Novel interactions of the 5-HT_{2A} and related receptors with intracellular signalling proteins

by

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for K

my angel

This thesis is dedicated to the memory of Melanie S Johnson

1954-2006

colleague, friend, confidant and mentor

Declaration of originality

I, Derek Robertson declare that, unless otherwise stated, this thesis represents my own work and was composed by me. No part of this work has been, or is being submitted for any other degree or qualification.

Derek Robertson

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Abbreviations:

5-HT	5-hydroxytryptamine, serotonin
$5\text{-HT}_{2A}\text{R}$	5-hydroxytryptamine 2A receptor
ACh	Acetylcholine
ARF	ADP-ribosylation factor
ARNO	ADP ribosylation factor nucleotide-binding site opener
BFA	Brefeldin A
BIG1	Brefeldin A-inhibited guanine nucleotide-exchange protein 1
CCh	carbachol
CHAPS	3-[(3-Cholamidoproply)dimethlammonio]-1-propanesulphonate
ct	carboxy-terminal tail domain
DAG	*sn*-1,2-diacylglycerol
GAP	GTPase-activating protein
GDP	Guanosine diphosphate
GEF	GTP-exchange factor
GPCR	G protein-coupled receptor
GRK	G protein-coupled receptor kinase
GST	glutathione-S-transferase
GTP	Guanosine triphosphate
GTPγS	Guanosine 5'-*O*-[γ-thio]triphosphate
HA	haemagglutinin epitope tag
i3	third intracellular loop domain
IP	immunoprecipitation
IP_3	*myo*-inositol-1,4,5-trisphoshate
IPTG	isopropyI-β-D-thiogalactoside
M_3R	M_3 muscarinic receptor
MAChR	muscarinic acetylcholine receptor
MEM	minimum essential medium
min	minute(s)
ml	millilitres
mM	millimolar
NMe-QNB	N-Methyl-quinuclidinyl benzilate
PACAP	pituitary adenylate cyclase-activating peptide

PBS	phosphate buffered saline
PH	pleckstrin homology
PIP_2	phosphatidylinositol-4,5-bisphosphate
PKC	protein kinase C
PLA_2	phospholipase A_2
PLC	phospholipase C
PLD	phospholipase D
RGS	regulator of G protein signalling
sPrC	protein C epitope tag with signal sequence
tm7	7^{th} transmembrane domain
NI IgG	non-immune mouse IgG
VIP	Vaso-active intestinal peptide
VPAC	VIP and PACAP receptors
EC_{50}	concentration required to evoke 50% of the maximum response
IC_{50}	concentration required to inhibit 50% of the response

The aim of this project is to further elucidate the pathways involved in the intracellular signalling mechanisms of the 5-HT_{2A} and related receptors. The G-protein coupled receptors (GPCRs) are named after their ability to interact with and signal through the trimeric G-proteins. The 5-hydroxytryptamine 2A receptor (5-HT_{2A}R) is a member of the group I family of rhodopsin-related GPCRs. The receptor is known to activate phospholipase C (PLC) via the heterotrimeric G proteins $G\alpha_{q/11}$, but has been shown to also signal through the phospholipase D (PLD) pathway in an ADP-ribosylation factor (ARF)-dependent manner, that appears to be independent of $G_{q/11}$. The M_3 muscarinic receptor, another member of the group I GPCRs, has also been shown to signal through both PLC (via $G\alpha$) and the alternative pathway of PLD activation via ARF. In this thesis, it has been shown that both these receptors interact directly with members of the ADP-ribosylation Factor (ARF) family of small G-proteins. Not only is there evidence to show that these receptors activate PLD signalling through the ARF family of proteins, as shown by *in vivo* signalling assays, but it can also be shown that the receptors interact directly with ARF. The 5-HT_{2A} receptor associates with ARF1, and the third intracellular loop domain of the M_3 muscarinic receptor associates with both ARF1 and ARF6, as shown by *in vitro* GST interaction assays.

Experiments undertaken to elucidate the exact criteria for this interaction suggest that a complex of proteins involving $G\beta\gamma$ for the M_3 muscarinic receptor, and arrestin for the 5-HT_{2A} receptor. The GDP/GTP status of the ARF involved plays a role in the ability of this interaction to take place. The conserved N/DPxxY motif in transmembrane domain 7 (tm7) of the Group I GPCRs also seems to affect the ability of the receptor to signal through ARF. Thus changing this motif altered the binding of ARF isoforms to the 5-HT_{2A} receptor.

The binding of novel interaction partners to the 5-HT_{2A} receptor was also investigated, with the discovery of that the glial protein S100B bound to the carboxy terminal domain of the 5-HT_{2A} receptor in a calcium dependent manner.

These findings have implications for the investigation of the signalling pathways of these and other related Group I type GPCRs

Publications

Some of the results presented in this thesis have been published as follows:

Papers

Derek N. Robertson, Melanie S. Johnson, Louise O. Moggach, Pamela J. Holland, Eve M. Lutz and Rory Mitchell. (2003)

Selective interaction of ARF1 with the carboxy-terminal tail domain of the 5-HT_{2A} receptor. *Molecular Pharmacology* **64** 5: 1239-1250.

Rory Mitchell, Derek N. Robertson, Pamela J. Holland, Daniel Collins, Eve M. Lutz and Melanie S. Johnson. (2003)

ADP-ribosylation factor-dependent phospholipase D activation by the M_3 muscarinic receptor. *Journal of Biological Chemistry* **278** 36: 33818-33830.

Melanie S. Johnson, Derek N. Robertson, Pamela J. Holland, Eve M. Lutz, and Rory Mitchell. (2006)

Role of the conserved NPxxY motif of the 5-HT_{2A} receptor in determining selective interaction with isoforms of ADP-Ribosylation Factor (ARF). *Cell Signalling* (accepted Febuary 2006).

Abstracts

Derek N. Robertson, Melanie S. Johnson, Derek A. McCulloch, Eve M. Lutz, Pamela Holland, Rory Mitchell. (1999)

Divergent pathways of phospholipase D activation in the human 5-HT_{2A} receptor and its $N^{376}D$ mutant. *Biochem. Soc. Trans.* **27**: A117.

Derek N. Robertson, Melanie S. Johnson, Pamela Holland, Rory Mitchell. (2001)

Association of small G protein ARF1 with the third intracellular loop of the M_3 muscarinic receptor. *Biochem. Soc. Trans.* **29**: A41

Melanie S. Johnson, Derek N. Robertson, Louise O. Moggach, Rory Mitchell. (2003)

Involvement of arrestin in 5-HT_{2A} receptor signalling through PLD. *Eu. J. Biochem.* **270** Suppl: PS01-0044.

Rory Mitchell, Derek N. Robertson, Stephen Miller, Melaine S. Johnson, Pamela Holland. (2003)

Interaction of ARF1 or ARF6 with the M_3 muscarinic receptor is facilitated by G beta/gamma. *Eu. J. Biochem.* **270** Suppl: PS01-0070.

Derek N. Robertson, Melanie S. Johnson, Louise O. Moggach, Pamela Holland, Eve M. Lutz and Rory Mitchell. (2003)

Differential interactions of ARF1 and ARF6 with domains of the 5-HT_{2A} receptor. *Eu. J. Biochem.* **270** Suppl: PS01-0085.

Contents

Chapter 1:

Introduction

G Protein-Coupled Receptors.

Cell surface receptors play an essential physiological role in allowing cells to recognise and react to external stimuli, allowing for cell-to-cell communication. Receptor-encoding genes account for 1-5% of known genomes, and are split into different families depending on their secondary structure and conserved domains. The largest of these families is the heptahelical, G protein-coupled receptor (GPCR) family, which accounts for >1% of the human genome, and contains over 1000 proteins. Members of this family of proteins are activated by many ligands (biogenic amines, peptide and non-peptide neurotransmitters, hormones, growth factors, odorant and taste molecules, light, ions, nucleotides, proteases), and act as the biological target for >50% of the therapeutic agents on the market (Flower, 1999; Marinissen and Gutkind, 2001). They share little sequence in common (although several motifs are conserved) but they do have a great deal of structural similarity. The GPCRs all follow a similar pattern of secondary structure, in that they all have an amino terminal extracellular domain, 7 transmembrane α-helical domains (giving rise to their alternative name, the 7TM receptors) which are connected by alternating intracellular and extracellular loop domains, and end in an intracellular carboxy terminal tail (Figure 1.1). These α-helical transmembrane domains have been shown, in the case of the photoreceptor rhodopsin (Palczewski *et al.*, 2000), to cluster together in cell membranes to form an anticlockwise bundle of α-helices, (viewed from the extracellular side) joined together by loops of various lengths. The third transmembrane spanning helix sits in an almost central position in the overall structure.

GPCRs classification

The GPCR super-family can be further divided into at least 6 families or types, and several systems of classification have been used, the previously most used of these systems uses classes A-F to identify the major groups (Kolakowski, 1994; Attwood and Findlay, 1994), which is used to classify all GPCRs, vertebrate and invertebrate (e.g. classes D and E represent fungal pheromone receptors)(Kolakowski, 1994). A more recent, post-genome system of classification analyses the sequences of GPCRs, and groups them on a phylogenetic basis. By comparison of the homology of the transmembrane spanning domains of the GPCRs, a dendrogram was constructed of the most related receptors (Fredriksson *et al.*, 2003)(Fig 1.2). This gives rise to five main families of GPCRs within the human genome. These are named for the best known member of each family, giving rise to the GRAFS nomenclature (glutamate, rhodopsin, adhesion, frizzled/taste2 and secretin) (Foord, 2002; Fredriksson *et al.*, 2003) (Figure 1.2).

Despite the shared structural topology of the seven transmembrane receptors, there is very little sequence similarity shown between these families beyond the predicted secondary structure (Probst *et al.*, 1992; Kolakowski, 1994). Many of the receptors within these families have been shown to couple to heterotrimeric G-proteins (Foord, 2002) but the rapidly increasing number of GPCR sequences being discovered by molecular techniques means that a considerable number have not yet been explicitly investigated.

Rhodopsin-like GPCRs

The family R (rhodopsin-like/class A/type I) GPCRs are the most studied of the groups, and includes receptors most like the photon receptor rhodopsin (Dixon *et al.*, 1986). This, the largest of the GPCR families, contains over 700 (241 non-olfactory) receptors in humans (Fredriksson *et al.*, 2003). This family can be further divided into 4 major subgroups, and comprise many different subfamilies, including: the α-group, containing the amine receptors (which in turn includes the serotonin or 5-HT receptors and the muscarinic receptors, as well as the adrenergic receptors, histamine receptors and dopamine receptors amongst others), opsins, melatonin receptors, prostaglandin receptors and MECA (melanocortin, endothelial differentiation, cannabinoid and adenosine-binding) receptors; the β-group which includes endothelin and neuropeptide Y receptors; the γ-group of opioid and somatostatin receptors and the δ-group of olfactory receptors (Dixon *et al.*, 1986; Kubo *et al.*, 1986; Strange, 1993; Drutel *et al.*, 1995; Narumiya and Fitzgerald, 2001; Foord, 2002; Ronnett and Moon, 2002; Fredriksson *et al.*, 2003). The first of these receptors to be cloned was the bovine rhodopsin receptor in 1983 (Nathans and Hogness, 1983)(Figure 1.3), with the sequence of the human rhodopsin receptor following in the next year (Nathans and Hogness, 1984). The β-adrenergic receptor followed shortly afterwards, and showed striking structural homology with the rhodopsin receptor (Dixon *et al.*, 1986). Since then a large number of GPCRs and their splice variants have been characterised from their gene products. The most studied of the members of the family R GPCRs include those for the biogenic

amines, the adrenergic (α and β-adrenergic) receptors, muscarinic receptors, dopamine receptors and 5-HT receptors (Lefkowitz *et al.*, 1976; Dixon *et al.*, 1986; Kubo *et al.*, 1986; Bonner *et al.*, 1987; Strange, 1993; Hoyer *et al.*, 1994; Hoyer *et al.*, 2002).

The classic family R GPCRs are those that bind "small molecule" transmitters (adrenaline, noradrenaline, dopamine, 5-HT, histamine and acetylcholine). The agonist binding sites for these molecules have been shown to be contained in a binding crevice formed by the transmembrane helices. The residues involved are buried deep within the receptor molecule as has been shown by spectroscopic analysis of the β_2-adrenergic receptor (Tota and Strader, 1990)(Figure 1.3). For the larger peptide agonists of families R and S the extracellular domains have been demonstrated to have a critical role, for example, the family R neurokinin receptors (NK) have been shown to bind their tachykinin ligands through the amino-terminus, a residue at the top of TM3 and residues at the top of TM7 (Fong *et al.*, 1992a; Fong *et al.*, 1992b), and in the case of the agonist substance P, the extracellular loops (Fong *et al.*, 1992b; Huang *et al.*, 1994). Other peptide agonists, for example angiotensin (Hjorth *et al.*, 1994; Feng *et al.*, 1995; Heerding *et al.*, 1997), neuropeptide Y (Walker *et al.*, 1994), GnRH (Davidson *et al.*, 1997), opioids (Wang *et al.*, 1995; Xue *et al.*, 1995; Valiquette *et al.*, 1996; Varga *et al.*, 1997; Pepin *et al.*, 1997), neurotensin (Labbejullie *et al.*, 1995) and others all have major interactions with the amino-terminal and predicted extracellular loop domains, and may to greater or lesser extents have additional interactions with the transmembrane domains, and a transmembrane binding crevice (Yamano *et al.*, 1995; Ozenberger and Hadcock, 1995; Monnot *et al.*, 1996).

Homology between the receptors of this family is relatively low overall, however, specific key regions or domains retain significant homology. The most conserved of these regions is an Aspartate-Arginine-Tyrosine (DRY) motif at the cytosolic interface at the end of transmembrane spanning domain 3 (TM3) (Probst *et al.*, 1992; Kolakowski, 1994). This motif has been implicated in the structural stability of the receptors (Rasmussen *et al.*, 1999), and has also been considered to be a conformational switch, allowing coupling to heterotrimeric G proteins. Mutations in these residues lead to constitutively active receptors in the case of the α_1-adrenergic receptor (Scheer *et al.*, 1997), the A_3 adenosine receptor (Chen *et al.*, 2001) and the GnRH receptor (Arora *et al.*, 1997). However, there is some contrary evidence, suggesting for example that mutation of the arginine of this motif in the β_2-adenergic receptor does not result in a constitutively active receptor (Seibold *et al.*, 1998).

In nearly all GPCRs there is a highly conserved disulphide bridge, where cysteine residues link the first extracellular loop (e1) to the second extracellular loop (e2) (Pedersen and Ross, 1985; Dixon *et al.*, 1987; Karnik and Khorana, 1990; Schertler *et al.*, 1993b) again conferring stability of the barrel structure of the GPCR. The majority of the family R GPCRs have a putative palmitoylation site on a cysteine in the proximal carboxy-terminal tail, which causes the formation of a putative fourth intracellular loop (i4) (fig 1.1). Another highly conserved motif in the family R GPCRs is an Asparagine Proline xxTyrosine (NPxxY), that is found at the junction of the α-helical transmembrane 7 (tm7) and carboxyl-terminal (ct) domains in a number of rhodopsin family GPCRs, and has been implicated as a determinant of ARF:receptor interactions and ARF-mediated signalling. Native receptors with an alternative DPxxY motif, or N to D mutant receptor show selective defects in this

pathway whereas native NPxxY containing receptors activate the PLD signalling pathway in an ARF-dependent manner (Mitchell *et al.*, 1998). However, it has not been clear whether this motif might be accessible as a direct docking site or whether instead it regulates access to a distinct site. What is known is that the proline (P) induces a kink, by disrupting the H-bonds in the helix as it emerges from the plasma membrane, however both NP and DP produce a perturbation of the helix that is significantly different from the minimal Proline-kink. They also induce a high degree of structural flexibility, making a large movement at the intracellular side of the TM7 likely to be achieved by a small repositioning of the H-bond network, especially the H-bond acceptors interacting with Asn (N) of the NPxxY motif (Konvicka *et al.*, 1998)

The first of these receptors, or in fact any of the GPCRs to have its crystal structure defined was the rhodopsin receptor (Palczewski *et al.*, 2000) showing the barrel arrangement of the transmembrane α-helices (Fig. 1.2).

Secretin receptor-like GPCRs

The family S family of GPCRs, otherwise known as the type II, Class B or Secretin-like receptors, is much smaller with around 25 members in total. Members of this group all bind to large peptide agonists and includes the subfamilies of the gastrointestinal peptide receptors (which includes the VPAC (vasoactive-intestinal peptide, (VIP) and pituitary adenylate cyclase activating peptide (PACAP) recognising receptors), as well as the receptors for secretin, glucagon, growth hormone receptor hormone, calcitonin and parathyroid hormone (Christophe *et al.*,

1988; Lutz *et al.*, 1993; Spengler *et al.*, 1993; Morrow *et al.*, 1993; Foord *et al.*, 2002). The family S receptors are renowned for having large extracellular amino-terminal domains, involved in recognition of their specific peptide agonists. Furthermore the family S GPCRs do not have the DRY motif that is found in the family R GPCRs, and neither the NPxxY motif, nor the putative fourth intracellular loop. However they do contain a disulphide bridge between the i2 and i3 domains, as is present in the family R receptors. One prominent characteristic of the family S GPCRs is a large (~100 residue) extracellular amino terminus, that is rich in cysteine residues, which presumably form a network of disulphide bridges (Ulrich *et al.*, 1998).

Receptors of the S family of GPCRs are thought to bind their peptide agonists substantially through their characteristically large amino-terminus (Di Paolo *et al.*, 1999; Unson *et al.*, 2002), however, there is additional evidence to suggest that the extracellular loops of these receptors are also involved. Nevertheless, there is no specific evidence at present to suggest a role of the transmembrane binding crevice in ligand binding by this family of receptors (Gaudin *et al.*, 1995; Unson *et al.*, 2002).

Receptors within family S GPCRs all appear to couple to activation of adenylyl cyclase through the G-protein Gs, although some members of this family can additionally activate phospholipase C via $G_{q/11}$ and some have been shown to activate PLD in a heterotrimeric G-protein independent manner, through the small G-protein ARF (Lutz *et al.*, 1999; McCulloch *et al.*, 2000; McCulloch *et al.*, 2001; Ronaldson *et al.*, 2002).

Glutamate receptor-like GPCRs

The family G GPCRs are a small family of receptors with around 15 members, and includes the metabotropic glutamate (mGlu) receptors, the metabotropic $GABA_B$ receptors, as well as calcium-sensing receptors, pheromone and group-1 taste receptors (Houamed *et al.*, 1991; Masu *et al.*, 1991; Conn and Pin, 1997; Kaupmann *et al.*, 1997; Couve *et al.*, 2000; Pin *et al.*, 2003).

Previously known as the class C GPCRs, members of this family have a large and definitive extracellular amino terminal domain (~500-600 amino acids) (Desai *et al.*, 1995), which takes the form of a so-called Venus Flytrap Module (VFTM) and is used for ligand recognition (Takahashi *et al.*, 1993; Ohara *et al.*, 1993). Additionally in all but the $GABA_B$ receptor, there is a conserved cysteine-rich domain connecting the VFTM to the seven transmembrane spanning part of the receptor (Pin *et al.*, 2003). The VFTM domain shares sequence similarity with a family of small molecule-binding bacterial periplasmic transport proteins (Ohara *et al.*, 1993), and it has been proposed that the family G GPCRs may have evolved from the fusion of an ancestral seven TM receptor with one of these periplasmic proteins (Felder *et al.*, 1999; Pin *et al.*, 2003). The Venus Flytrap Module is so called because it is composed of two regions (or lobes) each constructed of a β-sheet surrounded by two α-helices, separated by three linkers, which form a cleft into which the ligand binds (Sack *et al.*, 1989; Galvez *et al.*, 1999; Kunishima *et al.*, 2000). These lobes exist in either an open or closed conformation. The protein mostly exists in the open conformation when not bound to ligand, and is stabilised in the closed state when bound to ligand (Parmentier *et al.*, 2002). It is thought that part of the mechanism of

action of competitive antagonist ligands for this family of GPCR may be due to the interference with efficient closure of the VTFM lobes (Costantino and Pellicciari, 1996).

The family G receptors were the first GPCRs to be shown to form functional dimers *in vivo*. Subsequently, this has been shown to be the case for members of other families of GPCRs (Romano *et al.*, 1996; Robbins *et al.*, 1999; Milligan *et al.*, 2003). Both homodimers (in the case of the mGluR and Ca^{2+} sensing receptors) and heterodimers (e.g. the $GABA_B$ and taste receptors) can be formed and are required for full agonist activation (Romano *et al.*, 1996; Kuner *et al.*, 1999; Kunishima *et al.*, 2000; Pin *et al.*, 2003). Dimerisation of the receptors has been shown to involve the VFTM domains (formed by a disulphide bridge and a hydrophobic interaction), a putative transmembrane interaction and the carboxy terminal tail domains (Bai *et al.*, 1998; Robbins *et al.*, 1999; Ray *et al.*, 1999). It has been determined that the receptors do not require the binding of two agonist molecules to elicit activation (Galvez *et al.*, 2000). However X-ray crystallography data of the N-terminal domain suggests that the active state of the receptor may be more stable when the VFTM lobes of both receptors in the dimer are closed (Kunishima *et al.*, 2000; Pin *et al.*, 2003). Furthermore, the GPCR dimer appears to activate a single heterotrimeric G-protein at any one time (although, the functional dimer is still required for G-protein coupling) (Galvez *et al.*, 2001; Pin *et al.*, 2003). With regard to similarities with the other families of GPCRs, the family G receptors maintain the disulphide bridge structure between i2 and i3, but otherwise there are no other conserved residues (Probst *et al.*, 1992; Kolakowski, 1994).

Families F and A

The last two groups are named after the frizzled like and adhesion receptors. The family F, which includes the frizzled/smoothed receptors, the group-2 taste (TAS2) receptors, and the cAMP receptors from *Dictyostelium discoideum*, of which there are 4 currently known (Fredriksson *et al.*, 2003). The frizzled receptors control cell proliferation and polarity during metazoan development by mediating signals from secreted glycoproteins known as Wnt. The frizzled family of receptors have a 200 amino acid N-terminus with conserved cysteines that are likely to participate in Wnt binding (Fredriksson *et al.*, 2003).

The family A receptors, of which there are 24 members, are made up of adhesion receptors (or latrophilins), which are receptors that have the typical transmembrane spanning domain coupled to an amino-terminal domain that contains a functional adhesion motif, such as the EGF repeat or a mucin-like motif (Fredriksson *et al.*, 2003). The amino-terminal domains are vastly different in size, ranging from 200 residues up to 2800 residues in the case of the very large G protein coupled receptors (VLGRs) (Foord, 2002). Functional activities associated with these receptors include the definition of cell polarity, inhibition of angiogenesis and regulation of the immune system.

Heterotrimeric G-protein signalling

G protein-coupled receptors, as the name suggests, signal classically through activation of heterotrimeric guanine nucleotide-binding proteins, or G proteins. These proteins exist as heterotrimers, each consisting of an α, β and γ subunit. Within this superfamily of proteins there is considerable diversity, with each subunit

existing in several isoforms, with the Gα subunit being the most diverse. There are 20 known Gα subunits (~41kDa), also 6 Gβ subunits (~35kDa), and 12 Gγ (~10kDa) subunits. The βγ subunits bind tightly together and are regarded as a single functional unit *in vivo*. The number of possible combinations of G-protein subunits is large, introducing a further level of variation within the heterotrimeric G-proteins, and may yield part of the regulation that is necessary for the specificity of second messenger coupling and activation.

Upon binding of ligand to the extracellular domains of the receptor, a conformational change in the receptor causes the activation of the heterotrimeric G-proteins, and the signal downstream thereof. Upon activation, guanosine diphosphate (GDP) bound to the Gα subunit is exchanged for guanosine triphosphate (GTP), forcing the Gα subunit to undergo a conformational change in the switch II region that binds to the Gβγ subunit (Figure 1.5). This conformational change no longer allows for the binding of the Gβγ subunit to the Gα subunit, and the subunits are dissociated from each other. Both subunits are then free to interact with their respective effector molecules to activate or modulate various signalling pathways (Limbird *et al.*, 1980; Stadel *et al.*, 1981; Logothetis *et al.*, 1987) (Figure 1.4).

The Gα subunits are divided into 4 families, categorised and named from the second messenger pathways that the Gα subunits of the G-proteins couple to. These groupings are as named Gαs, Gαi/o, Gαq and Gα12/13. The Gs heterotrimeric G protein family includes Gαs and Gαolf G proteins. The Gs G protein was the first one characterised by Limbird and collegues, and has been shown to stimulate adenylate cyclase, which catalyses the formation of the second messenger cAMP

from ATP (Lefkowitz *et al.*, 1976; Limbird *et al.*, 1980; Sullivan *et al.*, 1986). This increase in cAMP levels subsequently activates protein kinase A to mediate other intracellular effects (Robison *et al.*, 1968; Walsh *et al.*, 1968; Miyamoto *et al.*, 1968). The Gs family of G proteins have also been shown to close K^+ channels (probably via a cAMP-dependent mechanism) (Madison and Nicoll, 1986; Pedarzani and Storm, 1995) and open cardiac L-type Ca^{2+} channels (mediated by a direct coupling of Gβγ subunits to the channel) (Fisher and Johnston, 1990). The hydrolysis of GTP to GDP by the dissociated Gα subunit can be specifically blocked by ADP-ribosylation of the subunit by the bacterial cholera toxin from *Vibrio cholerae*. Cholera toxin transfers the ADP-ribose group from nicotinamide adenine dinucleotide (NAD^+) to an arginine residue at position 178 in the Gα subunit (Hsia *et al.*, 1985). This permanently activates the G protein and it cannot then hydrolyse the bound GTP.

The Gαi/o family of G proteins consists of $G\alpha i_{1\text{-}3}$, $G\alpha o_{a\text{-}b}$, which are brain specific, Gαz and the photoreceptor Gαt (transducin) (Morris and Malbon, 1999). The Gαi/o subunits act to inhibit adenylate cyclase and thereby reduce the intracellular cAMP levels (Sullivan *et al.*, 1986; Yatani *et al.*, 1988). The Gi/o family act to open the G protein-coupled inwardly-rectifying K^+ channels (GIRK), and close neuronal N-type Ca^{2+} channels by direct Gβγ coupling (Logothetis *et al.*, 1987; Penington *et al.*, 1993; Krapivinsku *et al.*, 1995; Oh *et al.*, 1995; Herlitze *et al.*, 1996; Ikeda, 1996). Transducin (Gαt) acts to activate cGMP phosphodiesterase, which is the main effector mechanism in photoreceptor transduction (Morris and Malbon, 1999). With the exception of Gz (of which very little is known), Gαi/o subunits can be specifically inhibited by the action of pertussis toxin from *Bordatella pertussis*,

which transfers the ADP-ribose group from NAD^+ to a cysteine residue at position 350 on the Gα subunit to prevent the GDP/GTP exchange upon receptor activation (Hsia *et al.*, 1985).

The pertussis toxin insensitive Gq/11 family consists of Gαq, Gα11 and Gα14-16 proteins (Morris and Malbon, 1999). They can activate the beta isozymes of phospholipase C (PLCβ1-4), which hydrolyse PIP_2 to form the second messenger DAG and IP_3. IP_3 activates intracellular ionotropic IP_3 receptors on the endoplasmic reticulum to release the intracellular store of Ca^{2+} (Mak *et al.*, 1998). DAG directly activates protein kinase C, which phosphorylates many intracellular targets, including phospholipase D (PLD) (Merritt *et al.*, 1986b; Gierschik and Jakobs, 1987; Taylor *et al.*, 1991; Hammond *et al.*, 1997). Members of the Gq/11 G protein family have also been shown to modulate a novel K^+ current (Shi *et al.*, 2004) in addition to inhibiting the established GIRK current (Hill and Peralta, 2001).

