) Generation of an agonistic binding site for blockers of the M(3) muscarinic acetylcholine receptor. *Biochem J* **412**:103-112.

Tobin AB, Butcher AJ and Kong KC (2008) Location, location, location...site-specific GPCR phosphorylation offers a mechanism for cell-type-specific signalling. *Trends Pharmacol Sci* **29**:413-420.

Townsend-Nicholson A and Shine J (1992) Molecular cloning and characterisation of a human brain A1 adenosine receptor cDNA. *Brain Res Mol Brain Res* **16**:365-370.

Tran E and Ye F (2008) Duplexed label-free G protein--coupled receptor assays for high-throughput screening. *J Biomol Screen* **13**:975-985.

Tsai W, Morielli AD and Peralta EG (1997) The m1 muscarinic acetylcholine receptor transactivates the EGF receptor to modulate ion channel activity. *Embo J* **16**:4597-4605.

Tsang S, Wong SS, Wu S, Kravtsov GM and Wong TM (2009) Testosterone-augmented contractile responses to alpha1- and beta1-adrenoceptor stimulation are associated with increased activities of RyR, SERCA, and NCX in the heart. *Am J Physiol Cell Physiol* **296**:C766-782.

Urban JD, Clarke WP, von Zastrow M, Nichols DE, Kobilka B, Weinstein H, Javitch JA, Roth BL, Christopoulos A, Sexton PM, Miller KJ, Spedding M and Mailman RB (2007) Functional selectivity and classical concepts of quantitative pharmacology. *J Pharmacol Exp Ther* **320**:1-13.

Valant C, Gregory KJ, Hall NE, Scammells PJ, Lew MJ, Sexton PM and Christopoulos A (2008) A novel mechanism of G protein-coupled receptor functional selectivity. Muscarinic partial agonist McN-A-343 as a bitopic orthosteric/allosteric ligand. *J Biol Chem* **283**:29312-29321.

Valant C, Sexton PM and Christopoulos A (2009) Orthosteric/allosteric bitopic ligands: going hybrid at GPCRs. *Mol Interv* **9**:125-135.

Vanhaesebroeck B, Leevers SJ, Ahmadi K, Timms J, Katso R, Driscoll PC, Woscholski R, Parker PJ and Waterfield MD (2001) Synthesis and function of 3-phosphorylated inositol lipids. *Annu Rev Biochem* **70**:535-602.

Verbout NG, Lorton JK, Jacoby DB and Fryer AD (2007) Atropine pretreatment enhances airway hyperreactivity in antigen-challenged guinea pigs through an eosinophil-dependent mechanism. *Am J Physiol Lung Cell Mol Physiol* **292**:L1126-1135.

Versele M, Lemaire K and Thevelein JM (2001) Sex and sugar in yeast: two distinct GPCR systems. *EMBO Rep* **2**:574-579.

Violin JD, Ren XR and Lefkowitz RJ (2006) G-protein-coupled receptor kinase specificity for beta-arrestin recruitment to the beta2-adrenergic receptor revealed by fluorescence resonance energy transfer. *J Biol Chem* **281**:20577-20588.

Voigtlander U, Johren K, Mohr M, Raasch A, Trankle C, Buller S, Ellis J, Holtje HD and Mohr K (2003) Allosteric site on muscarinic acetylcholine receptors: identification of two amino acids in the muscarinic M2 receptor that account entirely for the M2/M5 subtype selectivities of some structurally diverse allosteric ligands in N-methylscopolamine-occupied receptors. *Mol Pharmacol* **64**:21-31.

Vorland M, Thorsen VA and Holmsen H (2008) Phospholipase D in platelets and other cells. *Platelets* **19**:582-594.

Wang JQ, Tang Q, Parelkar NK, Liu Z, Samdani S, Choe ES, Yang L and Mao L (2004) Glutamate signaling to Ras-MAPK in striatal neurons: mechanisms for inducible gene expression and plasticity. *Mol Neurobiol* **29**:1-14.

Wang Y, Deng X, Hewavitharana T, Soboloff J and Gill DL (2008) Stim, ORAI and TRPC channels in the control of calcium entry signals in smooth muscle. *Clin Exp Pharmacol Physiol* **35**:1127-1133.

Warne T, Serrano-Vega MJ, Baker JG, Moukhametzianov R, Edwards PC, Henderson R, Leslie AG, Tate CG and Schertler GF (2008) Structure of a beta1-adrenergic G-protein-coupled receptor. *Nature* **454**:486-491.

Wei H, Ahn S, Shenoy SK, Karnik SS, Hunyady L, Luttrell LM and Lefkowitz RJ (2003) Independent beta-arrestin 2 and G protein-mediated pathways for angiotensin II activation of extracellular signal-regulated kinases 1 and 2. *Proc Natl Acad Sci U S A* **100**:10782-10787.