The Gα12/13 G proteins have been shown to couple with small G-proteins via GEF p115RhoGEF, a GTPase activating protein (GAP) for the small G protein Rho (Jiang *et al.*, 1998; Kozasa *et al.*, 1998). G_{12} G-proteins have also been shown to couple to and activate PLD by a mechanism that may be Rho dependent (Hamm, 1998; Rumenapp *et al.*, 2001).

It was originally thought that the α-subunits of the G proteins could exclusively regulate their downstream effectors, hence the families of trimeric G proteins being named after the signalling cascade effected by the Gα subunit, but the Gα subunit is not the only molecule that activates intracellular signalling upon receptor activation. The Gβγ subunit has its own role in direct activation of at least three independent

signalling pathways. It has been proposed that Gβγ has the ability to activate phospholipase A_2 (PLA_2) based on reconstitution of purified retinal Gβγ with G protein–depleted retinal membranes (Jelsema and Axelrod, 1987). Phospholipase A_2 is an enzyme that hydrolyses the 2-acyl ester bond of membrane phospholipids to generate free fatty acid and lysophospholipids, for example arachidonic acid.

The Gβγ subunit (Figure 1.5) has since been shown to have several crucial roles in GPCR signalling. Firstly, it has been shown that the Gβγ subunit promotes the association of GDP bound Gα with ligand-bound receptor, thereby appearing to facilitate receptor dependent G-protein activation. Gαt has been shown to be able to bind directly to the rhodopsin receptor without Gβγ, however, the addition of Gβγ appears to increase the affinity of Gα association with its target receptor (Higashijima *et al.*, 1987; Phillips and Cerione, 1992; Heithier *et al.*, 1992). There is also evidence to suggest that Gβγ remains bound to rhodopsin, even after the Gαt subunit dissociates (Phillips and Cerione, 1992; Jian *et al.*, 2001). Gβγ has also been shown to interact directly with the β-adrenergic receptor (Heithier *et al.*, 1992), the α_2-adrenoceptor (Richardson and Robishaw, 1999), the rhodopsin receptor (Phillips and Cerione, 1992; Jian *et al.*, 2001), and the muscarinic M_2 (Azpiazu *et al.*, 1999; Hou *et al.*, 2000)and M_3 receptors (Wu *et al.*, 1998; Wu *et al.*, 2000).

PLCβ1, PLCβ2 and PLCβ3 (but not PLCβ4, PLCγ1, or PLCδ1), have been shown to be responsive to Gβγ (Lee *et al.*, 1992; Park *et al.*, 1993). Activation of PLCβ by purified Gβγ has shown that the interaction between PLCβ and Gβγ is direct, that the ability of the Gβγ subunit to bind to PLCβ is via a separate binding site from that of the Gα subunit (Park *et al.*, 1993). Evidence exists to show that in *Xenopus* oocytes,

the activation of PLCβ by the M_3 receptor was predominantly mediated by Gβγ, rather than Gα, since the PLC activation was inhibited by agents that specifically bound free Gβγ (Gα-GDP, GRKct), concluding that although receptor coupling specificity of the heterotrimer was determined by the Gα subunit, in this example, the predominant signaling molecule was Gβγ (Stehno-Bittel *et al.*, 1995).

G-protein and other interactions with intracellular receptor domains

Considerable insight has been gained into the specific domains and motifs of the GPCRs that are involved in the activation of the trimeric G-proteins over the past decade, and pivotal roles in this process have been established for the second (i2) and third (i3) intracellular loops, with some receptors also utilising proximal regions of the ct (Dohlman *et al.*, 1991; Savarese and Fraser, 1992; Koblika, 1992; Strader *et al.*, 1994; Wess *et al.*, 1997; Wess, 1997; Wess, 1998; Zhang *et al.*, 1999; Du *et al.*, 2000; Xie *et al.*, 2002). Mutagenesis studies of both the adrenergic and muscarinic receptors have clearly shown the third intracellular loop (i3) as the major determinant of coupling specificity among the different G protein α-subunits (Dohlman *et al.*, 1991; Savarese and Fraser, 1992; Koblika, 1992; Strader *et al.*, 1994; Wess, 1997; Wess, 1998). Point mutational analysis of various receptors has pinpointed this area as the amino-terminal part of i3, adjacent to TM5 (Strader *et al.*, 1994; Bluml *et al.*, 1994a; Bluml *et al.*, 1994b; Burstein *et al.*, 1996; Hill, Eubanks *et al.*, 1996) and the carboxy-terminal area next to TM6 (Liu *et al.*, 1995; Burstein *et al.*, 1995; Liu *et al.*, 1996). In the 5-HT_{2A}R, the i3 domain has been shown to be important for coupling to $G_{q/11}$ and the carboxyl terminal segment of the i3 in particular, may play a key role in the interaction (Roth *et al.*, 1998). The i2 domain has also been implicated in G-

protein specificity, but to a lesser extent than the i3 domain, although the i2 domain seems to be important in G-protein activation (Dohlman *et al.*, 1991; Savarese and Fraser, 1992; Kobilka, 1992; Strader *et al.*, 1994; Wu *et al.*, 1997; Wess, 1997; Wess, 1998; Oakley *et al.*, 2001).

The i3 domains has also been shown to provide docking sites for heterotrimeric G protein $\beta\gamma$ subunits (Wu *et al.*, 1998; Wu *et al.*, 2000). It has been known for some time that the arrestin family of proteins also interact directly with the GPCRs. These arrestins bind specifically to GPCRs phosphorylated by G-protein receptor kinases (GRK), an interaction which participates in the homologous desensitisation of the receptor by disrupting their coupling to G-proteins (Gurevich *et al.*, 1995). In these cases, arrestin does bind to the receptor domains at a low level, but this binding is considerably higher after GRK phosphorylation of the receptor domains (see Gurevich and Gurevich, 2004 and Lefkowitz and Whalen, 2004 for reviews). Arrestins also target the receptors for internalisation by virtue of their ability to interact with clathrin (See Carman and Benovic, 1998 for a review). Arrestin isoforms have been shown to bind to the i3 loop of the 5-HT_{2A}R, as well as the M_2 and M_3 muscarinic and α_2-adrenergic receptors. Arrestin bound to the 5-HT_{2A}R with a broader specificity than is shown at i3 of the M_2 and M_3 muscarinic receptors, where only non-visual arrestins are bound (Wu *et al.*, 1997; Gelber *et al.*, 1999).

The carboxy-terminal tail domains of various GPCRs have been shown to bind to a large number of proteins with various functional roles, including chaperone proteins, PDZ domain-containing proteins, as well as others that have no currently recognised binding motif.

GPCR Signalling Pathways: Phospholipase C

In the 1950s a novel enzyme was discovered that incorporated [^{32}P] into phospholipids upon carbachol stimulation of pigeon pancreatic cells (Hokin and Hokin, 1953). Later work by the Michell lab resulted in the characterisation of a membrane-associated enzyme named phospholipase C (PLC). PLC was shown to catalyse the formation of DAG and IP_3 from the membrane lipid phosphatidylinositol, which was coupled to an increase in intracellular Ca^{2+} levels. (Miyamoto *et al.*, 1968; Lapetina and Michell, 1972; Lapetina and Michell, 1973a; Lapetina and Michell, 1973b; Jones and Michell, 1974; Allan and Michell, 1975; Akhtar and Abdellatif, 1980).

Mammilian PLC isoforms are generally phosphatidylinositol specific (PI-PLC) and preferentially hydrolyse the phospholipids phosphatidyl (4,5)-bisphosphate (PIP_2) into 1,2 diacylglycerol (DAG) and myo-inositol (1,4,5)-trisphosphate (IP_3) (Griffin and Hawthorne, 1978; Ryu *et al.*, 1987; Berridge, 1993). DAG and IP_3 then mediate the activation of protein kinase C (PKC) and the release of Ca^{2+} from intracellular stores respectively (Streb *et al.*, 1983; Nishizuka, 1984b; Putney *et al.*, 1986). There are eleven mammalian isoforms of PLC, grouped into four main families. There are four subtypes of PLCβ (145-155 kDa), two subtypes of PLCγ (145-155 kDa), four PLCδ (~85 kDa) and a novel PLCε isoform (~86 kDa) (Suh *et al.*, 1988; Thomas *et al.*, 1991; Cockcroft and Thomas, 1992; Rhee and Choi, 1992; Berridge, 1993; Rhee and Bae, 1997; Wing *et al.*, 2003). PLC activation in response to agonist stimulated GPCRs was determined to act via a heterotrimeric G protein mechanism (Merritt *et al.*, 1986a), and subsequent analysis revealed that the PLC activity was due to the PLCβ isoform (Taylor *et al.*, 1991; Blank *et al.*, 1991; Shaw and Exton, 1992; Exton,

1993). PLCβ isoforms are activated by the Gq/11 family of heterotrimeric G proteins (Taylor *et al.*, 1991; Blank *et al.*, 1991; Lee *et al.*, 1992; Wu *et al.*, 1998). Purified PLCβ1 is more potently activated than PLCβ2 and PLCβ3 (which showed little difference in activation) by purified Gαq and Gα11 subunits (Hepler *et al.*, 1993; Smrcka and Sternweis, 1993). It has been shown that both the Gα and Gβγ subunits can activate members of the PLCβ family with different potencies – the activation of PLC by the Gβγ subunits of heterotrimeric G proteins is approximately 50-100 fold lower potency than that by the Gα subunits (Morris and Scarlata, 1997). However, as the PLCβ isoform has a GTPase-activating protein (GAP) effect for the Gα subunit (Berstein *et al.*, 1992; Paulssen *et al.*, 1996), the use of the non-hydrolysable GTP analogue (GTPγS) negates the GAP effect of PLC, and may account for the higher potency of Gα activation *in vitro* (Morris and Scarlata, 1997). The potency of PLCβ activation by Gβγ subunits is also isoform dependent, with PLCβ2 and PLCβ3 being more sensitive than PLCβ1 and PLCβ4 (Smrcka and Sternweis, 1993; Ueda *et al.*, 1994).

PLCβ is the only isoform subfamily that is directly activated by the Gq/11 family of heterotrimeric G proteins (Rhee and Choi, 1992) and this is thought to be due to PLCβ having an extended carboxy-terminal domain when compared to PLCδ and PLCγ (Lee *et al.*, 1992; Park *et al.*, 1993). Truncations of this tail portion abolished the ability of this enzyme ability to be activated by Gαq, but not Gβγ (Wu *et al.*, 1992; Park *et al.*, 1993). This suggests that this region contains a Gαq binding site. The Gβγ binding site is thought to be in the N-terminal pleckstrin homology (PH) domain, necessary for PIP_2 binding (Kuang *et al.*, 1996). There are recent reports

that suggest PLCε can be activated in response to GPCR stimulation, possibly via the Gα12/13 family of heterotrimeric G proteins (Wing *et al.*, 2003; Kelley *et al.*, 2004), as well as PLCε activation which may be mediated by an elevated level of intracellular cAMP (Evellin *et al.*, 2002).

The PLC family have a common main structure (Figure 1.6) consisting of an amino terminal pleckstrin homology (PH) domain, an EF-hand domain that acts as a flexible linking domain to the conserved catalytic core of the enzyme (the X/Y box) and a C2 region at the carboxy-terminal of the protein (Rhee and Choi, 1992). The PH domain is approximately 120 amino acid residues long, and is necessary for the association of PLC with phospholipids head groups in the membrane and specifically with PIP_2 (Paterson *et al.*, 1995; Lomasney *et al.*, 1994; Yagisawa *et al.*, 1998). The conserved catalytic core of the enzyme begins at approximately residue 300 and consists of two parts – the X region is approximately 147 residues and is connected to the Y region, which is approximately 118 residues in length. In PLCβ and PLCδ, this X/Y box is separated by 50-70 amino acids, however PLCγ has over 400 residues between the two regions, containing three src-homology (SH) domains (two SH2 domains and one SH3 domain)(Rhee and Choi, 1992; Essen *et al.*, 1996). The catalytic domain contains residues that are important for phosphoinositol hydrolysis (Lys-438, Lys-440, Ser-552 and Arg-549 in PLCδ) and also other residues that are important for catalysis (His-311 in PLCδ)(Ellis *et al.*, 1998). The C2 domain is similar to that found in the protein kinases C superfamily (where it is necessary for allosterically binding Ca^{2+} ions for activity), however it may primarily function as a stabilising domain, a mutations to prevent Ca^{2+} binding do not significantly alter the activity of the enzyme (James and Downes, 1997). The carboxy-terminal domain of

the enzyme, in addition to binding the Gαq subunit, has an intrinsic GTPase activating protein like (GAP-like) activity, which accelerates hydrolysis of the GTP on the active Gαq subunit back to the inactive GDP form (Paulssen *et al.,* 1996)

GPCR Signalling Pathways: Phospholipase D

Phospholipase D (PLD) activity was first described in plants (Hanahan and Chaikoff, 1948), and was originally thought to be present in eukaryotes only in simple forms such as *Dictyostelium discoideum* (Ellingson and Dischinger, 1984; Cubitt *et al.*, 1993) and plants (Dawson, 1967; Long *et al.*, 1967). PLD was characterised in mammals only in the 1970s (Saito and Kanfer, 1973). It has since been shown that PLD is widely present in mammalian cells, where it can be stimulated by a variety of extracellular signals, to hydrolyse its main substrate, phosphatidylcholine (PtdCho) to phosphatidic acid (PA) and choline. Phosphatidylcholine is the most abundant phospholipid constituent of mammalian membranes, providing about 50% of the total phospholipid content, and as much as 60% of the intracellular membrane content (Owen *et al.*, 1981; Lagarde *et al.*, 1982; Patton *et al.*, 1982).

The function of PLD activity has remained somewhat elusive. It was originally thought that the release of the choline group from phosphatidylcholine was an essential step in the synthesis of the neurotransmitter acetylcholine (Hattori and Kanfer, 1984; Zhao *et al.*, 2001) and whilst this may be the case in specific neuronal cells, the role of phosphatidic acid (PA) released during this hydrolysis as a second messenger in many peripheral tissues has also been established (Exton, 1990; English, 1996). Phosphatidic acid has been implicated in a variety of cellular functions, including a mode of Ca^{2+} mobilisation, apparently independent of that

caused by the action of IP_3 (English, 1996), cellular proliferation (Knauss *et al.*, 1990) and as a substrate for downstream prostaglandin synthesis (Marshall *et al.*, 1981). It has also been implicated in the activation of certain kinases (Ohguchi *et al.*, 1997). Furthermore, PA is known to be an important regulator of the mitogen activated protein kinase (MAPK) signalling mechanism (Ghosh *et al.*, 1996; Rizzo *et al.*, 1999).

PLD isoforms

There have been 2 isoforms of PLD isolated from mammalian tissues, the 124 kDa PLD1 and the 106 kDa PLD2 (Hammond *et al.*, 1995; Colley *et al.*, 1997; Kodaki and Yamashita, 1997). PLD1 occurs as two splice variants, PLD1a, and the shorter variant PLD1b which lacks the amino acids 565-624, both of which have similar regulatory properties (Rose *et al.*, 1995; Hammond *et al.*, 1997; Colley *et al.*, 1997; Park *et al.*, 1997).

Subcellular localisation of PLD has been shown to be mainly in the plasma membrane, as well as in the Golgi and nuclei. The localisation of PLD within the cell seems to be isoform specific. Studies indicate that PLD2 localises predominantly in the plasma membrane, whereas PLD1 is perinuclear (Golgi, endoplasmic reticulum and late endosomes)(Colley *et al.*, 1997; Park *et al.*, 2000; Du *et al.*, 2004).

Domain structures of PLD

The cloning of tha mammalian phosphatidylcholine specific PLDs, PLD1 and PLD2 led to investigations into the domain structure of the isoforms and to comparisons

with PLC to determine any regions of homology. It transpired that human PLD1 did not contain any specific domain regions in common with PLC family members, such as recognizable SH2 or SH3 domains and the PIP_2 interacting domain was distinct from that of PLCδ (Hammond *et al.,* 1995). The sequences of other PLD family members, such as the phosphatidylinositol-glycan specific PLD isolated from bovine sera (Scallon *et al.,* 1991), indicated that there were conserved domains within the PLD family group. One of these homologous domains is an invariant charged region with residues HxKxxxxD, known as the HKD motif, present at residues 455-490 and 892-926 in the human PLD1 isoform, which is thought to be important for catalytic activity (Hammond *et al.,* 1995). Subsequent studies using mutagenesis of the lysine (K) to an arginine (R) at position 898 have bourne out this hypothesis and the mutant human PLD1 K898R has been shown to be catalytically inactive (Sung *et al.,* 1997). The same mutation at the equivalent residue in the PLD2 isoform, K758R, elicits the same loss of catalytic activity (Sung *et al.,* 1997). It is though that a loop region within the enzyme allows PLD to fold, the amino- and carboxy-terminals are brought into closer proximity and the two HKD motifs can associate together to form a catalytic centre (Xie *et al.*, 2000; Leiros *et al.,* 2004). The two HKD motifs, along with two other areas of high homology within the PLD family, the IYIENQFF motifs, make up the conserved regions I, II, III and IV (Figure 1.6)(Morris *et al.*, 1996; Frohman *et al.*, 1999)

Mammalian PLD1 and PLD2 both have Phox homology domains, which are conserved regions found in many proteins, that facilitate protein-protein interactions (Frohman *et al.*, 1999). PLD1 has a Phox homology (PX) domain between residues 81-212 and PLD2 has a PX domain between residues 65-195 (Hammond *et al.*, 1995;

Lopez et al., 1998). The presence of the PX domain is critical for PLD activity (Frohman *et al.*, 1999; Sung *et al.*, 1999) and it is regions of the PX domain that may interact with the different PKC isoforms (Zhang *et al.*, 1999). In addition, the PX domains may be necessary for the modulation of the phospholipids-interacting PH domains (Sugars *et al.*, 2002; Ktistakis *et al.*, 2003).

The ability of PLD to localize to cellular membranes is influenced by fatty acid modifications of a PIP_2-interacting PH domain, present in both mammalian PLD isozymes (Sugars *et al.,* 1999; Sung *et al.*, 1999; Xie *et al.*, 2001). The PH domain of PLD1 is between residues 219-328 and within PLD2 is between residues 203-311; plalmitoylation occurs on two adjacent cysteine residues in the PH domain of both mammalian PLD isozymes to facilitate membrane association (Hammond *et al.*, 1995; Lopez *et al.*, 1998; Sugars *et al.*, 1999; Sung *et al.*, 1999; Xie *et al.*, 2001). Furthermore, PIP2 has additionally been shown to be necessary as a cofactor for PLD activity (Whatmore *et al.*, 1996) and, although the PH domain is required for PLD localization to membranes, the actual site of PIP_2 that is involved in functional activation has been contentious. It was originally proposed that the site of PIP_2 activation of PLD was the PH domain (Hodgkin *et al.,* 2000). However, experiments performed more recently would suggest that although the PH domain facilitates PLD localization with PIP_2-containing lipid membranes, the site of PIP_2 activation of PLD is present in an arginine and lysine rich sequence in the region 691-712 on PLD1 and 554-575 on PLD2 (Du *et al.*, 2003). This region, in addition to the PH and PX regions is also necessary for efficient PLD1 translocations to the plasma membrane upon cellular stimulation (Du *et al.*, 2003)

Activation of PLD

Activation of GPCRs can lead to downstream signalling through PLD through many routes. The most well defined regulation of PLD is through protein kinase C (PKC) of which there are 12 isoforms, and this pathway may well occur to some degree in most mammalian cells. However, another main route of PLD activation is through the small G-proteins ARF and Rho.

The activation of PLD due to PLC activity upon agonist stimulation of GPCRs, and the concomitant activation of PKC by DAG, a product of PLC activation, is well known (Cockcroft, 1984; Martinson *et al.*, 1989; Brown *et al.*, 1990). It was first shown in 1989 that the tumour–promoting phorbol esters, which activate PKC (Castagna *et al.*, 1982; Nishizuka, 1984a), led to the activation of PLD (Gelas *et al.*, 1989; Billah *et al.*, 1989; Huang and Cabot, 1990). It was later shown that PKC could indeed specifically activate PLD (Conricode *et al.*, 1992). Recombinant PKCα, from porcine brain has been shown to interact with and activate PLD1 synergistically with ARF and in a kinase-independent manner (Singer *et al.*, 1996). Moreover, the conventional PKCβ isoform, but not the PKCγ isoform, was shown to stimulate PLD activity, but with a lower potency than PKCα, whereas PKCδ, PKCε and PKCζ were ineffective (Conricode *et al.*, 1994). However the use of PKC inhibitors, such as staurosporine and bisindolylmaleimide has yielded information that agonist-dependent GPCR-stimulated PLD activity is dependent on catalytic activation of PKC in many cell types (Cook *et al.*, 1991; Plevin *et al.*, 1994; Meacci *et al.*, 1995; Ahmed *et al.*, 1995; Martinson *et al.*, 1995; Yeo and Exton, 1995; Rumenapp *et al.*, 1997). It

has been further demonstrated that both PLD1 and PLD2 are activated by PKC isoforms with similar potencies (Xie *et al.*, 2002; Chen and Exton, 2004).

The interaction of PKC with PLD has also been shown to lead to increased phosphorylation of PLD, which may switch off PLD activity (Hu and Exton, 2003; Chen and Exton, 2004). In addition to stimulation of PLD by PKCα, recently it has been reported that the competitive association of PKCδ with PLD1 may have a negative role in the activation of PLD. There is also evidence to suggest that PLD is negatively regulated by PKCδ in vivo, as cells containing both PKCα and PKCδ, elicited a lower PLD response and attenuated PKCα and PLD association compared to those containing PKCα alone (Hornia *et al.*, 1999; Oka *et al.*, 2003). However, another group has reported that PKCδ can activate PLD as well as PKCα (Hodgkin *et al.*, 1999). These conflicting reports demonstrate that there may be a number of distinct PKC isoform-dependent mechanisms for regulation of PLD activity and the extent to which these operate is likely to depend upon cellular context.

A major breakthrough in the understanding of cellular PLD regulation was made when a cytosolic factor of approximately 16 kDa was shown to reconstitute GTPγS evoked PLD activity in permeabilised and isolated membranes from HL-60 cells (Anthes *et al.*, 1991; Geny and Cockcroft, 1992; Geny *et al.*, 1993). This factor has since been shown to be the small G protein ADP-ribosylation factor (ARF) (Brown *et al.*, 1993; Cockcroft *et al.*, 1994). ARF has since been shown to activate PLD in a GTP-dependent manner (Hammond *et al.*, 1997). Recombinant ARF1 was shown to be effective in activating PLD in the presence of GTPγS (Brown *et al.*, 1993; Cockcroft *et al.*, 1994), as have ARF5 and ARF6 (Brown *et al.*, 1995; Caumont *et*

al., 1998). In addition to ARF proteins, RhoA and other members of the Rho family of GTPases have been shown to activate PLD in the presence of GTP (Bourgoin *et al.*, 1995; Ohguchi *et al.*, 1995; Kanaho *et al.*, 1996; Ohguchi *et al.*, 1996; Kim *et al.*, 1998; Meacci *et al.*, 1999; Genth *et al.*, 2003) including cdc-42 (Kato *et al.*, 1997) and Ral (Jiang *et al.*, 1995; Kim *et al.*, 1998; Frankel *et al.*, 1999). It was initially shown that the Rho-guanine nucleotide dissociation inhibitor (GDI), inhibited the GTP-dependent PLD activity in neutrophils (Bowman *et al.*, 1993), and it has been further shown that exogenously added RhoA reconstituted the GTP-stimulated PLD activity in Rho-GDI-treated rat liver membranes and HL60 cell membranes (Bowman *et al.*, 1993; Malcolm *et al.*, 1994). The ARF domain that is involved in the stimulation of PLD differs from that required for activation of cholera toxin (Zhang *et al.*, 1995), and has been narrowed to residues 35-94 (Liang *et al.*, 1997). This is similar to the site required for recruitment of adapter protein 1 (AP1) to the Golgi membranes by ARF. Adapter protein 1 is a subunit of clathrin-associated adapter protein complex 1 that plays a role in protein sorting in the late-Golgi/trans-Golgi network (TGN) and/or endosomes. The AP complex mediates both the recruitment of clathrin to membranes and the recognition of sorting signals within the cytosolic tails of transmembrane cargo molecules (see Robinson, 2004 for a review).

It has also been shown that PLD can be activated via ARF upon agonist stimulation. In human embryonic kidney cells stably expressing M_2 muscarinic receptors, the ARF-GEF-inhibitor brefeldin A (BFA) inhibited the stimulation of PLD by carbachol in intact cells, but not that induced by GTPγS in permeabilised cells (Rumenapp *et al.*, 1995). It has also been shown that BFA has an inhibitory effect on the activation of

PLD via receptors for platelet-derived growth factor (PDGF) and phorbol 12-myristate 13-acetate (PMA) on HIRcB fibroblast cells, and that the combination of GTPγS plus PDGF or PMA increased PLD activity, in permeabilised cells, in an ARF-dependent manner (Shome *et al.*, 1998). It has since been shown that activation of PLD via many different GPCR agonists can occur via an ARF (or Rho) dependent pathway. These include angiotensin II (AT_1) and endothelin 1 (ET-1) receptors in A10 smooth muscle cells (Shome *et al.*, 2000), the M_3 muscarinic and other receptors in 1321N1 astrocytoma cells, angiotensin II (AT_1) receptors in anterior pituitary gland but not the wild type gonadotrophin-releasing hormone (GnRH) receptors. Correspondingly ARF and RhoA could be co-immunoprecipitated with the angiotensin AT_1 receptor and the M_3 muscarinic receptor, but not the GnRH receptor (Mitchell *et al.*, 1998).

The isoform of PLD that is involved in the ARF-dependent responses was thought for several years to be PLD1 because of its activation *in vitro* by ARF (and Rho and PKC) (Hammond *et al.*, 1997; Park *et al.*, 1997). Nevertheless, recent evidence suggests that PLD2, and especially an amino-terminally-truncated form of PLD2 can also be activated by ARF (Lopez *et al.*, 1998; Sung *et al.*, 1999a). Both PLD1 and the truncated form of PLD2 are activated *in vitro* by ARF1 more effectively than by ARF6 (Sung *et al.*, 1999a). In contrast, PLD2 heterologously expressed in cells can be activated to a similar extent by constitutively active ARF1 and ARF6 (Du *et al.*, 2000). ARF-dependent PLD activity and GPCR-mediated PLD responses have been described in the plasma membrane compartment (Provost *et al.*, 1996; Morgan *et al.*, 1997), although the identity of the isoform responsible was not clear. PLD1 is largely associated with Golgi and other intracellular membranes (Colley *et al.*, 1997; Morgan

et al., 1997; Ktistakis *et al.*, 1999; Sung *et al.*, 1999b), but some is also associated with the plasma membrane, to a more predominant extent in some cell types, (Kim *et al.*, 1999; Freyberg *et al.*, 2001; Humeau *et al.*, 2001) and the enzyme can be recruited to the plasma membrane during exocytosis (Morgan *et al.*, 1997; Brown *et al.*, 1998) or in response to GPCR activation (Mitchell *et al.*, 2003). In contrast, PLD2 is more generally associated with the plasma membrane (Colley *et al.*, 1997; Liscovitch *et al.*, 2000) although it too may be associated with Golgi structures (Freyberg *et al.*, 2002).

It has since been suggested that both the small G proteins ARF and Rho, in conjunction with PKC act in a synergistic fashion to activate PLD (Kanaho *et al.*, 1996). Mutation studies, where PKC non-responsive alleles of PLD were constructed showed that the activation of PLD by PLD was synergistic with the activation of PLD by Rho A, in that a much higher response by PLD was observed when both PKC and Rho A were able to activate PLD than either PKC or RhoA acted individually (Zhang *et al.*, 1999). Furthermore, in studies with human neutrophils, where PLD activation in response to fMLP receptor stimulation was studied, cell permeabilisation by streptolysin O, which leads to the loss of cytosolic ARF, but not Rho proteins from the neutrophil cells, leads to a progressive inability of these calls to activate PLD. This ability is restored upon the re-addition of ARF, suggesting that Rho cannot act to substantially activate PLD on its own (Fensome *et al.*, 1998).

ADP Ribosylation Factor

ADP-ribosylation factors (ARFs) are 20 kDa GTPases of the Ras superfamily. ARF was first identified during the 1980s as a factor that stimulated the ADP-

ribosyltransferase activity of the cholera toxin A subunit (Kahn and Gilman, 1984). The ARF proteins all have a consensus sequence for GTP binding and hydrolysis, which are critical functions of ARF activity as a G protein (Moss and Vaughan, 1995), and this sequence is identical in all of the mammalian ARFs. It has been said that it is in this GTP binding domain, as well as the fact that the ARF proteins are myristoylated on an N-terminal glycine, that makes the ARF proteins more similar to the heterotrimeric G proteins than to the other 20 kDa Ras-like proteins in terms of secondary structure. ARF may indeed be an ancestor to both as it is present in the primitive parasite *Giardia lamlia* that lacks G_α subunits (Moss and Vaughan, 1993). Six ARF genes have been described, and the predicted protein sequences are highly conserved (100% identity for human and murine ARF1) (Boman and Kahn, 1995). There are 6 mammalian ARF proteins, which can be divided into 3 classes based on size, amino acid sequence, gene structure and phylogenetic analysis. Class I contains ARF1, ARF2 and ARF3; class II consists of ARF4 and ARF5; and class III consists of ARF6 alone (Moss and Vaughan, 1998). All isoforms of ARF can activate cholera toxin, and all isoforms of ARF can be shown also to activate PLD.

In man, there have been 5 ARF proteins identified (there is no ARF2 in humans). The human Class I ARFs are 96% identical, in resting cells they are mostly cytosolic in location, and they act to recruit coat proteins to the membranes of the Golgi apparatus. The Class I ARFs are expressed at higher levels than the other three ARF proteins in human tissues. The levels of ARF4-6 are often only about 10% of those of ARF1 and/or ARF3 (D'Souza-Schorey *et al.*, 1995; Peters *et al.*, 1995; Cavenagh *et al.*, 1996).

The two most abundant classes of cellular ARFs (Class I and III) are exemplified by ARF1 and ARF6, hence these members of the ARF family of proteins have had the most focus on them in studies of mammalian systems. GTP loading and GPCR activation can cause translocation of ARFs to Golgi and other unspecified membrane compartments, and marked translocation of ARF1 to the plasma membrane has been shown following activation of the M_3 muscarinic receptor (Mitchell *et al.*, 2003) and the 5-HT_{2A} and other receptors (Park *et al.*, 1997; Morgan *et al.*, 1997; Fensome *et al.*, 1998; Sung *et al.*, 1999b), thus both ARF1 and ARF6 are potentially available for interaction with plasma membrane GPCRs following agonist stimulation.

Very little is known of the functions of the class II ARFs, though their interactions with the effectors of other small GTPases suggest their potential role in co-ordinating membrane traffic in conjunction with additional small G proteins (Deretic *et al.*, 2005). ARF4 has been shown to have a role in the post-Golgi trafficking of rhodopsin, specifically recognising a C-terminal sorting sequence of rhodopsin and regulating its incorporation into rhodopsin transport carriers, which are specialised membrane carriers targeted to the rod cell outer segment (Deretic *et al.*, 2005).

Most members of the ARF family of proteins are predominantly cytosolic, with activation via GTP binding leading to increased membrane association. However, ARF6 is largely associated with the plasma membrane, and also to endosomes, and appears to modulate the assembly of actin cytoskeleton and endocytosis, as well as having a role in recycling endosomes to the plasma membrane (D'Souza-Schorey *et al.*, 1995; Peters *et al.*, 1995; Cavenagh *et al.*, 1996; Park *et al.*, 1997; Fensome *et al.*, 1998; Lopez *et al.*, 1998; Sung *et al.*, 1999a). ARF6 has been shown to interact specifically with adapter protein complex-2 (AP-2) and promotes its membrane

recruitment, thereby directly interacting with a route of endocytosis (Paleotti *et al.*, 2005). AP-2 plays a role in protein sorting in the late-Golgi/trans-Golgi network (TGN) and/or endosomes. The AP-2 complexes mediate both the recruitment of clathrin to membranes and the recognition of sorting signals within the cytosolic tails of transmembrane cargo molecules. The AP-2 complex seems to play a role in the recycling of synaptic vesicle membranes from the presynaptic surface (Laporte *et al.*, 1999; Laporte *et al.*, 2000; Laporte *et al.*, 2002 and see Robinson, 2004 for a review). This association suggests that ARF6 may have a role in clathrin coating of vesicles (Paleotti *et al.*, 2005).

ARF myristoylation

There is a known myristoylation site with all isoforms of ARF, on glycine 2 at the amino terminal end of the protein. Myristoylation allows for some GDP-GTP exchange at physiological Mg^{2+} levels, in the presence of phospholipid vesicles (Franco *et al.*, 1995a). Both myristoylated and non-myristoylated forms of $ARF_{GTP\gamma S}$ can bind to membrane phospholipids, but only myristoylated ARF_{GDP}, and not the non-myristoylated form has been shown to bind to the same membrane phospholipids (Franco *et al.*, 1996). ARF1 mutants with deletions of the first 13-17 residues of the N-terminus, and those with deletions of amino acids 3-7 (residues shown to be important in recognition by N-myristoyltransferase), had a greater affinity for GTPγS than for GDPβS (Franco *et al.*, 1995a). Therefore the N-terminus of ARF1 is a GTP-sensitive effector domain. In the presence of phospholipids, the myristoylated form of ARF1 has a higher affinity for GTPγS than for GDP. In the absence of phospholipids, the reverse is true (Randazzo *et al.*, 1995). It has been

suggested that the GTP activation of ARF proteins is a multi-part process, with myristoylation of the protein leading to membrane association. The myristoylated amino-terminal takes the shape of an alpha helix that associates with a hydrophobic cleft of the interswitch domain (amino acids 52-67 in ARF1) in the GDP bound form of ARF, but this association is weakened by association of the myristoyl group with the membrane, presumably allowing of the for greater access to the GTP binding site by GTP. This leads to the formation of ARF_{GTP}, which is more stable when associated with the membrane (Randazzo *et al.*, 1995; Franco *et al.*, 1995a; Franco *et al.*, 1996).

Regulation of ARFs

ARF proteins exist as GTP-dependent switches, and, as in other Ras-like proteins, the conformation of two regions of ARF, switch 1 (amino acids 40-51 of ARF1) and switch 2 (amino acids 68-81 of ARF1) differ between the GDP and GTP-bound state (Amor *et al.*, 1994; Greasley *et al.*, 1995; Goldberg, 1998; Goldberg, 1999; Menetrey *et al.*, 2000; Amor *et al.*, 2001; Pasqualato *et al.*, 2001; Amor *et al.*, 2002; Pasqualato *et al.*, 2002). Like all G-proteins, activation occurs when GDP is released from the protein and exchanged for a molecule of GTP. This exchange is facilitated in cells via a group of proteins known as guanine nucleotide exchange factors (GEFs), however it is possible that conformational changes in GPCR structure could allow the GPCR to act as a GEF for ARF, as is the case with the heterotrimeric G proteins, although this theorem has not been proven. Transition from the GDP-bound to GTP-bound form of ARF is achieved through the family of proteins known as ARF guanine nucleotide exchange factors, or ARF-GEFs, which consist of 14 proteins in 5 subfamilies. All of these proteins contain a Sec7 domain that catalyses the

exchange of nucleotide. Crystallographic studies reveal that the switch 1 and 2 regions of ARF form an interface with the Sec7 domain (Cherfils *et al.*, 1998; Beraud-Dufour *et al.*, 1998; Mossessova *et al.*, 1998; Goldberg, 1998; Cherfils and Chardin, 1999; Peyroche *et al.*, 1999). Nucleotide dissociation is favoured by disrupting the binding site for Mg^{2+} and the introduction of a glutamate residue from the Sec7 domain within 3 Å of the β-phosphate of GDP. The GEFs seem to have some specificity for the isoform of ARF they activate, which may lead to specificity of ARF activation within the cell depending on isoforms of ARF and the particular GEFs present. The GEF BIG1/2 is known to activate ARF1 and ARF3 preferentially, whereas the GEFs ARNO and GRP seem to preferentially activate ARF6 (Donaldson and Jackson, 2000; Jackson and Casanova, 2000) (Figure 1.7).

Deactivation of ARF_{GTP} is facilitated by a family of 16 GTPase-activating proteins or ARF GAPs. All of these proteins share a common GAP domain of 70 amino acids in length, which includes a zinc finger motif (CxxCx(16-17)CxxC) that is critical for GAP activity (Cukierman *et al.*, 1995). In addition to the zinc finger, all GAPs have a conserved arginine within the GAP domain. Mutation of this arginine to a lysine results in a 10^5 fold decrease in GTPase activity (Mandiyan *et al.*, 1999; Randazzo *et al.*, 2000) suggesting an arginine finger catalyst mechanism for GTP hydrolysis. ARF GAPs also display some selectivity for ARF isoforms. ARF GAP1 preferentially deactivates ARF1, ARF3 and ARF5, whereas ASAP1 and PAP work best on ARF1 and ARF5, and to a lesser extent deactivate ARF6. The ARF GAPs GIT1 and GIT2 work on ARF1, ARF3, ARF5 and ARF6 with seemingly equal effects (see Donaldson and Jackson, 2000 for a review). In the case of ARF and Rho, a group of proteins known as GDP dissociation inhibitors (GDIs) which retain

the G protein in an inactive state have also been discovered (Donaldson and Jackson, 2000; Nie *et al.*, 2003).

Muscarinic Receptors.

Cholinergic transmission takes place in via two classes of receptor, the nicotinic cholinergic family of ligand-gated ion channels, and the muscarinic family of GPCRs. The physiological effects of the prototypical muscarinic agonist muscarine (from the mushroom *Amanita muscaria*) and the antagonist atropine (from the plant *Atropa belladonna*) have been known for many years (Ford 1909; Dale 1914). More recent investigations have shown that there are 5 genes for the muscarinic receptors (m1-m5), which encode for highly related, yet distinct M_1-M_5 receptor subtypes. (Bonner *et al.*, 1987). Muscarinic receptors M_1, M_3 and M_5 have all been shown to activate the phosphoinositide pathway via G_q/G_{11} activation of PLC leading to an increase in the intracellular Ca^{2+} concentration, (Jones and Michell, 1974; Buckley *et al.*, 1989; Bonner, 1989) whereas M_2 and M_4 have been seen to couple through G_i/G_o G proteins, leading to the inhibition of adenylyl cyclase (Haga *et al.*, 1985; Bonner, 1989). In 1321N1 astrocytoma cells and transfected COS7 cells, the M_3 muscarinic receptor has also been shown to activate PLD in a heterotrimeric G protein-independent manner through the small G protein ARF (Mitchell *et al.*, 1998). However, in other cell types, protein kinase C (Martinson *et al.*, 1990) and tyrosine kinases have been shown to activate PLD, for example, in HEK293 cells, the tyrosine kinase inhibitor genistein abolished the carbachol-induced activation of PLD by the M_3 receptor (Schmidt *et al.*, 1994).

M_3 muscarinic receptor

The M_3 muscarinic receptor was first cloned in 1987 (Bonner *et al.*, 1987; Peralta *et al.*, 1987). It is distributed widely in neuronal cells and the CNS, as well as in the peripheral ganglia, in visceral and vascular smooth muscle, exocrine glands and the ciliary body of the eye. (Levey *et al.*, 1994; Doods *et al.*, 1994; de la Vega *et al.*, 1996; Zhang, 1996; Hoglund and Baghdoyan, 1997; Lau and Pennefather, 1998; Masuda *et al.*, 1998). The human M_3 receptor is 590 amino acids in length (approximately 66kDa in size) and like most of the family R GPCRs contains a number of predicted N-linked glycosylation sites on asparagine residues at positions 5, 6, 15 and 41 in the extracellular amino-terminal domain. Cysteine residues at positions 141 and 221 are presumed to form the extracellular disulphide bridge present in nearly all GPCRs and the receptor contains consensus palmitoylation sites on cysteine residues 561 and 563 in the intracellular carboxy-terminal tail domain (Peralta *et al.*, 1987; Bonner, 1989). Mutagenesis and modelling studies have demonstrated that the acetylcholine binding site is probably formed by multiple tyrosine and threonine residues within the transmembrane domain α-helices, which co-ordinate to provide a binding pocket on the internal face of the α-helix barrel. This ligand binding domain involves a conserved Asp-148, which co-ordinates the protonated nitrogen of acetylcholine, Tyr-149 in TM3, Thr-232 and Thr-235 in TM5, Tyr-507 in TM6 and Tyr-530 and Tyr-534 in TM7, mutations of which were shown to not appreciably affect receptor expression or G-protein coupling, but caused reduced agonist binding affinity (Wess *et al.*, 1991; Wess, 1993). A proline residue (201) in TM4, which does not appear to interact with the ligand, is also thought to be necessary for binding (Wess *et al.*, 1991; Wess, 1993).

The M_3 receptor is unusual in the fact it has a particularly large third intracellular loop (i3) domain (239 amino acids in length) R^{253}-Q^{491}, which has been previously mooted as a candidate area for binding of signalling proteins. The heterotrimeric G protein $G\beta\gamma$ subunit has been shown to bind to this M_3i3 domain at residues 331-333, and it has been put forward that the G protein coupled receptor kinase GRK2 phosphorylates the M_3i3 somewhere on residues 331-333 and 348-352 (Wu *et al.*, 2000), although this phosphorylation may not be necessary for receptor internalisation (Shockley *et al.*, 1999; Budd *et al.*, 2000). Another kinase has been shown to phosphorylate the M_3 receptor on the i3 domain. Casein Kinase 1 α (CK1α) has been shown to phosphorylate the M_3i3 in an agonist-dependent manner, however, this phosphorylation event does not lead to a desensitisation of the receptor, phosphorylation of the M_3i3 by CK1 α instead seems to dramatically potentiate the inositol phosphate response of the M_3 receptor, possibly playing a role in the control of the magnitude of the PLC response of the M_3 and other PLC-coupled GPCRs (Tobin *et al.*, 1993; Tobin *et al.*, 1997; Budd *et al.*, 2000).

5-HT receptors.

Serotonin, or 5-hydroxytryptamine (5-HT) is a biogenic amine whose multiple actions are mediated by a diverse group of receptors. Receptors for 5-HT are found in both the central nervous system and the peripheral nervous system, as well as in a number of non-neuronal tissues in the gut, cardiovascular system and blood. In evolutionary terms, 5-HT is one of the oldest neurotransmitters (first chemically characterised in 1948), and was identified as one of the major vasoconstricting

substances in defibrinated blood (Rapport *et al.*, 1947). In 1954, striking structural similarities were noticed between LSD and 5-HT (Woolley and Shaw, 1954). Based on this observation, and the fact that schizophrenia is characterised partially by hallucinosis, 5-HT was proposed as possibly being involved in the pathogenesis of schizophrenia (Woolley and Shaw, 1954). 5-HT has since been implicated in the aetiology of a much wider range of disease states including depression (Amamoto and Sarai, 1976), anxiety (Lesch, 1991a; Coplan *et al.*, 1992), social phobia (Denboer *et al.*, 1994), schizophrenia and obsessive-compulsive and panic disorders (Bender, 1978; Rammsayer and Netter, 1990; Lesch, 1991b; Coplan *et al.*, 1992); in addition to migraine, hypertension (Chemerinski *et al.*, 1980), pulmonary hypertension (Kanai *et al.*, 1993), eating disorders (Cooper, 1989), vomiting (Miller and Nonaka, 1992) and irritable bowel disorder (Talley, 1992).

The family of 5-HT receptors encompasses seven major subclasses, consisting of 14 different subtypes of receptors in total. All but one of these subtypes (5-HT_3, which is in fact a ligand-gated ion channel) signal through G protein-linked pathways (reviewed in (Raymond *et al.*, 2001; Hoyer *et al.*, 2002)). The classification of the 5-HT receptors began in 1957, when it was demonstrated that the functional responses of the guinea pig ileum, but not other tissues, to 5-HT could be blocked by morphine (Gaddum and Picarelli, 1957). Originally a nomenclature of M and D receptors was proposed, however, due to the non-specific effects of the ligands on other neurotransmitter systems, this system was not fully accepted (Lewis, 1960; Day and Vane, 1963). In 1976, a radioligand binding study of rat cortical membranes gave rise to the postulation that specific binding sites for 5-HT reflected the previously reported pharmacological receptors (Bennett and Snyder, 1976). The presence of

two distinct membrane binding sites for 5-HT was demonstrated in 1979, and these were named 5-HT_1 and 5-HT_2 (Pertouka and Snyder, 1979). The previously named M receptor was found to be distinct from these 5-HT_1 and 5-HT_2 receptors, and was subsequently renamed the 5-HT_3 receptor (Bradley *et al.*, 1986). The 5-HT_4 receptor was discovered shortly afterwards in the gastrointestinal tract and brain tissue. The cloning of the 5-HT_{1A} receptor was completed in 1988 (Fargin *et al.*, 1988) with the others following on in rapid succession. This cloning process led to the identification of a number of new receptors, initially named 5-HT_{1E}, 5-HT_{1F}, 5-HT_{5A}, 5-HT_{5B}, 5-HT_6 and 5-HT_7 (reviewed in Hoyer *et al.*, 1994; Hoyer *et al.*, 2002). In the post genomic era, hundreds of receptors have been identified either by functional studies, or by cloning, so to ensure a clear and comprehensive nomenclature for the existing 5-HT receptors, and any orphan receptors that may join this class, a new classification system has been proposed (Humphrey *et al.*, 1993; Hoyer *et al.*, 1994; Hartig *et al.*, 1996; Hoyer and Martin, 1997), which has lead to the currently recognised seven families of 5-HT receptor. The diversity of this group of GPCRs is further increased by the fact that some members of the 5-HT family (5-HT_4 and 5-HT_7) can exist as different splice variants, leading to further different isoforms of these receptors (Kilpatrick *et al.*, 1999).

The different subclasses of receptors for 5-HT transduce their signals via different heterotrimeric G proteins, with the 5-HT_1 group (and 5-HT_5) linking preferentially, to $G_{i/o}$,, and thereby inhibiting cyclic AMP (cAMP) production. 5-HT_2 receptors have been shown to preferentially signal through $G_{q/11}$ to increase inositol trisphosphate levels and intracellular Ca^{2+} levels via PLC. 5-HT_4, 5-HT_6 and 5-HT_7 all signal through G_s to increase production of cyclic AMP, however they are categorised as

separate receptor classes due to their limited (<35%) overall sequence identities (reviewed in Hoyer *et al.*, 2002 and Raymond *et al.*, 2001). The 5-HT_3 receptor differs entirely from the other members of this family in that it is not a 7 transmembrane domain receptor, but a ligand-gated ion channel.

5-HT_2 receptors

The 5-HT_2 subclass consists of the 5-HT_{2A}, 5-HT_{2B} and 5-HT_{2C} (originally 5-HT_{1C}) receptors. The 5-HT_2 receptors are all of a similar size, 451-479 amino acids, (~52 kDa) and display ~70% sequence homology in their transmembrane regions, and an overall sequence identity of 46-50%. The 5-HT_2 receptors share many characteristics, including some regions of sequence similarity, and considerable overlap in ligand recognition sites. Like most of the family R GPCRs, they display predicted glycosylation sites on asparagine residues in their extracellular domains (Asn 8, 38, 44, 51 and 54 for the 5-HT_{2A} receptor; Asn 203 in the 5-HT_{2B} receptor; Asn 39 and 204 in the 5-HT_{2C} receptor), as well as predicted palmitoylation on a cysteine residue in the proximal part of the carboxy-terminal tail. All three receptors have the cysteine residues required for putative disulphide bridges linking the extracellular loop 1 and 2 (e1 and e2) domains (Cys 95-Cys172 for the 5-HT_{2A}, Cys128-Cys207 for the 5-HT_{2B} and Cys127-Cys207 for the 5-HT_{2C}). The members of the 5-HT_2 subfamily all have highly conserved NPxxY motifs at the proximal region of the carboxy-terminal tail (Saltzman *et al.*, 1991; Cook *et al.*, 1994; Schmuck *et al.*, 1994; Xie *et al.*, 1996).

Residues thought to contribute to the ligand binding site of the 5-HT_{2A} receptor were first hypothesised through the use of 3D molecular modelling techniques, where two aspartic acid residues in the central core of the receptor (Asp120 and Asp155) were

postulated to interact with the protonated ligands 5-HT and ritanserin. During the simulations 5-HT had only a weak interaction with Asp155, but a strong interaction with Asp120, where the amino group on 5-HT bound tightly to the carboxylic side chain of the Asp residue (Edvardsen *et al.*, 1992). These findings were backed up the next year by functional studies following mutagenesis studies that showed not only these two residues, but a further aspartic acid at position 172 may be involved. This study showed that the Asp120 was important for allosteric activation of the guanine nucleotide-binding protein, Asp155 is necessary for high affinity ligand binding, and mutation of Asp172 lead to an approximately 5 fold decrease in the affinity of the receptor for 5-HT (Wang *et al.*, 1993).

Both the 5-HT_{2A} and the 5-HT_{2C} receptors are widespread in the central nervous system, whereas the 5-HT_{2B} receptor has a much more restricted distribution. 5-HT_{2A} receptors occur in high levels in the limbic forebrain, in particular in the frontal cortex and nucleus accumbens. 5-HT_{2C} receptors occur in moderate density throughout the forebrain and in the hindbrain and are particularly concentrated in the choroid plexus. 5-HT_{2B} receptors are scarce in the brain, and are found mostly in the stomach and other peripheral tissues (though their levels there are also low) (VanOekelen *et al.*, 2003; Hoyer *et al.*, 1994; Hoyer *et al.*, 2002, reviewed in Roth *et al.*, 1998).

The 5-HT_{2A} receptor in particular has been implicated as an important site of actions for drugs used in a variety of major psychiatric disorders, including: antipsychotics; antidepressants; anxiolytics; and antihistamines (which have 5-HT_2 antagonistic action), as well as in hallucinogenic drug actions such as that of *d*-lysergic acid

diethylamide (LSD) and similar compounds, which have an antagonistic effect on the 5-HT_2 receptors (Van Oekelen *et al.*, 2003).

The 5-HT_2 receptors signal through coupling to $G_{q/11}$ to activate PLC, leading to an increased accumulation of inositol phosphates and intracellular Ca^{2+}. In addition to $G_{q/11}$-mediated phospholipase C (PLC) activation, the 5-HT_{2A} receptor can activate other signalling pathways that may involve alternative direct coupling to the receptor. These include activation of phospholipase A_2 (PLA_2) (which may be mediated by a transduction mechanism other than $G_{q/11}$ (Berg *et al.*, 1998) and activation of tyrosine phosphorylation (correlating with evidence for direct association of the tyrosine kinase Jak2 with the carboxy-terminal tail domain of the receptor (Guillet-Deniau *et al.*, 1997) and ARF-dependent activation of phospholipase D (PLD) (which may involve direct GPCR:ARF interactions (Mitchell *et al.*, 1998)).

Despite their general structural and functional similarities, there may be differences in the mechanisms desensitisation of 5-HT_{2A} and 5-HT_{2C} receptors. In rat NIH 3T3 cells, receptor activation led to a decrease in surface levels of 5-HT_{2C}, but not 5-HT_{2A}, but both exhibited a decrease in inositol phosphate production over time, therefore they undergo differential desensitisation in the same cell type (Oekelen *et al.*, 2001). The 5-HT_{2A} receptor seems to be regulated by uncoupling from G proteins (Oekelen *et al.*, 2001). Receptor levels were actually increased during the key time frame, due to synthesis of new receptors. Newly synthesised receptors appear not to couple readily to G-proteins. These findings taken together seem to suggest that rat 5-HT_{2A} and 5-HT_{2C} receptors differ markedly in their ability to

couple to G-proteins to form a functionally active receptor (Oekelen *et al.*, 2001; Van Oekelen *et al.*, 2003).

All three of the 5-HT_2 receptors contain PDZ target motifs at the extreme end of their carboxy-terminal tail. In the case of the 5-HT_{2A} receptor, this PDZ target motif has been shown to bind to a PDZ domain protein named PSD-95, which can link to additional signalling pathways and regulate receptor trafficking (in HEK-293 cells) (Xia *et al.*, 2003). PSD-95 augmented the signalling of the 5-HT_{2A} receptor through $G\alpha_q$ to activate phosphoinositide hydrolysis, without altering the kinetics of the receptor desensitisation, but did however inhibit the agonist-induced internalisation (Xia *et al.*, 2003). PSD-95 has also been shown to bind to the ct of the 5-HT_{2C} receptor (in complex with several other synaptic structural proteins: Veli3, CASK and Mint1) (Becamel *et al.*, 2002) as well as to some extent to the 5-HT_{2A} receptor (Becamel *et al.*, 2004). The 5-HT_{2C} receptor also been shown to bind to MUPP1, a multi PDZ domain protein with unknown function (Marinissen and Gutkind, 2001; Bockaert *et al.*, 2003; Bockaert *et al.*, 2004).

General aims of the present study

This study will investigate members of separate sub-families of the family R GPCRs, focusing particularly on the M_3 muscarinic receptor, the 5-HT_{2A} receptor and the closely related 5-HT_{2C} receptor. Both the M_3 and 5-HT_{2A} receptors have previously been shown to be able to activate PLD via an ARF-dependent pathway (Mitchell *et al.*, 1998). Experiments with wild type and negative mutant ARF constructs will be used to discern the involvement of ARF1 and ARF6 in receptor-mediated activation of PLD, determining if this role is isoform specific, and indeed if there is any variation in specificity between receptor subtypes.

Using the *in vitro* technique of GST-fusion protein pull-down assays, the interactions of ARF isoforms with individual intracellular domains of the M_3 muscarinic and 5-HT_2 receptors will be analysed. This will determine the specificity of each isoform for the receptor domains, and also allow investigation into the mechanisms of this interaction as well as any modulation influences over these interactions of ARF with receptor domains. Experiments will be carried out to address the roles of $G\beta\gamma$, which has been shown to bind to the third intracellular loop of the M_3 receptor, and arrestins, which are known to interact with both third intracellular loop, and carboxy-terminal tail domains of family R GPCRs.

Finally this study will use a targeted proteomic approach in an attempt to discover novel interaction partners for the intracellular domains of the 5-HT_{2A} receptor, and begin to characterise any interactions discovered to elucidate the role of novel binding partners in 5-HT_{2A} receptor signalling.

These studies will develop the understanding of GPCR:ARF interactions, and will contribute to understanding the cellular and molecular mechanisms that determine this signalling pathway.