Weiss JM, Morgan PH, Lutz MW and Kenakin TP (1996) The cubic ternary complex receptor-occupancy model. III. resurrecting efficacy. *J Theor Biol* **181**:381-397.

Werry TD, Christopoulos A and Sexton PM (2006) Mechanisms of ERK1/2 regulation by seven-transmembrane-domain receptors. *Curr Pharm Des* **12**:1683-1702.

Werry TD, Gregory KJ, Sexton PM and Christopoulos A (2005) Characterization of serotonin 5-HT2C receptor signaling to extracellular signal-regulated kinases 1 and 2. *J Neurochem* **93**:1603-1615.

Werry TD, Stewart GD, Crouch MF, Watts A, Sexton PM and Christopoulos A (2008) Pharmacology of 5HT(2C) receptor-mediated ERK1/2 phosphorylation: agonist-specific activation pathways and the impact of RNA editing. *Biochem Pharmacol* **76**:1276-1287.

Werry TD, Wilkinson GF and Willars GB (2003) Mechanisms of cross-talk between G-protein-coupled receptors resulting in enhanced release of intracellular Ca2+. *Biochem J* **374**:281-296.

Wess J, Eglen RM and Gautam D (2007) Muscarinic acetylcholine receptors: mutant mice provide new insights for drug development. *Nat Rev Drug Discov* **6**:721-733.

Wess J, Han SJ, Kim SK, Jacobson KA and Li JH (2008) Conformational changes involved in G-protein-coupled-receptor activation. *Trends Pharmacol Sci* **29**:616-625.

Wickman K and Clapham DE (1995) Ion channel regulation by G proteins. *Physiol Rev* **75**:865-885.

Willoughby D and Cooper DM (2007) Organization and Ca2+ regulation of adenylyl cyclases in cAMP microdomains. *Physiol Rev* **87**:965-1010.

Wise A, Watson-Koken MA, Rees S, Lee M and Milligan G (1997) Interactions of the alpha2A-adrenoceptor with multiple Gi-family G-proteins: studies with pertussis toxin-resistant G-protein mutants. *Biochem J* **321 (Pt 3)**:721-728.

Worzfeld T, Wettschureck N and Offermanns S (2008) G(12)/G(13)-mediated signalling in mammalian physiology and disease. *Trends Pharmacol Sci* **29**:582-589.

Wylie PG, Challiss RA and Blank JL (1999) Regulation of extracellular-signal regulated kinase and c-Jun N-terminal kinase by G-protein-linked muscarinic acetylcholine receptors. *Biochem J* **338 (Pt 3)**:619-628.

Yamaguchi S, Tanabe K, Takai S, Matsushima-Nishiwaki R, Adachi S, Iida H, Kozawa O and Dohi S (2009) Involvement of Rho-kinase in tumor necrosis factor-alpha-induced interleukin-6 release from C6 glioma cells. *Neurochem Int*.

Yang C and Kazanietz MG (2007) Chimaerins: GAPs that bridge diacylglycerol signalling and the small G-protein Rac. *Biochem J* **403**:1-12.

Yedgar S, Lichtenberg D and Schnitzer E (2000) Inhibition of phospholipase A(2) as a therapeutic target. *Biochim Biophys Acta* **1488**:182-187.

Yu RC, Pesce CG, Colman-Lerner A, Lok L, Pincus D, Serra E, Holl M, Benjamin K, Gordon A and Brent R (2008) Negative feedback that improves information transmission in yeast signalling. *Nature* **456**:755-761.

Yuan J, Rey O and Rozengurt E (2006) Activation of protein kinase D3 by signaling through Rac and the alpha subunits of the heterotrimeric G proteins G12 and G13. *Cell Signal* **18**:1051-1062.

Zeng H, Zhao D and Mukhopadhyay D (2002) KDR stimulates endothelial cell migration through heterotrimeric G protein Gq/11-mediated activation of a small GTPase RhoA. *J Biol Chem* **277**:46791-46798.

Zhang L, Brass LF and Manning DR (2009) The Gq and G12 families of heterotrimeric G proteins report functional selectivity. *Mol Pharmacol* **75**:235-241.

Zhong H and Neubig RR (2001) Regulator of G protein signaling proteins: novel multifunctional drug targets. *J Pharmacol Exp Ther* **297**:837-845.

Zurn A, Zabel U, Vilardaga JP, Schindelin H, Lohse MJ and Hoffmann C (2009) Fluorescence resonance energy transfer analysis of alpha 2a-adrenergic receptor activation reveals distinct agonist-specific conformational changes. *Mol Pharmacol* **75**:534-541.