Figures

Figure 1.1

The topology of a typical G protein-coupled receptor

A two dimensional representation of the topology of a generic 7-transmembrane GPCR embedded in the plasma membrane. The figure shows the extracellular N-terminal domain, the 7 transmembrane spanning α–helices, linked by intracellular (i1-3) and extracellular (e1-3) loops, and the intracellular C-terminal (ct). The conserved cysteine bridge between e1 and e2 is shown in red. Palmitoylation of a conserved cysteine residue in the proximal part of the carboxy terminal tail, is present in most of the rhodopsin family GPCRs, and gives rise to a putative fourth intracellular loop (i4). In the folded protein, the 7 transmembrane domains form a barrel like structure, into which the agonist can bind from the extracellular surface. Furthermore, a short section of amphipathic α-helix, apparently running in the cytosolic compartment, parallel to the plane of the membrane has been identified distal to the seventh transmembrane spanning α-helix. This region which ends near a palmitoylated cysteine residue (occurring in most, but not all, of the family R GPCRs) is sometimes referred to as the fourth intracellular loop (i4 loop) or helix 8, and may be important for G protein coupling (Bourne, 1997; Wess, 1998; Palczewski *et al.*, 2000). The extracellular domains and in some cases parts of the TM regions are mostly involved in agonist recognition, and the intracellular domains seem to be involved in receptor regulation and signalling mechanisms (see Kroeze *et al.*, 2003 for a review).

Figure 1.1

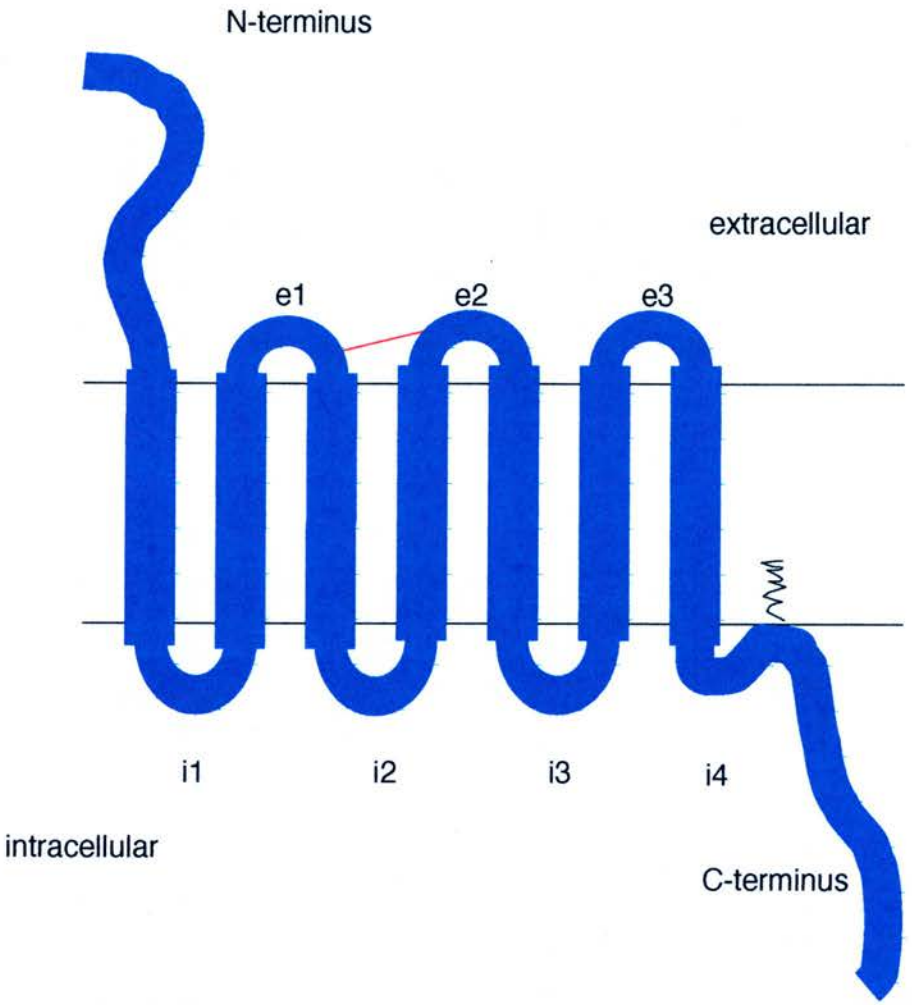

N-terminus
extracellular
e1
e2
e3
i1
i2
i3
i4
intracellular
C-terminus

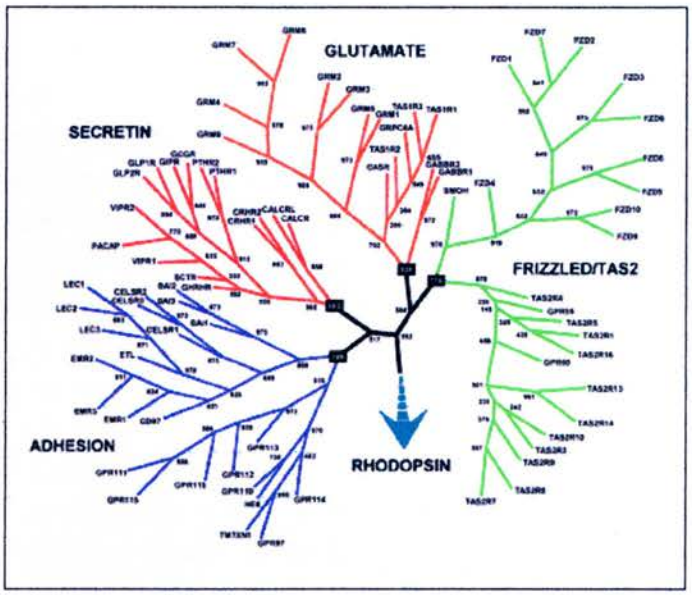
Figure 1.2

Phylogenetic relationship between the GPCRs in the human genome

The above dendrographs were calculated using the maximum parsimony method on 1000 replicas of the data set terminally truncated GPCR as described in Fredriksson *et al.*, 2003. The position of the rhodopsin family was established by including twenty random receptors from the rhodopsin family. These branches were removed from the figure 1.2 a, and are shown separately in figure 1.2 b.

Figure 1.2

a


GLUTAMATE
SECRETIN
FRIZZLED/TAS2
ADHESION
RHODOPSIN

b

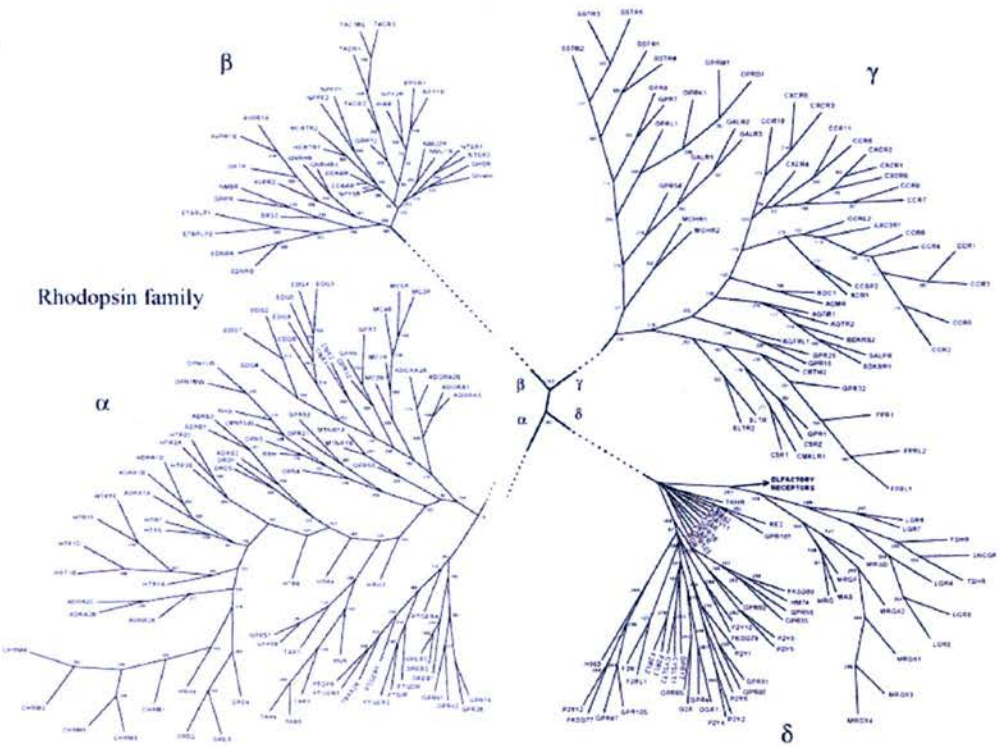

β
γ
Rhodopsin family
α
δ

Figure 1.3

The crystal structure of rhodopsin, a template for the G-protein coupled receptor

Figure 1.2 shows a diagram of the solved crystal structure of the seven transmembrane spanning helices of rhodopsin as seen from the sude, and from the extracellular views (Palczewski *et al.*, 2000). The helices are organised sequentially in an anticlockwise fashion with helix 3 being almost in the centre of the molecule. The seven transmembrane anticlockwise α-helix bundle is apparent (with helices labelled I-VII), as is the extracellular amino- and intracellular carboxy-terminal domains. the left hand image shows the extracellular view, containing the site of retinal attachment, which corresponds to the ligand binding site of Family R GPCRs.

Figure 1.3

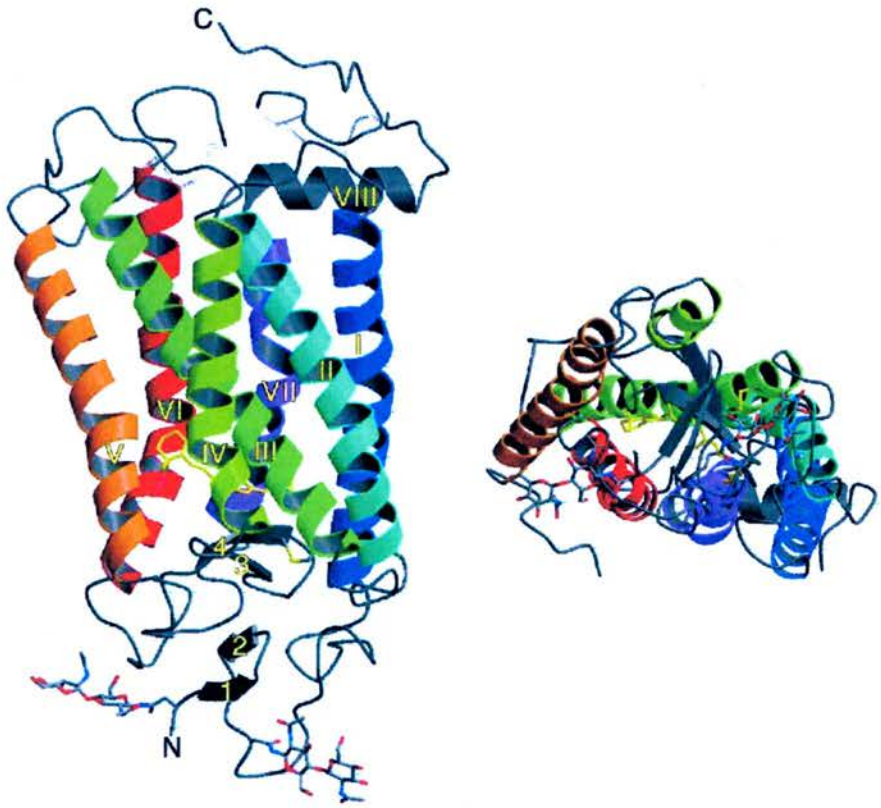


Figure 1.4

The activation/deactivation cycle of heterotrimeric G-proteins

Heterotrimeric G protein activity is regulated by the binding and hydrolysis of GTP by the α subunit. Only the GTP-bound α subunit is active, and contributes to the intracellular signalling of the GPCR by acting on the various effector proteins with the specificities described above. A GDP-bound α subunit is inactive. This figure shows the cycle of activation of trimeric G-proteins by GAPs (including ligand bound GPCRs) and deactivation via GTP hydrolysis by GEFs, or RGS proteins.

Figure 1.4

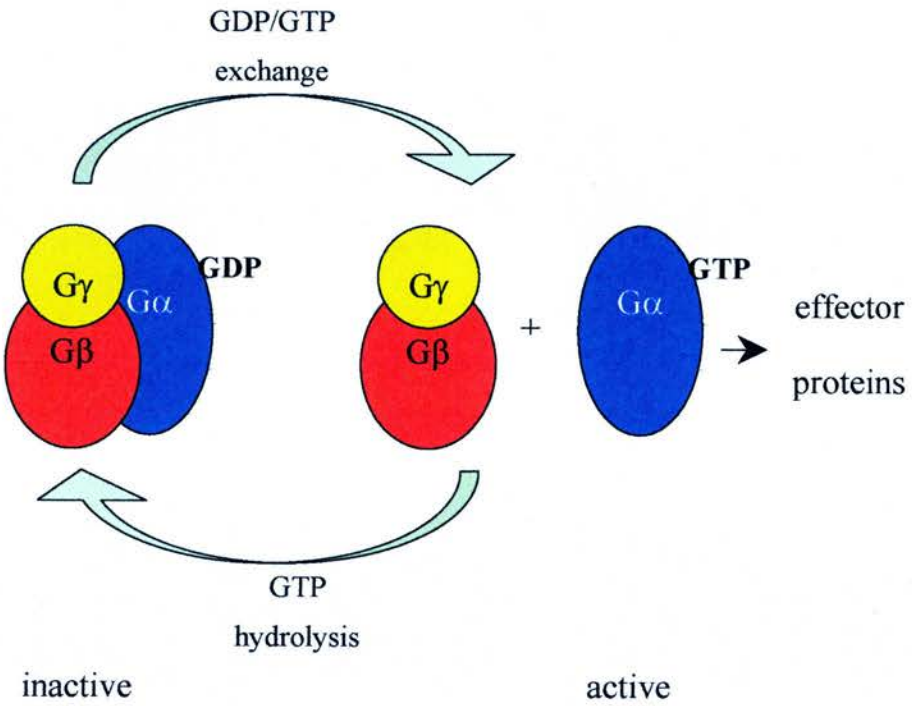

GDP/GTP
exchange
Gγ
Gα
Gβ
GDP
Gγ
Gβ
+
Gα
GTP
effector
proteins
GTP
hydrolysis
inactive
active

Figure 1.5

3D structure of the Gβγ heterodimer

Structure of the Gβγ heterodimer bound to the peptide SIGK (a small Gβγ binding peptide) (Davis *et al.,* 2005). Two views of the Gβγ-SIGK complex, shown in ribbon representation. The two representations are related by -90°. Gβ, blue, Gγ purple and SIGK is colored pink. The N- and C-termini of Gβ, and Gγ are labeled. The G protein beta subunit contains a distinctive 7 WD-40 repeat (tryptophan-aspartate repeat) structure. Each of the 7 repeats folds into a small antiparallel beta-sheet. These sheets are arranged around a central pseudosymmetry axis into a beta propeller. On the left panel, the seven blades of Gβ are labeled following the convention of Wall *et al.* (Wall *et al.,* 1995; Wall *et al.,* 1998). The SIGK peptide binds to two surfaces on $G\beta_1$, and the contact surface for SIGK on Gβγ is very similar to that occupied by the switch II region of Gα (Davis *et al.,* 2005).

Figure 1.5

Figure 1.6

The domain structures of the phospholipase C and D, and protein kinase C

The domain structures of the major GPCR pathway-coupled isozymes of PLC, PKC and PLD. The catalytic domains in each are indicated in red. The pleckstrin homology (PH) domains in PLC and PLD (blue) are important for associating with lipid membranes. The EF-hand and C2 regions in PLC and C2 region in PKC are important for Ca2+ ion binding. The P-box in PLCβ is important for interaction with the heterotrimeric G proteins. PKC contains and additional conserved C1 domain, where diacylglycerol and phorbol esters bind. PLD contains a phox homology (PX) domain and the conserved regions I-IV include the catalytic HKD domains. PLD contains an additional loop region, and allosteric PIP_2 site and the carboxy-terminal tail domain (CT) is important for regulating activity of the enzyme.

Figure 1.6

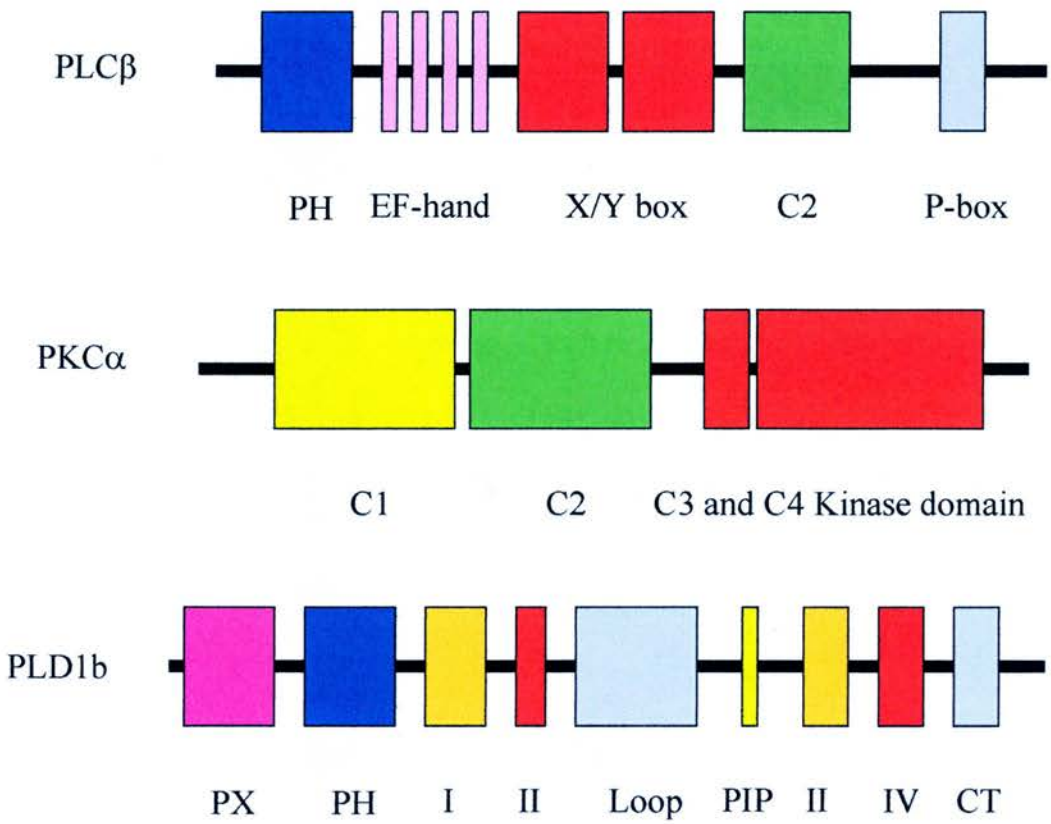
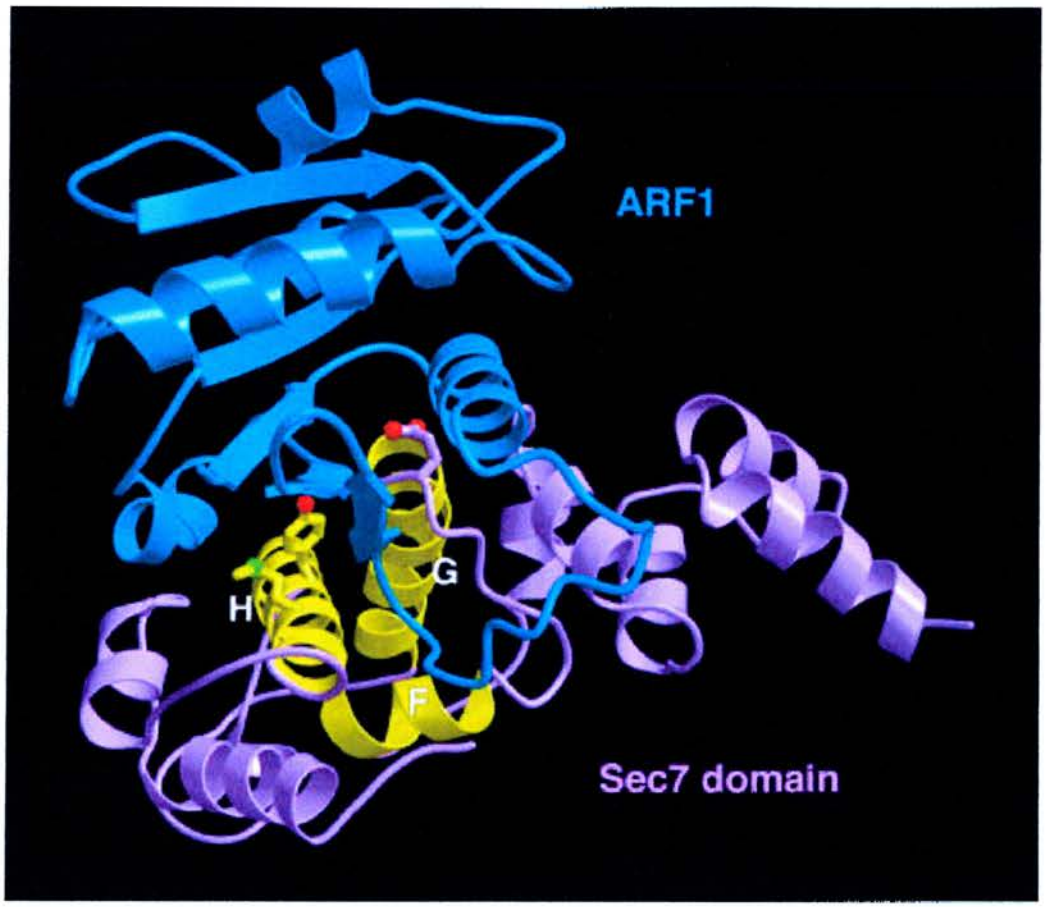

PLCβ
PH
EF-hand
X/Y box
C2
P-box
PKCα
C1
C2
C3 and C4
Kinase domain
PLD1b
PX
PH
I
II
Loop
PIP
II
IV
CT

Figure 1.7

Crystal structure of ARF1 bound to the Sec7-domain exchange factor

The mechanism by which ARF-family GTPases are activated to the GTP-bound state by exchange factors was revealed by the crystal structure of ARF1 complexed with a cognate exchange factor of the Sec7 family. The crystal structure shows how the exchange factor inserts amino-acid side chains directly into the ARF1 GTPase active site to exert a steric and electrostatic repulsive force that expels the bound nucleotide molecule (Goldberg, 1998).

Figure 1.7



Chapter 2:

Materials and Methods

Materials

All chemicals and reagents were from Sigma Chemical Company, (Poole, Dorset, UK) unless otherwise indicated. All restriction enzymes and appropriate buffers were from New England Biolabs, (NEB (UK) Ltd. Hitchin, Herts. UK), unless otherwise stated.

Plasmids

ARF1-HA, ARF6-HA, ARF1-HA G2A, ARF6-HA G2A, ARF1-HA Q71L and ARF6-HA Q67L, in plasmid pXS, were a kind gift from (Julie Donaldson, NIH). PLD1/2-HA (Mike Frohman, State University of New York). Arrestin 2 and arrestin 3 in pCMV5 and GRKct were a kind gifts from the Lefkowitz lab (Duke Medical Center). $G\alpha_q$-HA and hM_3 muscarinic receptor clones were obtained from ATCC (Manassas, Virginia, USA). Signal FLAG construct of the M_3 muscarinic receptor was made by Dr Eve Lutz (Dept of Biochemistry, University of Strathclyde, Glasgow). 5-HT_{2A} receptor clone SCS93 was a kind gift from Stuart Sealfon (Mount Sinai School of Medicine, New York).

Oligonucleotide Primers

Oligonucleotide primers were designed with the aid of GeneJockey (Biosoft Ferguson, MO, USA), and were synthesised by Invitrogen Life Technologies UK.

h2ARGINPLVY.fp: [5'-GGGTGATCAACCCACTAGTCTACACACTGTTCAA-3']

h2ARCT.rp: [5'-CAACTCAATTGTCACACACAGCTCACCTTTTCATT-3']

h2ARL406.rp: [5'-

CGGGAATTCTCACAATGGTTTTTTGTTTTCCTTGTACTGAC-3']

h2ARQ396.rp: [5'-GGGAATTCTCACTGAATATACCGTGAAAAGGCTGA-3']

Methods

Preparation of competent cells

Escherichia coli (*E. coli*) cells strains JM109 and BL21-RIL, were grown overnight at 37°C on M9 minimal media plates (5% glucose (w/v), 5mg/ml vitamin B1). Colonies were selected and grown up in 8ml of Luria Bertani (LB)-broth (10g/l tryptone, 5g/l yeast extract, 5g/l NaCl) for 8 hours. From this starter culture, 2.5ml was added to 250ml of LB-broth (+ 0.02M $MgSO_4$), and grown until the Optical Density (OD) of the culture was between 400 and 600. Cultures were centrifuged to pellet cells (3000g, 10 min, 4 °C) and the pellet resuspended gently in 0.4 volumes of ice cold sterile TFB1 (1M KOAc, 0.1M $CaCl_2$, 1M $MnCl_2$, 2M $RbCl_2$, 15% glycerol (v/v) in UHP H_2O, pH 5.8) before being incubated on ice for 5 minutes. Cells were again pelleted by centrifugation (3000g, 10 min, 4 °C), before being gently resuspended in 1/25th original culture volume in ice-cold sterile TFB2 (0.1M MOPS, 0.1M $CaCl_2$, 2M $RbCl_2$, 15% glycerol (v/v) in ultra high purity (UHP) H_2O pH 6.5). Cells were then incubated on ice for 15-60 minutes, before being aliquoted into

200µl lots into ice-cold 1.5ml Eppendorf tubes (Eppendorf) and snap frozen in a dry ice/ethanol bath. Cells were stored at -70 °C.

Transformation of competent cells

Approximately 100ng of cDNA was added to a 50µl aliquot of competent JM109 *E. coli* cells, mixed gently and incubated on ice for 30 minutes. The cells were then heat shocked for 45 seconds at 42°C, before being placed on ice for 2 minutes. 900µl of LB-broth was added, and the culture placed in a shaking incubator (Model G25 Incubator Shaker, New Brunswick Scientific Co. Inc. NJ, USA.) at 37°C for 45 minutes. 200µl of this suspension was plated out onto LB-agar plates (LB-broth + 1.2% bactoagar (w/v)) supplemented with ampicillin at 12.5µg/ml and/or tetracycline at 7.5µg/ml to select for plasmid-containing cells, and grown at 37°C in an incubator overnight.

Plasmid purification

Plasmid purification was carried out using the Qiagen QIAfilter Maxi kit (Qiagen Ltd. West Sussex, UK). Competent cells were transformed with cDNA as described above. A distinct colony was picked from the plates, and grown up for 8 hours in 3ml LB-broth (+ 12.5µg/ml ampicillin) at 37°C. 1 ml of this starter culture was transferred to 100ml of LB-broth (+ 12.5 µg/ml ampicillin) and grown overnight in a shaking incubator at 37°C. The culture was then centrifuged to pellet the bacterial cells (20 min, 7700g, 4°C) (Sorvall RC-5B Refrigerated Superspeed Centrifuge, Du Pont Instruments; Sorvall GSA rotor). The supernatant was removed, and the cells were resuspended in 10ml of Buffer P1 (Qiagen resuspension buffer; 50mM

Tris.HCl, pH 8.0; 10mM EDTA; 100μg/ml RNase), then 10 ml of Buffer P2 (Qiagen lysis buffer; 200mM NaOH, 1% SDS (w/v)) was added, and mixed gently, before incubating at room temperature for 5 minutes. 10 ml of ice-cold Buffer P3 (Qiagen neutralisation buffer; 3.0 M potassium acetate pH 5.5) was then added and the lysate mixed gently. The lysate was poured into the barrel of a QIAfilter Maxi Cartridge, and left to stand for 10 minutes, before being filtered into a QIAGEN-tip 500 column, which had been pre-equilibrated with 10 ml of Buffer QBT (Equilibration buffer; 750 mM NaCl; 50 mM MOPS, pH 7.0; 15% isopropanol(v/v); 0.15% Triton® X-100 (v/v)). After the lysate had flowed though the column, the column was washed through with 2 x 30ml Buffer QC (Wash buffer; 1.0 M NaCl; 50 mM MOPS, pH 7.0; 15% isopropanol (v/v)), and eluted by 15 ml Buffer QF (Elution buffer; 1.25 M NaCl; 50 mM Tris.HCl, pH 8.5; 15% isopropanol (v/v)). The cDNA was precipitated by the addition of 0.7 volumes (10.5 ml) of isopropanol, and then centrifuged (16000g, 30 min, 4°C) (Sorvall SS-34 rotor). The supernatant was removed, and the pellet washed in 5 ml of 70% ethanol (v/v), and recentrifuged (16000g, 30 min, 4°C). This step was repeated, and the pellet then allowed to air dry, before resuspension in an appropriate volume of TE buffer (10 mM Tris.HCl, pH 7.4; 1mM EDTA, pH 8.0; pH to 7.4).

Miniprep of cDNA

Small (3-5 ml) bacterial cultures, pre-transformed with cDNA were grown overnight at 37°C in LB broth (+ 12.5μg/ml ampicillin), before being centrifuged (5 min, 12000g) in 2ml tubes, to pellet bacteria, in an benchtop centrifuge (Eppendorf), resuspended in 300 μl resuspension buffer P1 (15 mM Tris.HCl, pH 8, 10 mM EDTA, which was sterilised and to which was added 100 μg/ml RNase A), and 300

µl lysis buffer P2 (0.2 N NaOH, 1% SDS (w/v)) was added, the tubes were then mixed carefully by inversion, and left at room temperature for 5 minutes to lyse fully. After this, 300 µl of ice-cold neutralisation buffer P3 (3 M KOAc, pH 5.5, autoclaved) was added, and the tubes inverted to mix. Tubes were centrifuged (20 min, 12000g) in a benchtop centrifuge, and the supernatant retained. To the supernatant, 800 µl isopropanol was added. The tubes were incubated on ice for 1 hour, and again centrifuged (20 min, 12000g, 4°C) and the resulting pellet washed in 1 ml 70% ethanol (v/v), centrifuged (5 min, 12000g, 4°C), and then air-dried. The pellet was finally resuspended in 40 µl TE buffer.

Cell culture

Cell lines were grown and maintained in a humidified atmosphere of 5% CO_2 (v/v) at 37°C at all times unless otherwise indicated. Culture medium was refreshed every 3-4 days, all flasks and 12 well plates for culturing and plastics were obtained from Greiner Bio-One (Gloucestershire, UK).

The cells were harvested by a brief incubation with 10 ml/175 cm^2 Hank's Buffered Saline Solution (HBSS) containing 10% (v/v) 10x trypsin-EDTA (Gibco), which was applied to the cell layer, agitated and aspirated after 20 seconds. After a further 10 minutes, the cells were washed off the flask surface with the appropriate medium and resuspended. The cells were then reseeded into flasks at a ratio of 1:3 or onto 12 well plates for assay purposes.

This method is added for the sake of completeness, all cell culture was performed by Pamela Holland.

Transient transfection of cDNA into mammalian cells

COS7 cells were trypsinised as described earlier and seeded into 75 cm^2 or 175 cm^2 flasks, or into 12 well plates at approximately 50% confluency for transfection the following day. Fugene-6 transfection reagent (Roche Diagnostics, East Sussex, UK) or genejuice (Novagen, Merck Biosciences, Nottingham, UK) was added to DMEM (with 4500 mg/l glucose, with l-glutamine, without pyruvate) according to manufacturers instructions and incubated for 5 minutes. cDNAs encoding proteins of choice or empty vector pcDNA3 (as a control) were added to the Fugene-6/DMEM or Genejuice/DMEM according to the manufacturers instructions and swirled gently to mix, then incubated for a further 15 minutes. The transfection mixture was added to the cell layer, swirled gently and the cells incubated at 37°C for 48 hours. Cells were made quiescent by replacing culture media with serum free media.

This method is added for the sake of completeness, all transient transfections of cDNA into mammalian cells were performed by Pamela Holland.

Construction of 5-HT_{2A} receptor carboxy terminal tail GST-fusion proteins

The human 5-HT_{2A}R carboxy-terminal tail constructs were PCR amplified using the proofreading KOD Polymerase (Novagen) from SCS93 (5-HT_{2A} receptor clone) with the following primer pairs: GST-5-HT_{2A}GINPLVYct (amino acids GIN376-V471) with primer pair h2ARGINPLVY.fp and h2ARCT.rp (see above for primer sequences); GST-5-HT_{2A}L406ct (GIN376-L406) with primer pair h2ARGINPLVY.fp and h2ARL406.rp; GST-5-HT_{2A}Q396ct (GIN376-Q396) with

primer pair h2ARGINPLVY.fp and h2ARQ396.rp, using *Taq* polymerase (Promega). The following temperature cycles were used: (94°C, 2 min) x1 cycle; (94°C; 1 min, 55°C; 1 min, 68°C ; 2.5 min) x40 cycles; (68°C, 10 min) x1 cycle, and then held at 4°C. The resulting PCR products were purified by Wizard cDNA clean-up resin (Promega), and subcloned into the pCR4Blunt TOPO vector using the ZeroBlunt® TOPO® PCR cloning kit for sequencing (Invitrogen). The resulting plasmids were transformed into competent JM109 *E. coli* strain, and plated out onto LB plates (+ 12.5μg/ml ampicillin), and incubated overnight at 37°C. Resulting colonies were grown overnight at 37°C in 5 ml LB broth (+ 12.5μg/ml ampicillin). Minipreps were performed on the cultures as described previously. Plasmids were checked by digests using the appropriate restriction enzyme pairs, and also by sequence analysis. Individual clones were isolated and the inserts checked by restriction digest with *EcoR*I and sequence analysis. Clones with the appropriate sequence were digested with the following restriction enzymes in order to subclone the inserts into the *Bam*HI/*Eco*RI site of the GST vector pGEX3x (Amersham/Pharmacia): *Bcl*1 and *Mfe*1 (GST-5-HT_{2A}GINPLVYct) in buffer 4; *Bcl*1 and *Eco*R1 (GST-5-HT_{2A}L406ct and GST-5-HT_{2A}Q396ct) in buffer 3; *Bam*H1 and Mfe1, for 1 hour at 50°C where *Bcl*1 was used, followed by 1 hour at 37°C. Plasmid constructs that were to be digested with the *Bcl*I enzyme had to be transformed into the methylase free *E. coli* strain JM110 (Stratagene) before isolation because this restriction enzyme is sensitive to DNA methylation. Plasmid digests were run on a 2% (w/v) low melting point agarose gel and the band of interest was cut out, and the DNA extracted from the gel by using the Qiaex II gel extraction kit (Qiagen).

Purification of DNA from gels

Each gel slice was weighed and 3x volume of solubilisation solution (Qiagen) added along with the Qiaex II resin. The mixture was incubated at 50°C for 10 mins with intermittent mixing to ensure the resin stayed in suspension. This was then microfuged for 30 secs and the resin/captured DNA pellet washed once in 500 µl of solubilisation solution (Qiagen), then twice in 500 µl wash buffer. The pellets were briefly air-dried and the DNA eluted by incubation for 10 minutes with sterile UHP water at 50°C. Extracted DNA was subcloned into the *Bam*HI/*Eco*RI site of pGEX-3X plasmid, and again cloned inserts were checked by sequence analysis.

Expression of GST proteins

GST-5-HT_{2A}i3 (pGEX-3X), GST-5-HT_{2A}ct (pGEX-2T), GST-5-HT_{2A}103ct, GST-5-HT_{2A}K385ct, GST-5-HT_{2C}ct, GST-5-HT_{2C}i3, GST-M_3ct (All kindly provided by Eve Lutz, University of Strathclyde), the GST-5-HT_{2A}L406ct and GST-5-HT_{2A}Q396ct (pGEX-3X) described above, GST-M_3i3 (kindly provided by Stephen M. Lanier, Louisiana State University Health Sciences Center, New Orleans) constructs, as well as a control GST fusion protein of the STREX insert of the large conductance Ca^{2+}- and voltage-activated K^+ (BK) channel, in the pGEX-5X1 vector (kindly supplied by Mike Shipston, University of Edinburgh) and GST (PGEX-3X) alone were transformed into the BL21-RIL strain of *E. coli* bacterial cells, as detailed above. A single colony was selected from the plate, and grown overnight at 37°C in 20 ml of 2xYT medium (16 g/l tryptone, 10 g/l yeast extract, 5 g/l NaCl) with 2% (w/v) glucose and 12.5 mg/ml ampicillin. From this starter culture, 4 ml was taken and added to 400 ml of 2xYT medium (+ 2% glucose (w/v); 12.5 µg/ml ampicillin) and grown until the culture had reached an OD_{600} of 0.6-0.8, expression of the fusion

proteins was then induced by the addition of 0.1 mM isopropyI-β-D-thiogalactoside (IPTG) and the culture left for a further 3 hours at 37 ^{0}C. Cells were harvested by centrifugation (10 min, 7700g, 4°C; Sorvall GSA rotor), the supernatant removed and the cells resuspended in 10 ml BugBuster reagent (Novagen, CN-Biosciences, Nottingham, UK) and left for 10 min to lyse. The culture was again centrifuged (20 mins, 16000g, 4°C; Sorvall SS-34 rotor) and the supernatant, containing the GST fusion proteins, was added to glutathione-Sepharose beads which had been prewashed in 1 ml PBS, to give a final bed volume of beads of 200µl (Amersham-Pharmacia Biotech AB). The beads were incubated with an amount of bacterial supernatant that would ensure equal amounts of protein for each construct, for 20 minutes at room temperature to allow binding of the GST fusion proteins to the beads. The matrix formed was then washed three times with 2 ml each PBS and used immediately in the protein interaction assay.

Preparation of protein enriched extracts

COS7 cells were transfected with various constructs (ARF1/6-HA; ΔARF1/6-HA; HA-PLD1b/2; arrestin 2/3; $GRK2_{495-689}$; $G\alpha_q$) through the use of genejuice ® transfection reagent (Novagen) according to manufacturers instructions, in 175 cm^2 flasks. Cells were washed with 10 ml Earle's Balanced Salt Solution (EBSS; Life Technologies) before being scraped into 2 ml ice-cold extraction buffer (2 µg/ml aprotinin, 1 mM 4-(2-aminoethyl)-benzenesulphonylfluoride (AEBSF) (Alexis Biochemicals, San Diego, CA, USA), 1 mM dithiothreitol (DTT), 1 mM pepstatin, 1 mM Na orthovanadate, 1 mM NaF, 50 µg/ml soyabean trypsin inhibitor (SBTI) in PBS). In the case of the extracts containing overexpression of either of the PLD-HA

isoforms, to allow the PLD-HA to become soluble, 1% (w/v) CHAPS, 1% (w/v) deoxycholate and 20% (v/v) glycerol (for stability) were added to the above buffer. The cells were then homogenised (Ystral polytron, (Scientific Industries Intl. UK Ltd, Loughborough, UK) setting 3, 15 sec) before being centrifuged, (12000 g, 20 min, 4 °C). The supernatant was aliquoted and stored at -40 °C until required.

In vitro protein interaction assays

COS7 cellular extracts enriched in the various constructs made as described above (*Preparation of protein enriched extracts*), and/or pure protein (Gβγ subunit from bovine brain) (Calbiochem) were incubated with GST-fusion proteins bound to glutathione-Sepharose beads, as described in the *Expression of GST Proteins* section above (Amersham Pharmacia Biotech), in 250 µl Buffer A (20 mM Tris HCl pH 7.5, 0.6 mM EDTA, 1 mM DTT, 70 mM NaCl, 0.05% Tween 80 (v/v)) for 90 min at 4°C, with rolling. In some experiments, GTPγS (100 µM) was added to the incubations. The beads were washed 4 times in 1 ml Buffer A and the retained proteins removed from the beads with x2 Laemmli buffer (2% SDS (w/v), 715 mM mercaptoethanol 20 mM Tris HCl, pH 7.4)(Laemmli, 1970) before SDS-PAGE and immunoblotting (see below). Input levels of both putative interacting proteins and fusion proteins (monitored by GST immunoreactivity) were carefully balanced by use of the ScanAnalysis densitometric imaging program (Biosoft, Cambridge, UK). to ensure comparability between samples.

In the case of the rat brain homogenate, loosely-bound proteins were eluted from the column with a high salt elution buffer (50mM Tris, 1M NaCl, 1mM EDTA, 1mM

EGTA, 5% glycerol (v/v)) before any proteins remaining were removed with x2 Laemmli buffer as above.

The quality of wild type and mutant receptor GSTs was routinely monitored by anti-GST blots/protein staining.

Preparation of rat brain homogenate

Male rat brains were kindly provided frozen by Susan Fleetwood-Walker (Royal Dick Vet School, University of Edinburgh). Each brain was homogenised in 8ml of homogenisation buffer (2 μg/ml aprotinin, 1 mM 4-(2-aminoethyl)-benzenesulphonylfluoride (AEBSF) (Alexis Biochemicals, San Diego, CA, USA), 1 mM dithiothreitol (DTT), 1 mM pepstatin, 1 mM Na orthovanadate, 1 mM NaF, 50 μg/ml soyabean trypsin inhibitor (SBTI) in PBS). The brains were homogenised (Ystral polytron (Scientific Industries Intl.) setting 3, 1 min) before being centrifuged (12000g, 30 mins, 4°C). The cytosol was then incubated for 90 min with 1ml glutathione-Sepharose beads that had been preincubated with GST protein alone, to remove any proteins that may bind non-specifically to the GST part of the construct of interest, before the brain lysate was introduced to each of the target GST-constructs. See *Expression of GST Proteins* above.

Protein interaction assays with homogenised rat brain extracts

2 ml of homogenised and solubilised rat brain as described above (*Preparation of Rat Brain Homogenate*) was added to each of columns that contained 500 ml of glutathione-Sepharose beads that had been bound to the GST constructs of interest (*Expression of GST Proteins*) to give a final reaction volume of 2.5 ml and incubated

at 4°C for 16 hours. Columns were washed twice in homogenisation buffer detailed above, and then proteins were eluted from the column with 1ml of a high salt elution buffer (50mM Tris, 1M NaCl, 1mM EDTA, 1mM EGTA, 5% glycerol (v/v) pH 7.5) before any proteins remaining were removed with x2 Laemmli buffer as above, and the eluates run on SDS-PAGE gells as described below (*Western Blots*).

Western blots

Western blots were carried out on samples from immunoprecipitation and GST-fusion protein interaction assays. Either 20% or 12.5% precast homogeneous Phastgels (Amersham Pharmacia Biotech) were used. SDS-PAGE and electroblotting onto polyvinylidene difluoride membranes (Immobilon-P; Immobilon-P^{SQ}; Millipore, Watford, UK) were performed on a Phastsystem apparatus (Amersham Pharmacia Biotech). Membranes were blocked overnight at 4°C in 5% Marvel (Premier Brands UK Ltd, Spalding, Lincs UK)/PBS (w/v). The detection antibodies used were rabbit polyclonal anti-HA (Santa Cruz Biotechnology; Autogen Bioclear, Calne, Wilts, UK, catalogue number sc-805) at a 1 in 500 dilution, goat polyclonal anti-GST (Amersham Pharmacia Biotech, catalogue number 27-4577-01) at a 1 in 300 dilution and mouse monoclonal anti-PrC (Roche, clone HPCA, catalogue number 1814508) at 1 in 1000 dilution. Primary antibodies were incubated with the membranes for 90 minutes in 0.1% TWEEN®-20/PBS (v/v) at room temperature with agitation, washed 5 x 5 minutes in 0.1% TWEEN®-20/PBS (v/v) followed by incubation for 2 hours with preabsorbed secondary antibodies conjugated to HRP (Chemicon Intl. Ltd., Harrow, UK) in secondary antibody buffer (0.25% BSA (w/v) / 0.1% TWEEN®-20 (v/v) in PBS), followed by a further 5 x 5

minute washes in 0.1% TWEEN® 20/PBS (v/v). Visualisation of antibody bands was achieved using Luminol (New England Biolabs (NEB) Hitchin, UK) as per manufacturers instruction, and blots were exposed to ECL film (Amersham Pharmacia Biotech).

Some Western blots were also run on the larger NuPAGE *Surelock*™ Mini-cell (Invitrogen). Samples were prepared by the addition of 4μl loading buffer (0.04% (w/v) bromophenol blue in glycerol), and samples were centrifuged to mix, and then 20μl of each sample loaded onto gels and run under reducing conditions using either 10% or 4-12% gradient Bis-Tris precast gels (Invitrogen) and either NuPAGE® 1x MES or 1x MOPS running buffer (Invitrogen) at 200V for 1 hour. Transfer of proteins was to P^{SQ} transfer membranes (Immobilon), which were previously washed, 1 x 1 minute in 100% (v/v) methanol, 2 x 5 minutes in UHP H_2O, to remove residual methanol, and then immersed in 1x NuPAGE transfer buffer (Invitrogen) containing 10% (v/v) methanol. Following separation, the gel was placed on the membrane and transfer of proteins carried out using X Cell II blot module (Invitrogen) at 30V for up to 2 hours. Gels were stained using Colloidal Coomassie Blue stain (Invitrogen).

Densitometric analysis of ECL images from Western blots was carried out using a flat bed scanner and the ScanAnalysis program (Biosoft, Cambridge, UK).

MALDI-TOF mass spectrophotometry

Bands visualised by Colloidal Coomassie Blue were cut from these gels and sent for MALDI-TOF mass spectrometry "fingerprinting", carried out by the Edinburgh Protein Interaction Centre (EPIC) centre (University of Edinburgh, Swann Building, Kings Buildings, Edinburgh, UK.).

Protein searches were made in both Swissprot and NCBI protein databases for candidate proteins. Likely matches were identified on the following criteria: the number of peptides matched; extent (%) of sequence coverage; the molecular weight search (MOWSE) score (Pappin *et al.*, 1993); and the molecular mass of the protein identified. "Matched peptides" is a measure of the number of peptides from the digest that are matched to database listings of peptides known to result from digestion of the particular protein. The percentage coverage is the "matched peptides" expressed as a percentage of the total protein sequence. For proteins under 100kDa, a minimum of 20% is generally required. The MOWSE score is a number providing a probability value for a potential hit, as first described by Pappin (Pappin *et al.*, 1993). The higher this number, the greater is the likelihood of a correct identification. Although MOWSE score does not take account of relative peak abundance, it does provide reliability weightings for the results based on variability in the data obtained. A hit of over 10^{-4} suggests a good match for a protein of under 100kDa in size.

Phospholipase D assays

Cells were plated out into 12 well plates ($1x10^5$ cells/well), and 24 hours later transfected with plasmids containing the cDNA for proteins or peptides of interest. 18 hours prior to experiment, cells were labelled with [^{3}H]palmitate (specific activity 47.5 Ci/mmol) (Perkin Elmer Biosciences, Hounslow, UK) (1.5 μCi/well) and deprived of Ultra Ser-G (USG), synthetic serum, or normal calf serum by transferring to DMEM (1 ml per well). After washing once in 0.75 ml MEM containing HEPES (25 mM, pH 7.5) (Gibco Life Technologies) with 1% (w/v) fatty acid-free bovine serum albumin, cells were preincubated for 20 min in 1 ml MEM containing HEPES (25 mM, pH 7.5) with 0.5% fatty acid-free bovine serum albumin. Various inhibitors or antagonists were added up to 20 minutes before the addition of butan-1-ol (30 mM). The butanol was added immediately prior to the cells being incubated for the appropriate time period in various concentrations of appropriate agonist (5-hydroxytryptamine or carbachol). Reactions were terminated by removal of MEM medium and cells being scraped into 0.5 ml ice-cold methanol.

Phospholipids were extracted by placing the contents of each well into 2 ml vials (Chromacol, BDH), and adding to each vial 500 μl chloroform and 400 μl distilled water to give a final ratio of methanol/chloroform/water of 1:1:0.8 by volume. Vials were thoroughly mixed by vortex, and centrifuged (5000g, 20 min, RT) (Jouan RC 10.22 centrifugal evaporator; Jouan) to allow the sample to separate into aqueous and organic layers. The upper aqueous layer was removed, and 250 μl of the non-aqueous layer was transferred into a separate Chromacol vial, and centrifuged (5000g, 30 min, 30°C) under vacuum conditions, to ensure the evaporation of the

sample carrier. The sample was dissolved in 50 µl in a mix of chloroform and methanol (19 parts $CHCl_3$: 1 part CH_3OH).

The samples were separated on Whatman LK5D thin layer chromatography silica plates (Whatman, Maidstone, Kent, UK) with the solvent being the organic phase of a mixture of ethyl acetate/ 2,2,4-trimethylpentane/acetic acid/water in the ratios of 110:50:20:100. The area corresponding to the [^{3}H]phosphatidyl butanol ([^{3}H]PtdBut), which had been previously identified using iodine staining of phosphatidyl butanol standard (Avanti Polar Lipids, USA), was scraped from the plates in 6 x 0.5 cm bands. Each band was placed into a miniature 6 ml polyethylene vial (Packard Bioscience) and 4 ml of Emulsifier-Safe LSC cocktail (Packard Bioscience) added. Samples were thoroughly mixed, and counted (4 min count per sample) on a Beckman LS 5801 Series scintillation counter (Beckman).

Phospholipase C assays

Cells were plated out into 12 well plates ($1x10^5$ cells/well), and 24 hours later transfected with plasmids containing the cDNA for proteins or peptides of interest. 18 hours before the experiment, cells were deprived of USG by transferring to Earle's Balanced Salt Solution (EBSS) containing 10 mM HEPES (pH 7.5) and 0.18% glucose (w/v) (37°C, in an 5% CO_2/95% O_2 environment), and labelled with [^{3}H]inositol (specific activity 25 Ci/mmol) (Perkin Elmer Biosciences) (0.75 µCi/well). The medium was changed for EBSS containing 10 mM HEPES, 0.18% (w/v) glucose and 0.2% (w/v) bovine serum albumin and washed once in the same medium. Cells were preincubated for 20 min with 10 mM LiCl, before being incubated for 30 min with various concentrations of 5-HT. Reactions were

terminated by the removal of medium and the addition of 1 ml ice cold 10 mM formic acid (Almaula *et al.*, 1996; Bohm *et al.*, 1997) and the cells were left on ice for at least an hour to ensure lysis. [^{3}H]Inositol phosphates ([^{3}H]InsP) were separated following addition to a 1 ml Dowex anion exchange resin (1x8 resin, formate form, 200-400 mesh) (Bio-Rad). A stepwise gradient of ammonium formate/formic acid was used to separate and then elute the [^{3}H]InsPs as previously described (Berridge *et al.*, 1983). The samples in the columns were washed with 15 ml UHP water, then by 5 ml of 50 mM ammonium formate. The [^{3}H]InsPs were eluted by addition of 10 ml of 1 M ammonium formate/0.1 M formic acid to the column, and collected in scintillation vials (Zinsser Analytic GmbH). The columns were regenerated by the addition of 5ml 2 M ammonium formate/0.1 M formic acid, and washed through with 15 ml UHP water.

500 µl of collected eluates were distributed into miniature 6 ml polyethylene vials (Packard Bioscience), two for each sample, and 4 ml of Emulsifier-Safe LSC cocktail for aqueous samples (Packard Bioscience) added. The samples were shaken briefly, and left overnight, before being counted (4 min count per sample) on the Beckman LS 5801 Series scintillation counter (Beckman).

Data Analysis

All values were expressed as mean ± standard error of mean (SEM) from data obtained from a number (n) of individual experiments. Statistical analyses were carried out using the Wilcoxon test unless otherwise stated. The concentration of drug which could produce 50% of the maximum response (EC_{50} value) and the concentration of inhibitors which inhibit 50% of a stimulus-evoked response (IC_{50})

were assessed by fitting the data with a non-linear, error weighted, iterative curve fitting programme, Fig.P (Biosoft, Cambridge, UK), using the logistic Hill equation.

In the case of signalling experiments, when an experiment has been said to have been repeated n times, each experiment has been repeated from the stage of transfection of cells with appropriate plasmids onwards. In the case of pull down assays, each repeat of an experiment was repeated from the stage of plasmid transduction onwards.

In Glutathione Sepharose pull-down assays for the GST-5-HT_2 constructs, normalisation of data was achieved by taking a ratio of the GST-construct present in each blot to the amount of protein binding (e.g. ARF-HA or PLD-HA) present, both being measured by chemoluminescence. This gave a figure, which would take into account small fluctuations in GST-construct levels across the different columns of each experiment. Within each experiment, the binding to the wild type GST-5-HT_{2A}ct construct was taken to be 1, and the binding of proteins to the other constructs compared to this value, this allowed for direct correlations to be made between different experiments. The extent of proteolytic cleavage of the GST constructs following isolation was shown to have no effect on the interpretation of the results gleaned by this comparison.

Chapter 3:

The interaction of the small G protein ARF with domains of the M_3 muscarinic receptor

Introduction

It has been previously shown that a number of the family R (group I) GPCRs can signal through non-trimeric G protein pathways to stimulate downstream effectors, in particular PLD. PLD activation has also been shown upon G-protein mediated responses after agonist stimulation of muscarinic receptors (Martinson *et al.*, 1990; Sandmann *et al.*, 1991; Nieto *et al.*, 1994; Schmidt *et al.*, 1994). PLD can be activated via PKC, protein tyrosine kinase, PI 3-kinases, and possibly by the elevation of intracellular Ca^{2+} levels (reviewed in Exton, 1999). The activation of PLD by tyrosine kinases has been shown in many studies as a product of the activation of growth factor receptors which have an intrinsic tyrosine kinase activity (Rydzewska and Morisett, 1995; Natarajan *et al.*, 1997). For example PLD2 (but not PLD1) was seen to form a complex with the EGF receptor, and was tyrosine phosphorylated upon receptor activation (Slaaby *et al.*, 1998). Tyrosine kinase activation of PLD has also been shown following activation of insulin receptors (Shome *et al.*, 2000; Slaaby *et al.*, 2000) and receptors for the T-cell antigen of mast cells (Exton, 1997) as well as in several other cell systems (e.g. PLD activation by thrombin in human platelets, by vasopressin or TPA in rat fibroblasts, PGDF in A10 smooth muscle cells. Reviewed in Natarajan *et al.*, 1996).

The best established signalling pathway for the M_3 muscarinic receptor is via the heterotrimeric G protein $G_{q/11}$ leading to activation of PLC. Almost every GPCR that is known to activate PLC also activates PLD, and indeed 4 muscarinic receptor isoforms have been shown to activate PLD in HEK cells ($M_{1\text{-}4}$). However in the case

of both the M_1 and M_3 receptor-mediated stimulation of PLD, this activation may be independent of PLC activation, as inhibition of PLC activation by neomycin does not drastically change the PLD response in the same cells (Sandmann *et al.*, 1991). When activation of PLD does occur via PLC, this may be due to several signalling events caused by these receptors. The cellular signals generated by PLC activation are expected to lead to activation of protein kinase C (PKC), which is known to activate PLD. Diacylglycerol will directly activate PKC isoforms, and inositol 1,4,5-triphosphate will cause Ca^{2+} mobilisation and therefore further stimulate the Ca^{2+}-dependent PKC isoforms α, β and γ (Exton, 1997; Rumenapp *et al.*, 2001). The possibility of PLD being activated directly by Gq cannot be ignored, however there is no evidence to suggest this might be the case. The small G proteins ARF and RhoA have been implicated in PLD activation by the M_3 receptor in HEK cells (Rumenapp *et al.*, 1995; Schmidt *et al.*, 1997), and indeed stimulation of the M_3 receptor in 1321N1 cells and in transfected COS7 cells has been suggested to activate PLD through the more direct pathway of activation of the small G proteins of the ARF and Rho families, in a trimeric G protein-independent manner (Mitchell *et al.*, 1998). Furthermore, PLD responses elicited by U46619 (an agonist of the native thromboxane A_2 (TP) receptor which is present in to 1321N1 cells) and PMTx (*Pasturella multocida* toxin, which was been shown to cause a concentration-dependent and $G_{q/11}$-mediated activation of PLC) have been shown to be inhibited by the PLC inhibitor U73122 in 1321N1 cells, suggesting an indirect PLC-dependent route of activation. In contrast carbachol-induced activation of the M_3 receptor in these cells (and in COS7 cells transfected with the M_3 receptor) is unaffected by U73122, suggesting that the route of activation here is not reliant on the classical

$G_{q/11}$ coupling to PLC (Mitchell *et al.*, 2003). Alternative pathways of PLD activation by the M_3 receptor may play a greater role in other cell types, depending on the relative expression of different components

ARF and Rho have each been implicated in M_3 receptor-mediated PLD activation in particular cell types. However the exact role of the ARF family of small G proteins has not been fully investigated. To further investigate the activation pathway of PLD activation via ARF, a variety of approaches were taken, including the use of assays of phospholipase D (PLD) and phospholipase C (PLC) activity for the M_3 muscarinic receptor expressed endogenously in 1321N1 human astrocytoma cells, and heterologously in COS7 cells. It has been previously shown that the $\beta\gamma$ subunit of trimeric G proteins, but not the $G\alpha$ (Wu *et al.*, 1998) and the G protein receptor kinase GRK2 (Wu *et al.*, 1998) can both bind to the 3rd intracellular loop of the M_3 and M_2 muscarinic receptors. Therefore to investigate the ability of the ARF protein to bind to the M_3 receptor *in vitro* glutathione S-transferase pull-down assays using specific domains of the M_3 muscarinic receptor were carried out. Further experiments then explored the possibility that the additional presence of $G\beta\gamma$ might influence the efficiency of ARF binding to domains of the M_3 receptor.

Results

The role of ARF1 and ARF6 in PLD activation by the sFM_3 receptor expressed in COS7 cells

In order to elucidate which ARF isoforms were mediating the M_3 receptor response, experiments using both native M_3 receptors in 1321N1 cells and the signal sequence FLG-tagged M3 receptor construct (sFM_3) transfected into COS7 cells were carried out. To elicit a response from the M_3 receptor, the muscarinic agonist carbachol was used. Carbachol is not selective for the M_3 subtype of muscarinic receptor, however, no PLD or PLC response to carbachol by COS7 cells was elicited in the absence of the transfected sFM_3 receptor, and there are no other known muscarinic receptors in the 1321N1 cell line (Wall *et al.,* 1991). In 1321N1 cells, carbachol caused concentration-dependent increases in both [^{3}H]PtdBut and [^{3}H]InsP production (Figure 3.1). The EC_{50} values for these PLD and PLC responses were similar (10.2 ± 2.0 and 8.1 ± 1.7 μM respectively). In the COS7 cells transfected with sFM_3 receptor, carbachol caused concentration-dependent activation of PLD and PLC with EC_{50} values of 9.4 ± 2.2 and 1.2 ± 0.1 μM respectively (Figure 3.2). Brefeldin A (BFA) is an inhibitor of one subclass of ARF-GEFs (BIG1/2), and has been shown to have relative selectivity to inhibit the activation of ARF1 over ARF6 (Morinaga *et al.*, 1999). BFA caused a significant inhibition of the carbachol-induced PLD response in 1321N1 cells, at both 10μM and 200μM carbachol (showing that ARF-dependent PLD activation does not only occur at high occupancy of the receptor, and the linkage to signalling via ARF makes an important contribution at all concentrations, not only at high doses of agonist). However BFA had no effect on

the ability of the M_3 receptor to activate PLC upon exposure to carbachol (Fig 3.3). Co-transfection of negative mutant ARF1 or ARF6 constructs, where the site of GDP-GTP exchange has been mutated to ensure the construct remains in the GDP-bound form (making the constructs functionally negative for all known functions of ARFs) caused inhibition of carbachol-induced activation of PLD, but not PLC (Fig 3.2). Both T31N-ARF1 and T27N-ARF6 caused significant inhibition of carbachol-induced PLD activation throughout the concentration-response curve (Fig. 3.2b). The co-transfection of wild type ARF1 or ARF6 had no significant effect on the activation of PLD by carbachol stimulation of the sFM_3 receptor (Figure 3.2b). None of the ARF constructs significantly modified basal PLD activity (Figure 3.2b and data not shown). In cells transfected with the sFM_3 receptor alone, 200 μM carbachol caused 5.18 ± 0.50 fold of basal [^{3}H]PtdBut production, whereas co-transfection with T31N-ARF1 gave a 2.80 ± 0.21 fold response, co-transfection with T27N-ARF6 gave a 3.26 ± 0.48 fold response and co-transfection with a combination of the ARF1/6 mutant constructs resulted in a 1.68 ± 0.28 fold response (n=8) (Fig. 3.4). Where various constructs were omitted from the transfections, empty vector was used in substitution. The negative mutant ARF values were significantly less than responses to carbachol alone and the combination showed a further significant reduction due to the interruption of activation of PLD by each of the individual isoforms of ARF. A small residual component of sFM_3 receptor-mediated [^{3}H]PtdBut production remained in the presence of both T31N-ARF1 and T27N-ARF6 (Fig 3.4).

Figure 3.4 also illustrates the BFA-sensitivity of carbachol-induced [^{3}H]PtdBut production with or without the negative mutant ARF1/6 constructs present. Controls

showed inhibition of responses by BFA with an IC_{50} of 64.1 ± 16.3 μM. The remaining PLD activation in the presence of T27N-ARF6 remained sensitive to BFA with an IC_{50} of 29.8 ± 17.1 μM. In contrast, the residual responses in the presence of T31N-ARF1, or both T31N-ARF1 and T27N-ARF6, were no longer reduced by BFA. This suggests that the sFM_3 receptor can utilise both ARF1 and ARF6 for activation of PLD, but the BFA-sensitivity of the response reflects predominantly a role of ARF1 (Fig 3.4).

Physical association of ARF1/ARF6 with GST fusion proteins of sFM_3 receptor domains

The question of whether ARF1 or ARF6 could participate in some form of direct complex with the receptor was investigated by looking for *in vitro* interactions with GST-fusion proteins of the M_3 receptor i3 and ct domains. These domains were selected as the most likely candidates because the i3 region is thought to be the site of key interactions with subunits of heterotrimeric G proteins and the ct region contains the NPxxY motif that is critical in determining linkage to ARF-dependent PLD responses (Mitchell *et al.*, 1998). GST constructs of these two domains, the M_3i3 (G^{308}-L^{497}) and the M_3ct (N^{540}-L^{590}) were attached to glutathione Sepharose beads, and exposed to cytosolic extracts from COS7 cells enriched with ARF1-HA or ARF6-HA under conditions that were optimised in previous studies (Wu *et al.*, 1998). Proteins captured by the fusion protein constructs were removed from this affinity matrix and separated by SDS-PAGE.

Figure 3.5 shows the relative sizes of the M_3 receptor i3 and ct domain constructs used in these glutathione S-transferase pull-down experiments, and shows the levels present in a typical assay. As a positive control, Gβγ purified from bovine brain (Calbiochem) was used, as Gβγ has been previously shown to bind specifically to the third intracellular loop of the M_3 muscarinic receptor (Wu *et al.*, 1998; Wu *et al.*, 2000, Fig 3.6). Figure 3.6 shows that indeed Gβγ did bind specifically to the GST-M_3i3 construct, but not to the control GST-BK_{STREX} exon or GST alone. Cytosolic extracts containing equivalent amounts of ARF1-HA and ARF6-HA were introduced to assays containing GST constructs of the M_3i3, M_3ct, BK_{STREX} and GST alone. ARF1-HA bound specifically to the GST constructs of the 3rd intracellular loop, and the carboxy-terminal tail domain of the M_3 muscarinic receptor (Fig. 3.7a). Both M_3 receptor constructs also bound ARF6-HA. ARF6-HA bound well to the GST-M_3i3 construct, but bound to the GST-M_3ct with a lower affinity, when compared to a similar input of ARF1-HA (Fig. 3.7b). The levels of each GST construct were shown to be similar by Coomassie Blue staining and by GST-immunoreactivity and any adjustments needed to ensure fully balanced construct inputs were made on the basis of comparing the levels of the specific construct bands of interest. These data are representative of at least 4 separate experiments.

Potential interaction of ARFs and heterotrimeric G protein subunits in their association with GST-fusion proteins of M_3 receptor domains

The question of whether of Gβγ might have an effect on ARF binding to the M_3 muscarinic receptor was investigated because two reports in the literature have

suggested that ARF can bind directly to Gβγ subunits, although probably with lower affinity than to Gα subunits (Colombo *et al.*, 1995; Franco *et al.*, 1995b). To investigate firstly whether any functional evidence could be found to implicate a role of Gβγ in ARF-dependent PLD responses, experiments were conducted on cell signalling responses. The peptide encoding the C-terminal domain of GRK2 ($GRK2_{495-689}$) has previously been shown to sequester Gβγ (Koch *et al.*, 1994) and has been used effectively to suppress Gβγ-mediated signalling responses in cells. The plasmid encoding the $GRK2_{495-689}$ peptide was transfected into COS7 cells in the presence of the sFM_3 receptor, and assays of PLC and PLD activity were used to determine the effect of the GRKct peptide on carbachol-induced activation of PLD. The peptide had no effect on the ability of the sFM_3 receptor to activate PLC (Fig. 3.8a). The ability of the sFM_3 receptor to activate PLD in the presence of the $GRK2_{495-689}$ peptide was inhibited to give a reduced maximal response, and the extent of the reduction was similar to the inhibition caused by 100μM BFA (Fig. 3.8b). The PLD activity remaining in the presence of the $GRK2_{495-689}$ peptide was shown to be BFA insensitive (Fig 3.8c), suggesting that the $GRK2_{495-689}$ peptide may be blocking the activation of PLD via ARF1.

The ability of Gβγ to directly contribute to the mechanism of ARF activation of PLD was further investigated *in vitro* by comparing binding of ARF (with or without Gβγ present) to GST-fusion protein constructs of the 3rd intracellular loop and the carboxyl terminal tail domains of the M_3 receptor. GST constructs were incubated with equivalent amounts of ARF1-HA and ARF6-HA in either the presence or absence of Gβγ. The HA-tagged ARFs were supplied as cytosolic extracts from

COS7 cells transfected with these constructs and the Gβγ was purified from bovine brain (Calbiochem) and used at a concentration of 30nM. Figure 3.9a shows the effects of Gβγ on the binding of ARF1 to the M_3 receptor constructs. In the case of both control constructs, GST-BK_{STREX} and GST alone, there was no apparent difference in the basal binding of ARF1-HA due to the addition of Gβγ. In the case of both the GST-M_3i3 and GST-M_3ct construct, the addition of 30nM Gβγ clearly increased the ability of ARF1-HA to interact with both receptor domains. In the case of ARF6-HA, the addition of Gβγ had no apparent effect on the ability of ARF6-HA to bind to the control constructs, and only a slight increase of ARF6-HA binding to the GST-M_3ct construct was seen (however, the basal level of binding of ARF6-HA, in the absence of Gβγ, to the ct construct was lower than that to the M_3i3). The ability of ARF6-HA to bind to the M_3i3 construct was however clearly increased in the presence of Gβγ. The data shown in figure 3.9 are representative of 3 separate experiments.

It has previously been shown that $G\alpha_{q/11}$ does not bind directly to either the M_2i3 or M_3i3 receptor domains in a manner that shows lasting association (Wu *et al.*, 1998). However, the effect of addition of $G\alpha_{q/11}$ on the binding of ARF1-HA and its facilitation by Gβγ was investigated here by addition of cytosolic extracts from COS7 cells over-expressing ARF1-HA to glutathione S-transferase pull-down assays containing the M_3 receptor i3 in the presence of either Gβγ, $G\alpha_{q/11}$ or both. No binding of $G\alpha_{q/11}$-HA was seen to the M_3i3 receptor domain construct (data not shown). The introduction of $G\alpha_{q/11}$ led to a decrease in the basal level of binding of ARF1-HA to the M_3i3 (Fig. 3.10), this may well be due to the $G\alpha_{q/11}$ binding to free

cytosolic Gβγ, and therefore diminishing the ability of this free cytosolic Gβγ to aid ARF1-HA binding to the GST-M_3i3. The addition of $G\alpha_{q/11}$ and Gβγ together had an intermediate effect, between that of ARF1-HA alone and that of Gβγ alone (Fig. 3.10). The ability of $G\alpha_{q/11}$ to modulate the binding of ARF1-HA in the presence of Gβγ cannot be defined in more exact terms here because the experimental setup makes it not possible to add $G\alpha_{q/11}$, which was in cytosolic extract, and therefore of an undefinable amount, in stoichiometric levels to Gβγ, which was purified and added amounts could be calculated exactly (30 nM was added to each column in these experiments). Figure 3.10 is representative of 3 individual experiments.

The role of Gβγ was further investigated by examining the participation of GRK2 in the M_3i3:ARF1:Gβγ complex. It has been shown previously that Gβγ can interact with GRK2 to mediate the binding of the kinase to the M_3 receptor (Koch *et al.*, 1993). To investigate whether this interaction might inhibit or aid the binding of ARF1 to the M_3i3 domain, pilot glutathione S-transferase experiments were carried out where ARF1-HA binding was attempted in the presence of GRK2, and GRK2 and Gβγ. It was observed that the ability of ARF1-HA to bind to the 3rd intracellular loop of the M_3 receptor was increased in the presence of GRK2, and a further increase was seen in the presence of GRK2 and Gβγ (Fig 3.11). These observations suggested that the presence of GRK2 could further augment ARF1-HA binding to the M_3i3-GST-fusion protein but were not pursued further because of the difficulty in achieving precisely balanced inputs of a range of different reagents which would be necessary to make formal comparisons using this approach.

Discussion:

A brefeldin A-sensitive route of PLD activation for the M_3 receptor and some, but not all other family R GPCRs:

The M_3 muscarinic receptor shows BFA-sensitive activation of PLD when expressed as a native receptor in 1321N1 cells (Mitchell *et al.*, 2003) or heterologously in COS7 cells. Time-course experiments have shown a rapid desensitisation of M_3 receptor PLD and PLC responses in 1321N1 cells (Nieto *et al.*, 1994; Mitchell *et al.*, 2003). The addition of BFA led to no change in the activation of PLC by carbachol, but in the presence of BFA, the initial rate and maximal extent of the PLD response was diminished although the profile of desensitisation remained the same, therefore the mechanism of activation of PLD is not involved with the mechanism for desensitisation of the receptor (Mitchell *et al.*, 2003). In contrast, it has been shown that the PLD responses of several other family R GPCRs, for example the TP receptor in 1321N1 cells, as well as the P_{2U} receptor and N376D-mutant 5-HT_{2A} receptor in COS7 cells, are BFA-insensitive (Mitchell *et al.*, 2003). These receptors differ from the M_3 receptor in that they contain a DPxxY sequence in the carboxy-terminal tail, whereas the M_3 receptor contains an NPxxY motif. BFA inhibited M_3 receptor PLD responses in 1321N1 and COS7 cells with IC_{50} values around 50 μM. BFA-sensitivity of M_3 receptor PLD responses in HEK 293 cells has been reported previously, but with some 2-3 fold lower potency (Rumenapp *et al.*, 1995) as we confirmed in transiently transfected HEK 293 cells (IC_{50} of 157 ± 23 μM, n=4). The lower potency in HEK 293 cells may reflect greater involvement of an alternative

tyrosine kinase-dependent pathway (Schmidt *et al.*, 1994). The PLD responses of angiotensin II type 1A and ET-1 receptors were strongly inhibited by BFA in A10 smooth muscle cells, (Shome *et al.*, 2000), whereas fMLP and ATP receptor responses in differentiated HL60 cells, and bradykinin and sphingosine 1-phosphate receptor responses in A549 adenocarcinoma cells, were not (Guillemain and Exton, 1997; Meacci *et al.*, 1999). The extent to which a GPCR demonstrates BFA-sensitive PLD responses in different cell types may well be influenced by the cellular expression of the various pathway components. The concentrations of BFA that selectively inhibit M_3 receptor PLD responses here exceed those needed to disrupt the integrity of Golgi membranes (Donaldson *et al.*, 1990; Guillemain and Exton, 1997; Meacci *et al.*, 1999), but are similar to those that inhibit the ARF-GEFs, BIG1 and BIG2 (Morinaga *et al.*, 1999). However, the cell surface expression of the M_3 receptor has been shown to be unaffected at this concentration of BFA (Mitchell *et al.*, 2003), as has the ability of the receptor to signal through PLC. Furthermore, the subcellular location of carbachol-induced [^{3}H]PtdBut production in sFM_3 receptor-containing COS7 cells has been shown to be predominantly in the plasma membrane fraction and furthermore, while the response involved a movement of both PLD1 and ARF1 to this site, the translocation of these proteins was not prevented by BFA (Mitchell *et al.*, 2003). Therefore the effects of BFA on PLD responses of particular receptors appear to be the result of a specific inhibition of the signal transduction pathway rather than a general disruption of protein trafficking.

ARF1 and ARF6 involvement in PLD activation by the sFM_3 receptor, but not other GPCRs:

There are 3 subtypes of the ARF family. The two main classes of cellular ARFs are exemplified by ARF1 and ARF6, which are thought to have characteristically distinct subcellular distributions. The Group II ARF subtype is exemplified by ARF5, however very little is known of the functions of ARF5, and no link has been made to the activation of PLD by ARF5 *in vivo*. This being said, recent evidence has suggested that another of the group II ARF isoforms, ARF4, can bind to the ct domain of the rhodopsin receptor (Deretic *et al.*, 2005). The role of different subtypes of ARF in sFM_3 receptor PLD activation was investigated by co-transfection with the sFM_3 receptor of either wild type ARF1 or ARF6, or their dominant-negative constructs, T31N-ARF1 and T27N-ARF6 (Peters *et al.*, 1995). Neither wild type ARF construct had a significant effect on PLD activation by carbachol, suggesting that the cellular levels of endogenous ARFs are probably not a limiting factor. However, dominant-negative ARF1 and ARF6 constructs each inhibited PLD responses without modifying PLC responses. Effects of negative mutant ARF1 and ARF6 in combination were cumulative, suggesting that the two ARF isoforms might each play a distinct role. Although negative or positive mutants of ARFs can disrupt Golgi and other vesicular trafficking (Peters *et al.*, 1995; D'Souza-Schorey *et al.*, 1998; Beraud-Dufour and Balch, 2001; Donaldson and Radhakrishna, 2001; Kuai and Kahn, 2002) it has been shown that neither the levels of specific cell surface binding sites for [^{3}H]oxotremorine-M (an M_3 receptor ligand) (Mitchell *et al.*, 2003) nor sFM_3 receptor PLC responses were affected by the ARF constructs. Similarly, the abilities of the angiotensin II and ET-1 receptors to

stimulate PLD responses in A10 cells were inhibited by both T31N-ARF1 and T27N-ARF6 constructs (Shome *et al.*, 2000). In contrast, the responses of two DPxxY-containing receptors, the P_{2U} receptor and the N376D-mutant 5-HT_{2A} receptor were unaffected by T31N-ARF1, but were clearly inhibited by T27N-ARF6 and PKC inhibitors (P_{2U} receptor) or by PKC inhibitors (N376D-mutant 5-HT_{2A} receptor)(Mitchell *et al.*, 2003). This suggests that ARF6 and PKC may be important in alternative pathways that underlie the BFA-insensitive [^{3}H]PtdBut production seen with some GPCRs. The presence of dominant-negative ARF1 significantly decreased the BFA-sensitive activation of PLD upon carbachol stimulation. However, the addition of dominant-negative ARF6 did not affect the ability of BFA to inhibit the remaining PLD response after carbachol stimulation. This suggests that an ARF1-dependent pathway from the receptor, probably involving the BFA targets, BIG1/2, is responsible for the BFA-sensitivity. Correspondingly, it has been shown that BIG1/2 can act as effective, BFA-sensitive, ARF-GEFs for ARF1 but not ARF6 (Morinaga *et al.*, 1999) and that *in vivo* functional effects of ARF6 are often BFA-insensitive (Frank *et al.*, 1998; Franco *et al.*, 1999). In the case of the sFM_3 receptor in COS7 cells, the PLD response seems to be mediated by ARF1 through a BFA-sensitive pathway, and by ARF6 in a BFA-insensitive manner.

Physical association of both ARF1 and ARF6 with the M_3 receptor through the i3 and ct domains:

Immunoprecipitation experiments carried out in the lab to determine the ability of wild type ARF1-HA and ARF6-HA to bind to the sFM_3 muscarinic receptor in COS7 cells showed that low levels of ARF1-HA and ARF6-HA were associated with the

sFM_3 receptor under basal conditions, while the amount of associated ARF1-HA but not ARF6-HA clearly increased when cells were incubated with carbachol (Mitchell *et al.*, 2003). In an alternative immunoprecipitation procedure where sFM_3 receptor association with ARF1-HA/ARF6-HA was measured as [^{3}H]NMe-QNB binding, low levels of basal co-immunoprecipitated binding sites were observed, but increased association of the sFM_3 receptor with ARF1-HA and to a lesser extent ARF6-HA was revealed following carbachol stimulation (Mitchell *et al.*, 2003).

Based on this evidence, GST-fusion proteins were used here to further investigate the interaction of ARF1 and ARF6 with the M_3i3 and M_3ct receptor domains. The carboxy-terminal tail of the receptor contains the NPxxY motif which has been shown to influence the ability of the family R GPCRs to activate PLD through an ARF1-dependent pathway (Mitchell *et al.*, 1998), making the ct domain a candidate for direct ARF interaction. However, the ct of the M_3 receptor is relatively short, at only 43 amino acids. The third intracellular loop of the M_3 receptor is much longer in comparison (239 aa), and is known to contain sites for interaction with heterotrimeric G proteins, arrestins, Gβγ and the kinases GRK2 and CK1-α (Wu *et al.*, 1997; Budd *et al.*, 2000; Wu *et al.*, 2000). It has been shown previously that i3 domain splice variants of the PAC_1 receptor show marked differences in their BFA-sensitive activation of PLD but not other signalling responses (McCulloch *et al.*, 2001) suggesting that M_3i3 may potentially contribute part of the binding interface for ARF docking to the receptor, along with the M_3ct. Indeed specific binding of each ARF was demonstrated to the M_3i3 GST-fusion protein, and also to the M_3ct GST-fusion protein, with ARF6-HA binding to a lesser extent than the ARF1-HA to the M_3ct. No binding of either ARF was observed to the control constructs.

Role of Gβγ modularity in the binding of ARF1 and ARF6 to M_3 receptor domains:

It has been previously shown that there is a direct interaction between the third intracellular loop of the M_3 receptor and purified G-protein βγ subunits (Gβγ), but not with the Gα subunit (Wu *et al.*, 1998; Wu *et al.*, 2000), and that Gβγ acts as an apparent docking module for the kinase GRK2 through the PH domain within the GRK2ct domain (Wu *et al.*, 1998). There is also evidence for a direct interaction between the small G protein ARF1 and the $G_t\beta\gamma$ subunit (Franco *et al.*, 1995b), where ARF1 is recruited to retinal membranes, and to isolated phospholipid membranes (in its GDP-bound form) by Gβγ in the absence of the $G\alpha_{GDP}$ subunit. Gβγ interacts with ARF1 through a sequence in the "switch II" domain of ARF1. The switch II domain was first described in Gα as an α-helical domain on the effector binding surface of Gα that may provide a GDP-dependent binding site for Gβγ (Conklin and Bourne, 1993). This domain consists of 2 flexible loops, and undergoes a dramatic conformational change between GDP and GTP-bound states, where in the GTP bound form, the α2 helix binds tightly to the α3 helix of the same domain of Gα (Lambright *et al.*, 1994). It has been shown that there is some sequence homology between the ARF1 switch II domain and that of the Gα subunit, particularly in the α2 helix, which has a conserved RxxWxxxF sequence ($RxxWxxxF^{211}$ in Gα; $RxxWxxxF^{81}$ in ARF1 and ARF5; $RxxWxxxY^{76}$ in ARF6). The RxxWxxxF motif does not exist in the small G-protein Ras, and indeed it has been shown that Ras does not bind Gβγ under similar conditions (Franco *et al.*, 1995b). This sequence may be an analogue of the PH (Pleckstrin homology) domain

which also contains the RxxWxxxI sequence, and has been shown to be present in many signalling proteins, some of which bind Gβγ (Touhara *et al.*, 1994; Franco *et al.*, 1995b). With this in mind, the question of whether Gβγ may affect the binding of ARF1 and ARF6 to the M_3 receptor domain constructs was investigated.

Assays of cellular PLD and PLC activity showed that the Gβγ-sequestering agent $GRK2_{495\text{-}689}$ (Eichmann *et al.*, 2003) had no effect on the M_3 muscarinic receptor's ability to activate PLC. However, the same agent decreased the ability of the M_3 muscarinic receptor to activate PLD to an extent similar to that caused by the ARF-GEF inhibitor, BFA. It has also been shown that in the presence of the $GRK2_{495\text{-}689}$ construct, BFA did not reduce the residual PLD response, revealing the presence of a BFA-insensitive (possibly ARF6-mediated) component to carbachol-induced PLD activation. These data show that Gβγ increases the ability of ARF1 to bind to both the M_3i3 and M_3ct GST constructs, and also increases the binding of ARF6 to the M_3 receptor domain constructs, although the effect on the binding of ARF6 to the M_3ct did not appear to be as marked.

Several reports indicate that the G-protein receptor kinase GRK2 interacts with the 3rd intracellular loop of the muscarinic receptors (Kameyama *et al.*, 1993; Nakata *et al.*, 1994; Haga *et al.*, 1996) including the M_3 receptor (Debburman *et al.*, 1995; Wu *et al.*, 1998), and binds to the βγ subunits of trimeric G proteins, through the PH domain at the carboxy-terminal end of GRK2 (Koch *et al.*, 1993; Carman *et al.*, 2000). It has been shown here that the $GRK2_{495\text{-}689}$ peptide, which codes for the carboxy-terminal domain of GRK2 can be used to block the activation of PLD through ARF1 upon stimulation of the M_3 receptor. However, it is unclear whether

this is due to any direct blocking of the ability of Gβγ to bind to ARF1, or if it was due to the inability, following Gβγ sequestration, of the cells to form the correct complex of proteins to ensure ARF1 activation of PLD. Figure 3.11 shows that both Gβγ and GRK2 act in a distinct facilitatory fashion to increase the ability of ARF1-HA to bind to the GST-M_3i3 construct. This suggests that an active complex of Gβγ, GRK2 and ARF1 may be formed upon agonist activation of the M_3 receptor, through which the activation of PLD is achieved. The variety of binding sites on Gβγ for ARF/$G\alpha_{q/11}$, GRK2 and other partners (for example the adenylyl cyclases AC1 and AC2 (Chen *et al.*, 1997)) suggest that Gβγ may be a vital partner in this complex, and may act to recruit ARF1 and GRK2. However, the activation of PLD via ARF upon M_3 muscarinic receptor activation by carbachol appears to be independent of activation of the trimeric G proteins, at least in 1321N1 and COS7 cells. The detailed mechanisms of how this complex is brought about, in what order the proteins are recruited, and indeed, if they are all necessary for activation of the PLD pathway *in vivo*, will therefore be targets for future study.

In conclusion, the present experiments describe an ARF-dependent activation of PLD by the M_3 muscarinic receptor that appears to be essentially independent of conventional routes of GPCR signalling. Instead, both ARF1 and ARF6 can associate physically with the receptor, as shown by GST-fusion protein experiments, and both may have a role in the carbachol-induced activation of PLD by the M_3 receptor, as shown by the use of dominant negative constructs of ARF1/6. The range of GPCR motifs and cellular factors that determine receptor selectivity for these different pathways of PLD activation remain to be determined, however the Gβγ subunit (and

possibly also the docking of GRK2) has been shown to increase the ability of ARF1/6 to bind to the M_3i3 and M_3ct receptor domains.

Figures

Figure 3.1

Carbachol-induced PLC and PLD responses mediated by native M_3 receptors in 1321N1 cells

The data show the concentration-dependence of [^{3}H]PtdBut (PLD) responses (●) and [^{3}H]InsP (PLC) responses (■) elicited by the cholinergic receptor agonist carbachol in 1321N1 human astrocytoma cells which natively express the M_3 muscarinic receptor, but not any other muscarinic or nicotinic subtype of cholinergic receptor. Carbachol elicited concentration-dependent responses of PLC and PLD with similar EC_{50} values of 8.1 ± 1.7 and 10.2 ± 2.0 μM respectively. Values are means ± SEM, n=4-12.

Figure 3.1

Carbachol-induced PLC and PLD responses mediated by the native M_3 receptor in 1321N1 cells

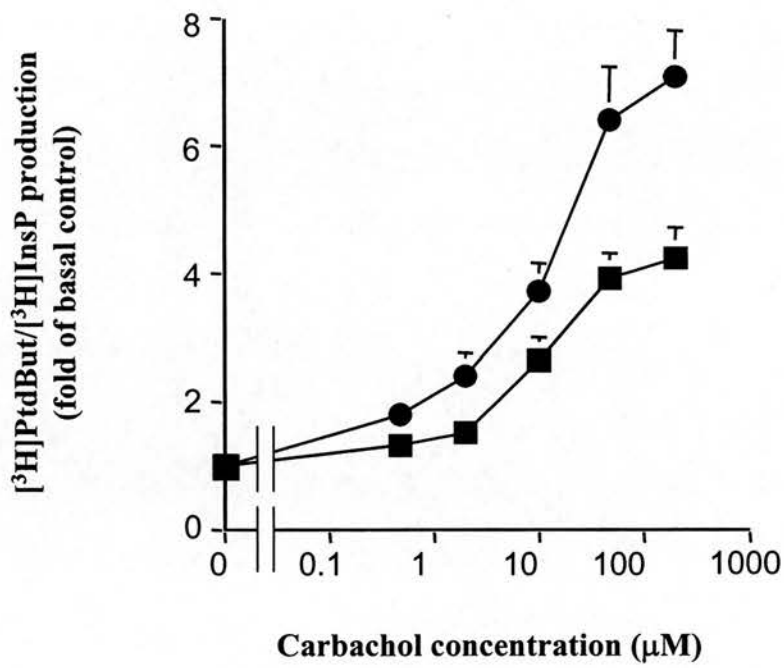


Figure 3.2

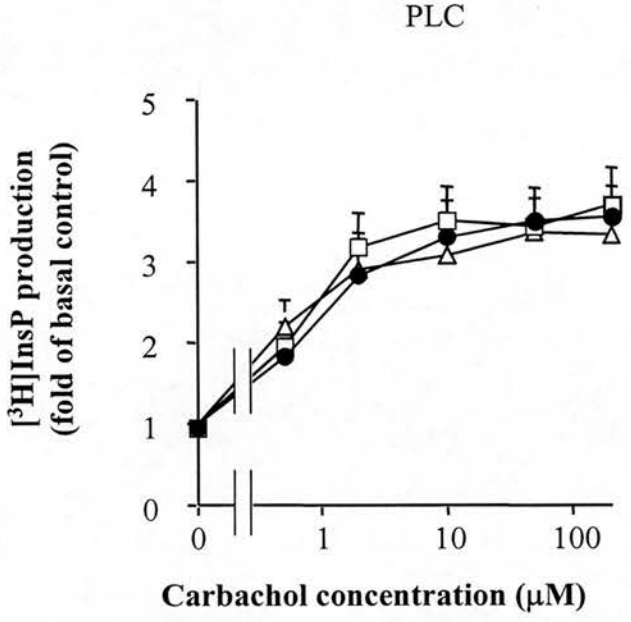
PLD and PLC responses of the sFM_3 receptor transfected into COS7 cells and the effects on these of wild type and dominant negative constructs of ARF1 and ARF6

COS7 cells were co-transfected with sFM_3R and a range of ARF constructs, including the wild type ARF1-HA, wild type ARF6-HA, and the dominant negative forms T31N-ARF1-HA and T27N-ARF6-HA. The control (●) dose response curves showed EC50 values for PLC and PLD activation to be 1.2 ± 0.1 and 9.4 ± 2.2 μM respectively. In the case of the $[^3H]$InsP (PLC) responses (3.2a), none of the ARF constructs (ARF1-HA (■), ARF6-HA (Δ), T31N-ARF1-HA (▲) or T27N-ARF6-HA(Δ)) showed any consistent deviation from the response of the control (●) experiments (sFM_3R plus empty pcDNA3 plasmid). In the $[^3H]$PtdBut production (PLD) response experiments, (3.2b), wild type ARF1-HA (■) and wild type ARF6-HA (▲) did not cause any apparent difference from responses in the presence of the control vector (●). However, in the presence of the negative mutant forms of both ARF1-HA (□) and ARF6-HA (Δ), the ability of the sFM_3R to activate PLD was significantly reduced at carbachol concentrations of 2-200μM ($p<0.05$, Wilcoxon test)

Figure 3.2

Dominant negative constructs of ARF1 and ARF6 inhibit PLD but not PLC responses of the sFM_3R in transfected COS7 cells

a



b

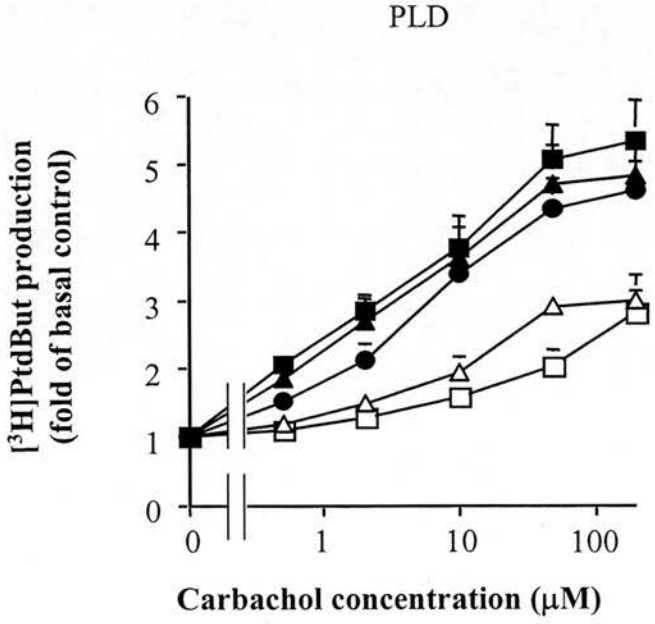


Table 3.1

EC_{50} and E_{max} values for PLC and PLD responses of the sFM_3R in transfected COS7 cells

Table 3.1 a shows the EC_{50} and E_{max} values for the PLC response of the sFM_3 receptor in COS7 cells, as shown in figure 3.2a. The table also shows the same values in the presence of the negative mutant forms of ARF1 and ARF6. There is no significant difference in these values in the presence of the mutant ARF isoforms when compared with the control. The results shown on this table are from a total of 6 separate experiments.

Table 3.2 b shows the same values for PLD activation, as shown in figure 3.2b. The table shows values for control (●) experiments, as well as with additional wild type ARF1 (■) and ARF6 (▲), and mutant forms of ARF1 (□) and ARF6 (Δ). Values could not be obtained for EC_{50} and E_{max} for PLD responses in the presence of mutant ARF1 (□) due to the fact that a sigmoid curve fit could not be determined as no clear maximum was reached with the concentrations of carbachol used, however, the E_{max} value for PLD response in the presence of ARF6 (Δ) was significantly less than the corresponding control value ($p< 0.05$ Wilcoxon test). Results are taken from 6 individual experiments.

Table 3.1

EC_{50} and E_{max} values for PLC and PLD responses of the sFM3R in transfected COS7 cells

a **PLC response**

	EC_{50} (nM)	Emax (fold of basal)
control (●)	1.21 ± 0/11	3.60 ± 0.33
Δ⁻ ARF 1 (□)	0.90 ± 0.23	3.74 ± 0.42
Δ⁻ ARF6 (Δ)	0.77 ± 0.28	3.35 ± 0.40

b **PLD response**

	EC_{50} (nM)	Emax (fold of basal)
control (●)	9.2 ± 2.2	4.890± 0.45
wild type ARF 1 (■)	5.1 ± 3.2	5.46 ± 0.60
wild type ARF6 (▲)	5.7 ± 3.6	4.98 ± 0.49
Δ⁻ ARF 1 (□)	nd	nd
Δ⁻ ARF6 (Δ)	10.8 ± 2.9	3.04 ± 0.29 *

Figure 3.3

Effects of brefeldin A (BFA) on carbachol-induced PLD and PLC responses in 1321N1 cells

This figure shows the concentration-dependence of BFA effects on PLD responses to 200 μM carbachol (●) and 10 μM carbachol (▲) as well as PLC responses to 200 μM carbachol (○) in 1321N1 cells. BFA caused statistically significant inhibition of the PLD responses to carbachol at concentrations of 50-200 μM BFA ($p<0.05$, Wilcoxon test).

Figure 3.3

Effects of brefeldin A on carbachol-induced PLD and PLC responses in 1321N1 cells

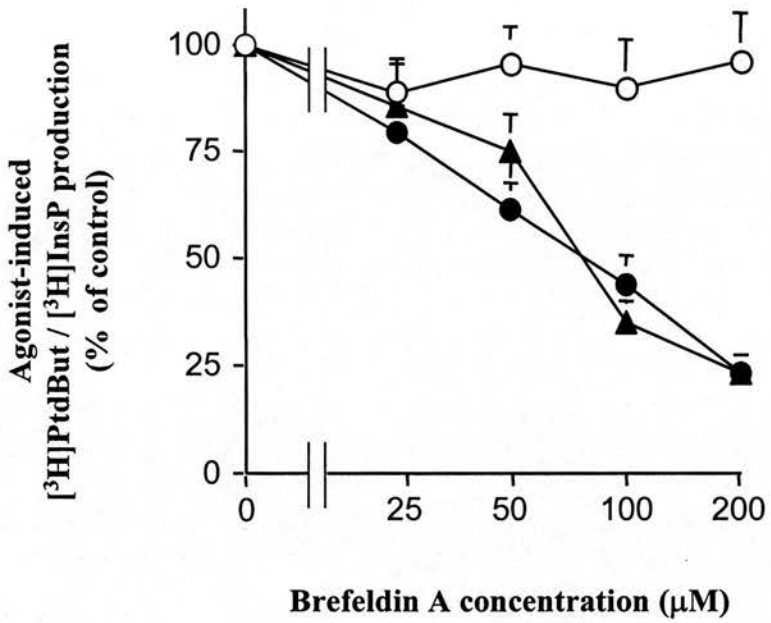


Figure 3.4

Effects of dominant negative ARF1 and ARF6 constructs on the brefeldin A (BFA) inhibition of carbachol-induced PLD responses of the sFM_3 receptor transfected into COS7 cells

Shows the concentration-dependence of BFA effects on PLD responses to 200 μM carbachol in the presence of control vector (●), T31N-ARF1 (■), T27N-ARF6 (▲) and T31N-ARF1 plus T27N-ARF6 (▼). (○) Shows the effects of BFA on PLD responses of cells transfected with sFM_3 receptor, but no ARF constructs, in the absence of carbachol stimulation. All controls for transfections contained equivalent levels of empty vector. The carbachol-evoked PLC responses of sFM_3 receptor-transfected cells were unaffected by BFA (data not shown). BFA (50-200 μM) caused significant inhibition of the PLD responses to carbachol only in the presence of control vector or of the negative mutant form of ARF6 ($p<0.05$, Wilcoxon test). The fact that the presence of T31N-ARF1 but not T27N-ARF6 pre-empts any inhibitory effect of BFA suggests that BFA inhibition may reflect a contribution of ARF1 rather than ARF6 in these M_3 receptor-mediated PLD responses.

Figure 3.4

Effects of dominant negative ARF1 and ARF6 constructs on the brefeldin A inhibition of carbachol-induced PLD response of the sFM_3 receptor transfected into COS7 cells

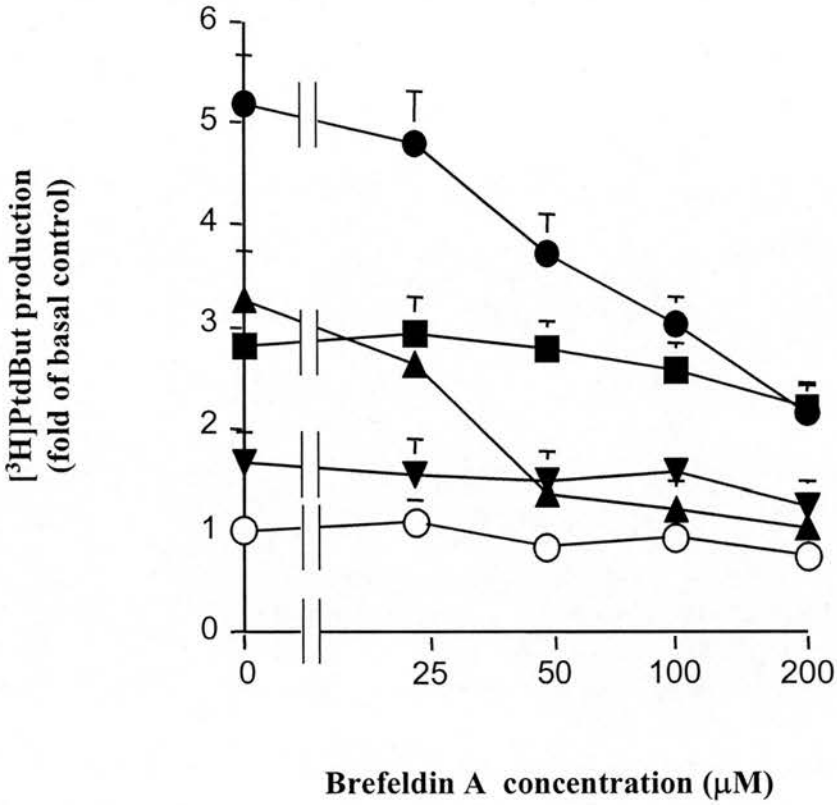


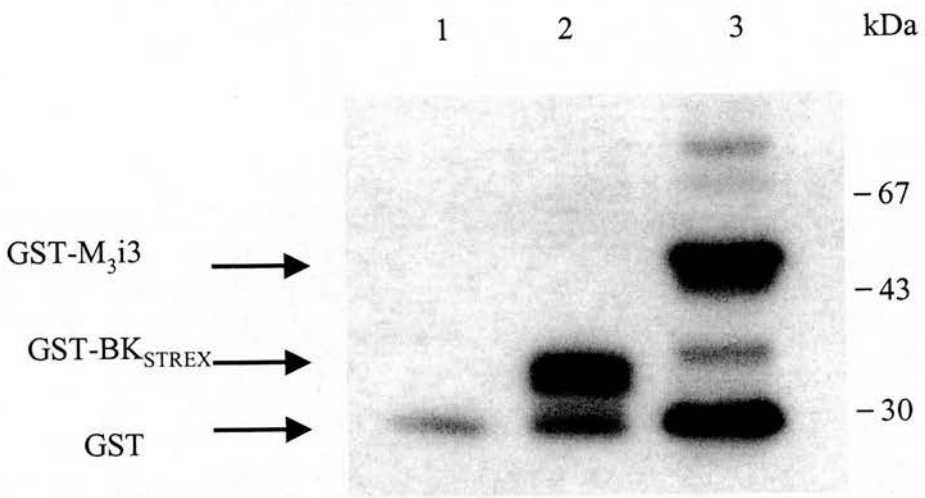
Figure 3.5

The relative sizes and input levels of GST, GST-BK and GST-M_3i3 fusion proteins used for pull-down assays with ARF1/6

GST alone, GST-BK_{STREX} and GST-M_3i3 constructs were separated on 20% homogeneous gels, blotted onto PVDF membrane, and incubated with anti-GST IgG, then HRP linked secondary antibody, before being visualised via enhanced-chemiluminescence (ECL). Lane 1 is GST protein, lane 2 is GST-BK fusion protein and lane 3 is GST-M_3i3 fusion protein. This shows that GST has a MW of ~29kDa and that, as would be predicted from the sequences of the inserts in the constructs, GST-BK has a MW of ~35kDa and GST-M_3i3 has a MW of ~49kDa.

Figure 3.5

The relative sizes and input levels of GST, GST-BK and GST-M_3i3 fusion proteins used for pull-down assays with ARF1/6



Probed for: GST

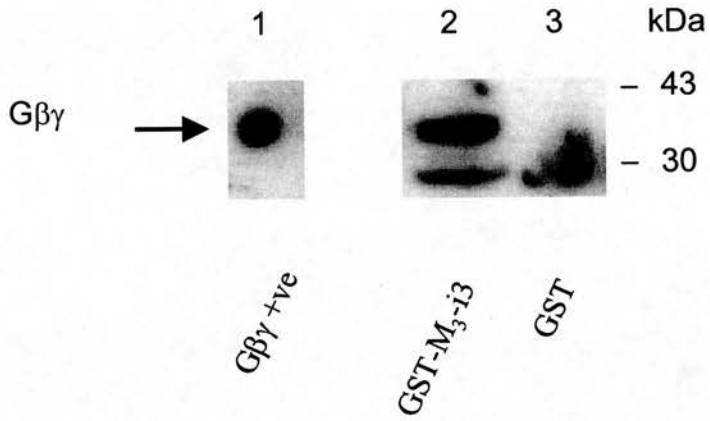
Figure 3.6

Purified Gβγ binds to the GST-M_3i3 construct but not GST alone

Purified Gβγ isolated from heterotrimeric brain G-protein (Calbiochem) (30nM) was incubated with the control GST or GST-M_3i3 constructs. Lane 1 shows Gβγ as a positive control, lane 2 shows GST-M_3i3 incubated with Gβγ and lane 3 shows GST incubated with Gβγ protein. This illustrates that Gβγ binds strongly to the M_3i3 construct and does not bind to GST protein alone.

Figure 3.6

Purified Gβγ binds to the GST-M_3i3 construct but not to GST alone



Probed for: Gβ

Figure 3.7

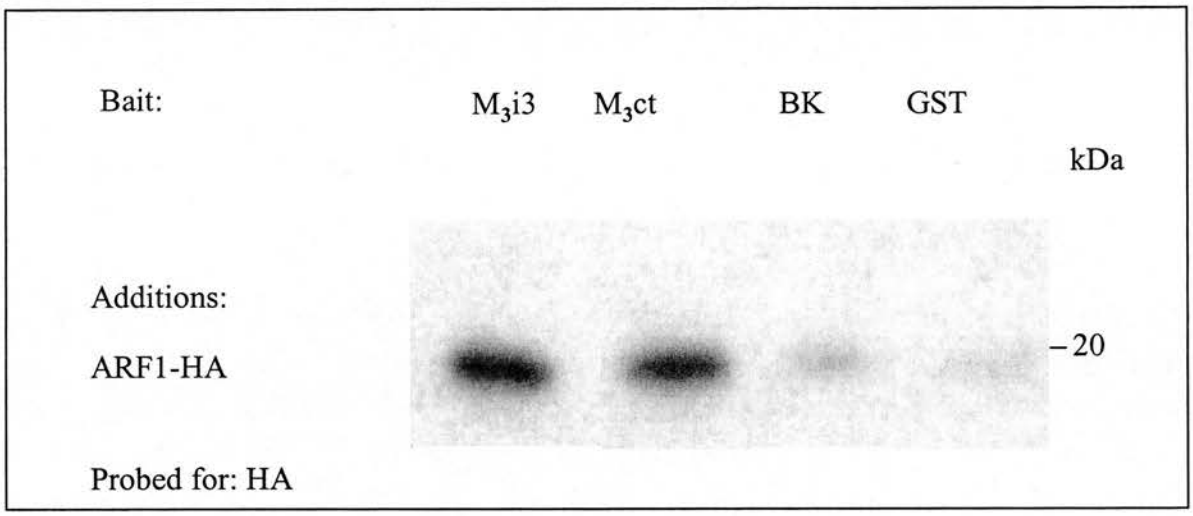
ARF1-HA and ARF6-HA bind specifically to both the M_3i3 and M_3ct GST-fusion proteins but not to control constructs

GST-M_3i3, GST-M_3ct, GST-BK_{STREX} or GST alone constructs were incubated with equal amounts of ARF1-HA and ARF6-HA. Figure 3.7a shows the ability of ARF1-HA to bind to both the GST-M_3i3 and the GST-M_3ct, but not to the GST-BK_{STREX} or GST alone. Figure 3.7b shows that ARF6-HA binds to the GST-M_3i3, and to a lesser extent to the GST-M_3ct, but not to either the GST-BK_{STREX} or to GST alone.

Figure 3.7

ARF1-HA and ARF6-HA bind specifically to both the M_3i3 and M_3ct GST-fusion proteins but not to control constructs

a



b

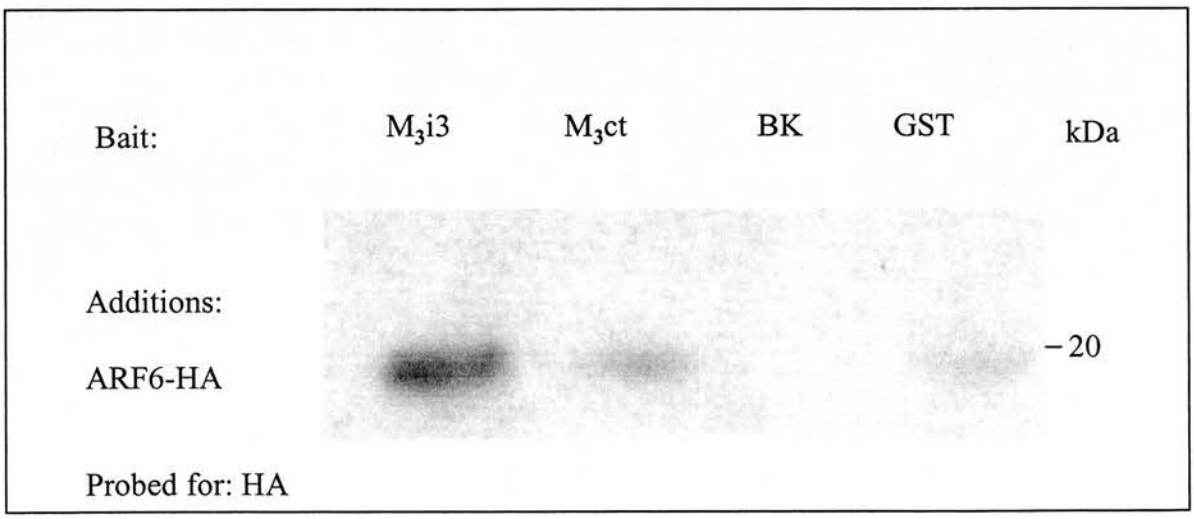


Figure 3.8

Effects of the Gβγ-sequestering $GRK2_{495-689}$ construct and brefeldin A (BFA) on PLD and PLC responses of the sFM_3 receptor transfected into COS7 cells

Figure 3.8 also shows the effects of the carboxy-terminal domain of GRK2 on the ability of the sFM_3R to activate PLC and PLD. In these experiments, the addition of $GRK2_{495-689}$ had no effect on the ability of the sFM_3R to activate PLC (3.8a▲). However, the PLD activation by sFM_3R in these COS7 cells was reduced in the presence of the $GRK2_{495-689}$ construct (3.8b▲).

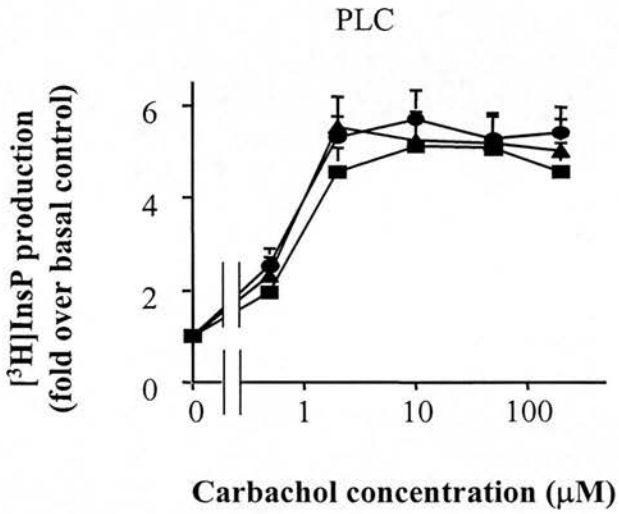
In these experiments, 100μM BFA had no effect on the ability of the sFM_3R to activate PLC when the receptor was transfected into COS7 cells (3.8a ■) when compared to control experiments (●), however, the ability of the sFM_3R to activate PLD in COS7 cells in the presence of BFA (3.8b ■) was reduced when compared to the control experiments (●).

Figure 3.8c shows the effect of BFA on the carbachol-induced PLD response of cells co-transfected with the sFM_3 receptor and the Gβγ-sequestering agent $GRK2_{495-689}$. Under the control conditions (●), BFA inhibited the carbachol-elicited PLD response at carbachol concentrations of 50-200μM ($p<0.05$ Mann-Whitney U-test). The response in the presence of the $GRK2_{495-689}$ (■) was reduced overall, but the remaining response was then no longer affected by the presence of BFA (○).

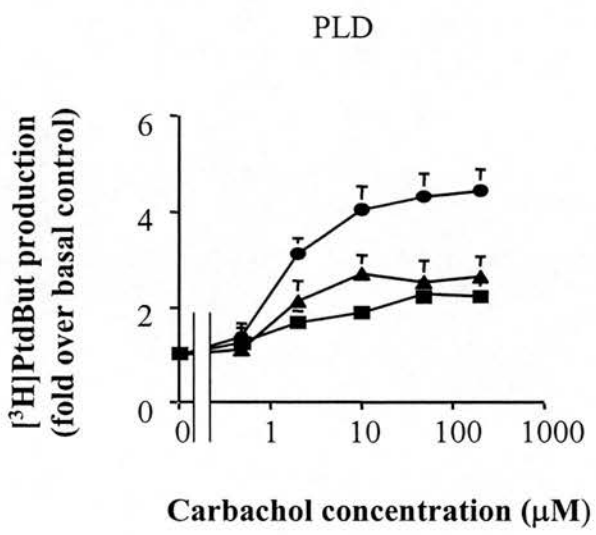
Figure 3.8

Effects of brefeldin A (BFA) and the Gβγ-sequestering $GRK2_{495-689}$ construct on PLD and PLC responses of the sFM_3 receptor

a



b



c

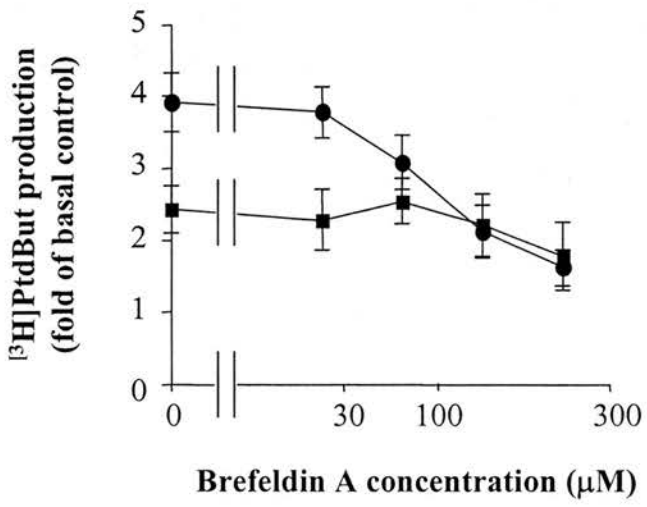


Table 3.2

EC_{50} and E_{max} values for PLD and PLC responses of the $_s$FM3 receptor in transfected COS7 cells in the presence of BFA and $GRK2_{495-689}$

Table 3.1a shows the EC_{50} and E_{max} values for the PLC response of the sFM_3 receptor in COS7 cells, as shown in figure 3.8a. The table also shows the same values in the presence the Gβγ sequestering agent $GRK2_{495-689}$ (▲) and the ARF-GEF inhibitor BFA (100μM) (■). There is no significant difference in these values in the presence of these agents when compared with the control response. The results shown on this table are from a total of 6 separate experiments.

Table 3.2b shows the same values for PLD activation, as shown in figure 3.8b. The table shows values for control (●) experiments, as well as with the addition of $GRK2_{495-689}$ (▲) and BFA (100μM) (■). The E_{max} value for PLD response in the presence of both $GRK2_{495-689}$ (▲) and BFA (100μM) (■) was significantly less than the corresponding control value ($p< 0.05$ Wilcoxon test). Results are taken from 6 individual experiments.

Table 3.2

EC_{50} and E_{max} values for PLD and PLC responses of the $_s$FM3 receptor in transfected COS7 cells in the presence of BFA and $GRK2_{495-689}$

a **PLC response**

	EC_{50} (nM)	Emax (fold of basal)
control (●)	0.59 ± 0.31	5.40 ± 0.58
$GRK2_{495-689}$ (▲)	0.63 ± 0.28	5.22 ± 0.27
BFA 100μM (■)	0.98 ± 0.33	4.71 ± 0.49

b **PLD response**

	EC_{50} (nM)	Emax (fold of basal)
control (●)	2.13 ± 0.42	4.76 ± 0.55
$GRK2_{495-689}$ (▲)	1.82 ± 0.56	3.01 ± 0.40 *
BFA 100μM (■)	5.76 ± 2.91	2.48 ± 0.35 *

Figure 3.9

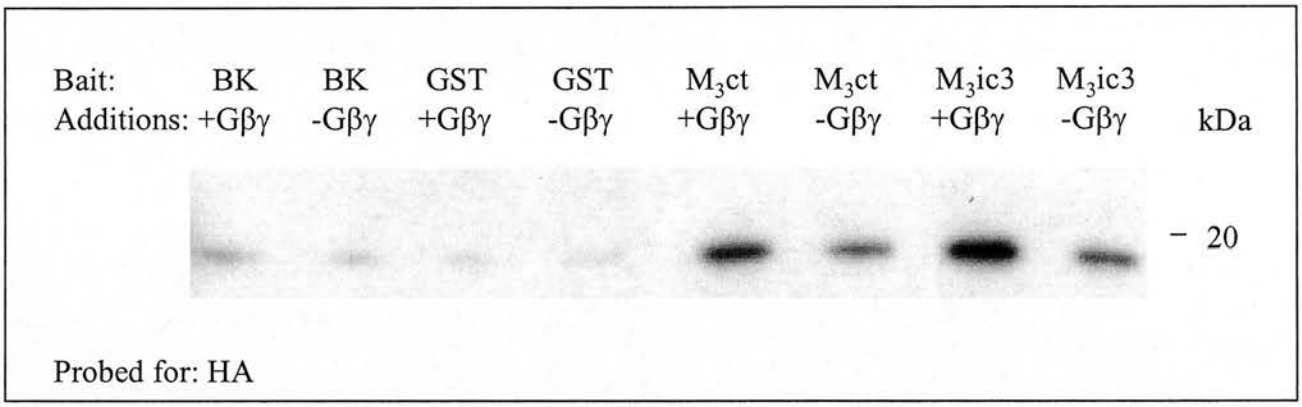
Effect of Gβγ on ARF1-HA and ARF6-HA binding to the M_3i3 and M_3ct GST-fusion proteins

Equal amounts of ARF1-HA and ARF6-HA were added to glutathione-Sepharose columns loaded with GST-BK_{STREX}, GST alone, GST-M_3ct or GST-M_3i3, in the presence or absence of Gβγ (30nM). In the presence of Gβγ, the binding of ARF1-HA to both the GST-M_3ct and the GST-M_3i3 constructs was clearly increased, but not to the GST-BK_{STREX} construct or to GST alone. ARF6-HA showed a similar increase in binding to the GST-M_3i3 construct in the presence of Gβγ, however the binding of ARF6-HA to the GST-M_3ct construct was not as robust, and any increase in binding in the presence of Gβγ is therefore less obvious.

Figure 3.9

Effect of Gβγ on ARF1-HA and ARF6-HA binding to the M_3i3 and M_3ct GST-fusion proteins

a ARF1-HA association



b ARF6-HA association

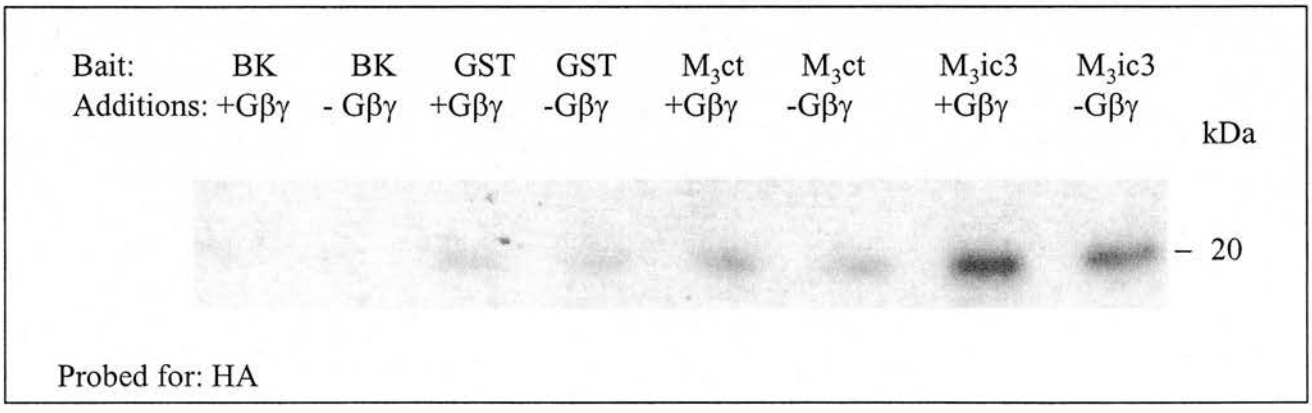


Figure 3.10

Effect of $G\alpha_q$ on ARF1-HA binding to the M_3i3 GST-fusion proteins with or without $G\beta\gamma$

Figure 3.10 shows a representative image (n= 4) of the extent of ARF1-HA binding (HA immunoreactivity) to the GST-M_3i3 in the presence of purified $G\beta\gamma$, $G\alpha_q$-enriched COS7 extracts or $G\beta\gamma$ and $G\alpha_q$ together. Equivalent amounts of transfected COS7 cytosolic extracts were included to ensure balanced amounts of cytosol were present in all samples. The lower panel shows the grey-scale density of each band in this experiment (representing associated ARF1-HA) as measured by Scan-Analysis (Elsevier software UK), with the value for ARF1-HA alone taken to equal 1, and the other bands compared to this.

Figure 3.10

Effect of $G\alpha_q$ on ARF1-HA binding to the M_3i3 GST-fusion protein

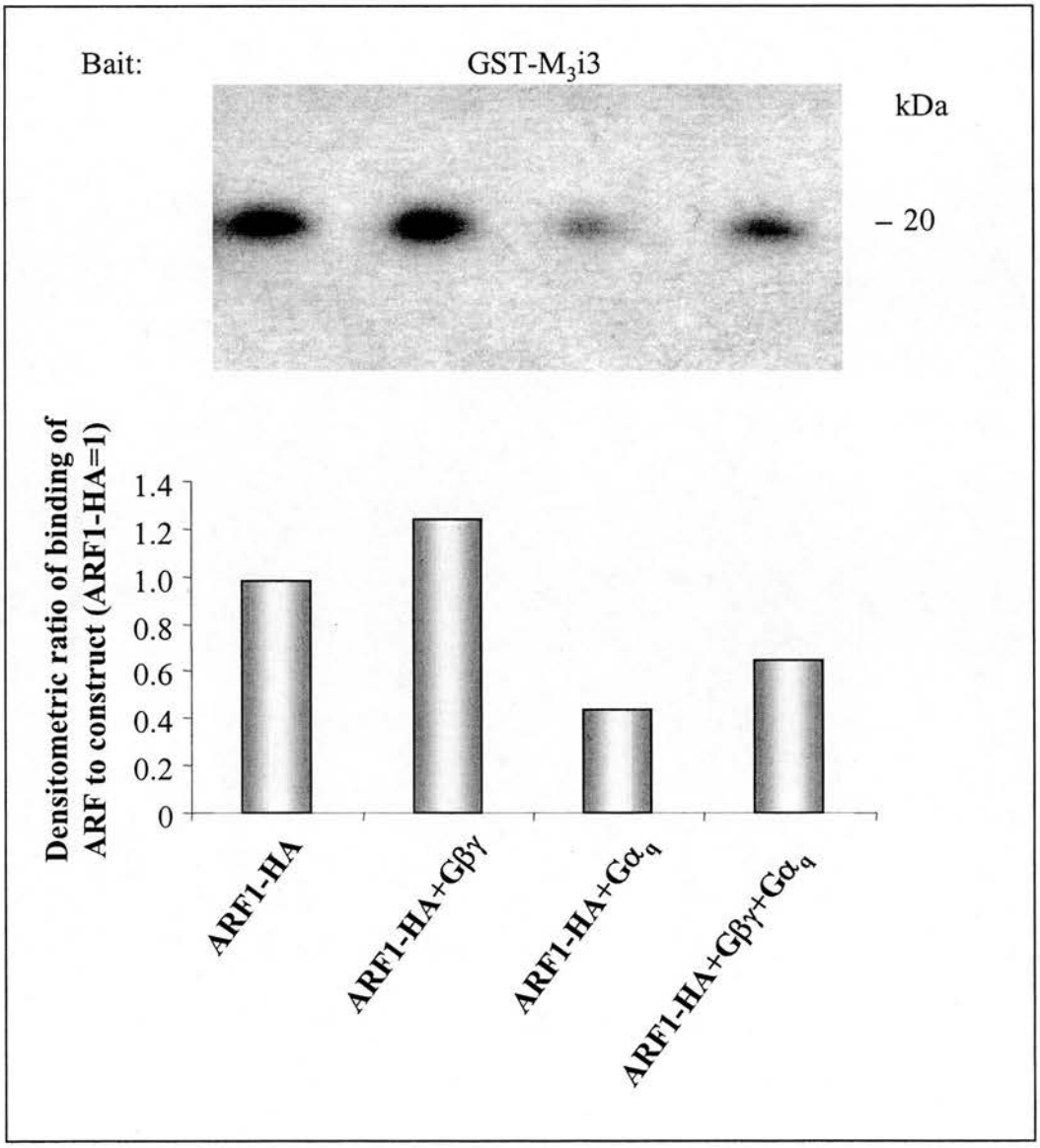


Figure 3.11

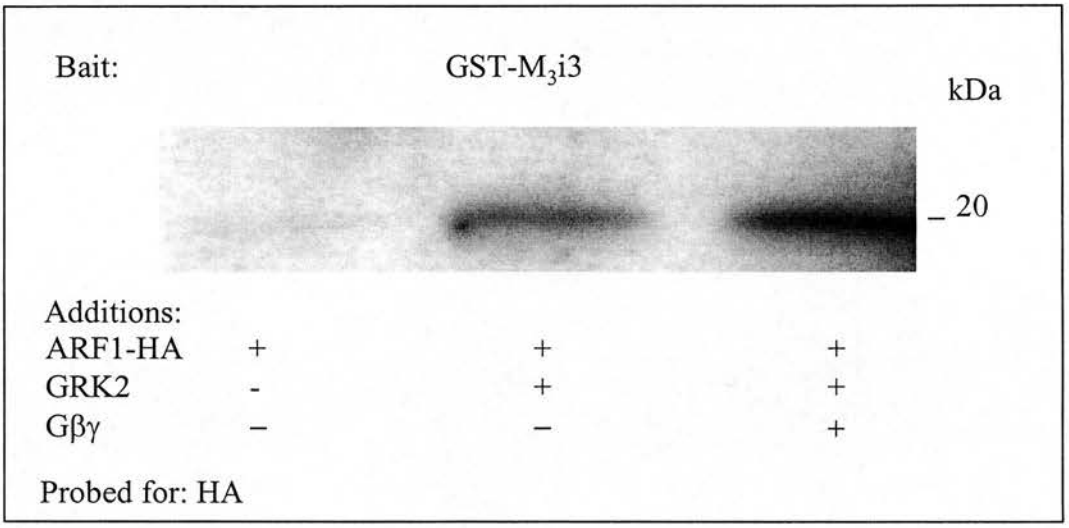
Effect of GRK2 and Gβγ on ARF1-HA binding to the M_3i3 GST-fusion protein

This figure shows the effect of the addition of COS7 cells extracts overexpressing GRK2 as well as the additional influence of purified Gβγ (30nM) on the binding of a submaximal amount of ARF1-HA (from COS7 cell cytosolic lysate) to the M_3i3 GST-fusion proteins. Input amounts of GRK2-enriched extract were balanced with COS7 cell extracts from cells containing empty pcDNA3 vector. Figure 3.11a shows the binding of ARF1-HA under these conditions to the GST-M_3i3 constructs, and 3.11b shows binding to the control construct, GST-BK_{STREX}.

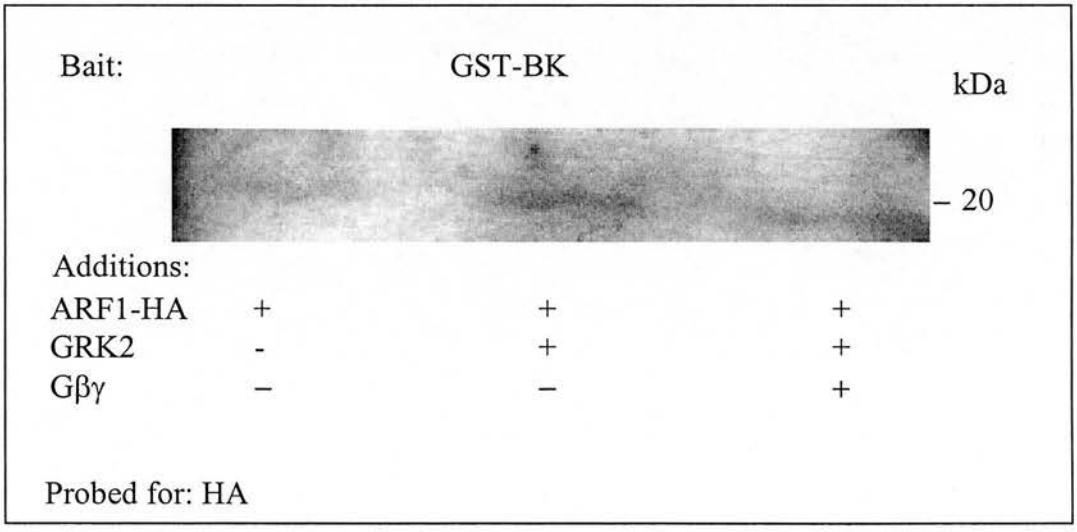
Figure 3.11

Effect of GRK2 and Gβγ on ARF1-HA binding to the M_3i3 GST-fusion protein

a



b



Chapter 4:

The association of ARF and other signalling proteins with domains of the 5-HT_{2A} receptor.

Introduction

The 5-hydroxytryptamine 2A receptor (5-HT_{2A}R) is known to activate phospholipase C (PLC) via the heterotrimeric G proteins $G_{q/11}$ (Hoyer *et al.*, 1994), and has also been shown to be able to couple to phospholipase A_2 (PLA_2)-mediated arachidonic acid release (Berg *et al.*, 1994; Berg *et al.*, 1996). Since the 5-HT_{2A} receptor (and 5-HT_{2C}) can activate both PLC and PLA_2 in an agonist-dependent manner but with different profiles of efficacies for different agonists (Berg *et al.*, 1998) it is hypothesised that this differential coupling is mediated via receptor coupling to different trimeric G-proteins. It has been further suggested that the mechanism of PLA_2 activation may be through a complex signalling mechanism involving both $G\alpha_{i/o}$ associated $G\beta\gamma$-mediated ERK1,2 activation and $G\alpha_{12/13}$-coupled, Rho-mediated p38 activation (Berg *et al.*, 1998; Kurrasch-Orbaugh *et al.*, 2003a; Kurrasch-Orbaugh *et al.*, 2003b). The 5-HT_{2A} receptor has also been shown to activate tyrosine phosphorylation (correlating with evidence for association of the tyrosine kinase JAK2 with the ct domain (Guillet-Deniau *et al.*, 1997)). Another important signalling cascade activated by the 5-HT_{2A} receptor is the activation of the phospholipase D (PLD) pathway in an ADP-ribosylation factor (ARF)-dependent manner, that appears to be independent of $G_{q/11}$ or $G_{i/o}$ (Mitchell *et al.*, 1998; Mitchell *et al.*, 2003).

GPCR interactions with heterotrimeric G proteins often (but not exclusively) appear to involve the third intracellular loop (i3) (Wess *et al.*, 1997). The i3 domains of various GPCRs have been shown to provide docking sites for heterotrimeric G

protein βγ subunits (Wu *et al.*, 1998) as well as arrestins (Wu *et al.*, 1997; Mukherjee *et al.*, 1999; Gelber *et al.*, 1999), GPCR-kinases (GRKs) (Wu *et al.*, 1998) and indeed ARFs (McCulloch *et al.*, 2001; Ronaldson *et al.*, 2002). In a number of GPCRs, other intracellular loops and ct domains have also been implicated in interactions with heterotrimeric G proteins, and indeed it seems likely that there are multiple sites of interaction, suggesting that the interaction of G proteins and GPCRs may involve motifs on both the i3 and the ct domains (Taylor *et al.*, 1994; Hamm, 2001). In the 5-HT_{2A}R, the i3 domain has been shown to be important for coupling to $G_{q/11}$ and the carboxyl terminal segment of the i3 in particular, may play a key role in this interaction (Roth *et al.*, 1998). The carboxyl terminal tail of the rhodopsin receptor has been shown to interact with the α and γ subunits of transducin (Ernst *et al.*, 2000; Marin *et al.*, 2000), and the NPxxY motif and the seven residues downstream have been shown to influence the heterotrimeric G protein activation of both rhodopsin and the 5-HT_{2C} receptor (Prioleau *et al.*, 2002; Fritze *et al.*, 2003).

It has previously been shown that members of the arrestin family can bind to various intracellular domains of the family R GPCRs, and this may also involve multiple interaction sites (Oakley *et al.*, 2000). High affinity binding of arrestins to GPCRs generally requires both receptor activation and phosphorylation by GRKs or other kinases (Gurevich *et al.*, 1995). However, arrestins can also bind to non-phosphorylated sites (Wu *et al.*, 1997; DeGraff *et al.*, 2002; Mukherjee *et al.*, 2002). The binding of arrestins has been documented to non-phosphorylated forms of the i3 loops of the M_3 and M_2 muscarinic receptors, and the $\alpha_{2A/D}$ adrenoreceptor (Wu *et al.*, 1997) and indeed arrestin isoforms have been shown to bind to the i3 loop of the 5-HT_{2A}R, with a broader specificity than is shown at i3 of the M_2 and M_3 muscarinic

receptors, where only non-visual arrestins are bound (Wu *et al.*, 1997; Gelber *et al.*, 1999). Additionally, a range of the family R GPCRs (β_2 adrenergic receptor, mu opioid receptor, endothelin type A receptor, dopamine D1A receptor, and α1b adrenergic receptor) have been shown to bind arrestin 3 with a higher affinity than arrestin 2 through the carboxy-terminal tail, whereas no interaction was observed with arrestin 1 (visual arrestin) (Oakley *et al.*, 2000), however, the third intracellular loop of these receptors was not investigated in depth in this study.

The specific conserved NPxxY motif that is found at the junction of the tm7 and ct domains in a number of rhodopsin family GPCRs, has been implicated as a determinant of ARF:receptor interactions and ARF-mediated signalling since native receptors with an alternative DPxxY motif, or N to D mutation (for example in the 5-HT_{2A} receptor) display selective reduction in this pathway (Mitchell *et al.*, 1998; Xu *et al.*, 1999). However, it has not been clear whether this motif might be accessible as a direct docking site or whether instead it regulates access to a distinct site.

There is evidence to suggest that PLD may be able to bind directly to some of the family R GPCRs. The amino terminal domain of PLD2 has been found to interact with the carboxy-terminal tail domain of the μ-opioid receptor by yeast two-hybrid screening and co-immunoprecipitation experiments, whereas PLD1b did not associate with the receptor in this investigation (Koch *et al.*, 2003). This association of PLD2 and the μ-opioid receptor was also suggested to be necessary for ARF1 to bind to the μ-opioid receptor. The proposed arrangement involved ARF1 binding to the PLD2 which in turn associated with the receptor, however agonist stimulation increased the association of ARF1 with the μ-opioid receptor, suggesting a

conformational change of the receptor may facilitate binding of ARF (Koch *et al.*, 2003). The physical association of both PLD1 and PLD2 with the muscarinic M_3 receptor has also been shown by co-immunoprecipitation assays in other work in this laboratory (Collins, D. and Johnson, M.S. unpublished), although signalling may be preferentially through PLD1 (Mitchell *et al.*, 2003).

In the case of the M_3 muscarinic receptor, both ARF1 and ARF6 play a role in the activation of PLD upon receptor activation (Chapter 3). The aim of this investigation was to assess the role of ARF in the PLD signalling of the 5-HT_{2A} receptor, elucidating the relative involvement of ARF1 and ARF6 isoforms, including the location and mechanism of their potential interaction with the receptor. The influence of interactions with some other possible binding partners for ARF and the 5-HT_{2A} receptor was also examined, as well as the ability of PLD itself to bind to the 5-HT_{2A} receptor.

Results

The role of ARF1 and ARF6 in PLD activation by the 5-HT_{2A} receptor.

The role of ARF1 and ARF6 in the activation of PLD by the 5-HT_{2A} receptor was investigated. Figure 4.1 illustrates functional signalling responses of the sPrC-5-HT_{2A}R expressed in COS7 cells. [^{3}H]Ketanserin binding experiments indicated that the sPrC-5-HT_{2A}R was expressed in COS7 cell membranes at a mean level of 0.84 ± 0.04 pmol/mg total protein with an K_i value of 1.96 ± 0.08 nM (with K_d data from GPCR database, as calculated by the Cheng-Prussoff equation)(Robertson *et al.*,

2003). The receptor produced robust PLC and PLD activation responses to 5-HT stimulation, similar to the untagged receptor (Bohm *et al.*, 1997; Mitchell *et al.*, 1998) but with slightly greater potency, the EC_{50} values for PLC and PLD responses being 5.0 ± 2.2 and 6.8 ± 1.9 nM respectively (Fig. 4.1 a,b). The EC_{50} value for PLC activation by the untagged receptor in similar experiments was found to be 28 ± 2 nM (unpublished observations) and 22 ± 5 nM (Bohm *et al.*, 1997). The effects of co-transfection of ARF mutants were investigated on 5-HT-induced signalling events mediated by the sPrC-5-HT_{2A}R expressed in COS7 cells. PLC activation was unaffected by co-transfection of either T31N-ARF1-HA or T27N-ARF6-HA; (mutant constructs of the ARF isoforms that have a dysfunctional GTP binding domain (Peters *et al.*, 1995)), (Fig. 4.1a). However T31N-ARF1-HA, but not T27N-ARF6-HA, significantly inhibited 5-HT_{2A}R-mediated PLD activation (Fig. 4.1b). Figure 4.1c shows the inhibitory effect of BFA (a blocker of the BIG1/2 class of ARF GTP-exchange factor (GEF) (Morinaga *et al.*, 1999)) on the PLD response of the 5-HT_{2A} receptor. When only the receptor was expressed, BFA caused a concentration-dependent inhibition of 5-HT-induced PLD activation, with significant inhibition at 50 μM and above. In cells additionally expressing T27N-ARF6-HA, BFA was also inhibitory throughout a similar concentration range. However, in cells expressing T31N-ARF1-HA, the residual 5-HT-induced PLD response became insensitive to BFA. The PLC response of the 5-HT_{2A}R was unaffected by BFA (data not shown).

These findings demonstrate that a negative mutant construct of ARF1, but not ARF6, inhibits the activation of PLD, but not PLC, by the 5-HT_{2A}R. This indicates a selective functional role for the ARF1 isoform in the PLD signalling pathway of the

5-HT_{2A}R. PLD, but not PLC responses of the 5-HT_{2A}R were correspondingly reduced in a concentration-dependent manner by BFA. Further evidence consistent with a functional role for ARF1 in BFA-sensitive PLD responses can be taken from the experiments assessing the BFA-sensitivity of 5-HT_{2A}R PLD responses in cells co-expressing negative mutant ARFs, T31N-ARF1-HA or T27N-ARF6-HA. The inhibitory effect of the negative mutant ARF1 construct pre-empted any further inhibition by BFA, suggesting that they both acted within the same pathway, whereas negative mutant ARF6 was without effect. Other GPCRs may show different selectivity for ARF isoforms. In COS7 cells expressing the M_3 muscarinic receptor and in A10 smooth muscle cells, PLD responses to carbachol and to angiotensin II or ET-1 respectively were attenuated by T31N-ARF1 and by T27N-ARF6 (Xu *et al.*, 1999; Shome *et al.*, 2000), whereas some other GPCRs such as P_{2u} and $PAC_{1\text{-}hop1}$ receptors may show partial selectivity for ARF6 over ARF1 (Xu *et al.*, 1999; Ronaldson *et al.*, 2002).

To investigate whether a physical interaction of either ARF1 or ARF6 with the receptor occurs upon activation, co-immunoprecipitation of HA-tagged ARF isoforms with the sPrC-5-HT_{2A}R was carried out. Significant levels of ARF1-HA (and to a much lesser extent, ARF6-HA) appeared to be specifically associated with the PrC-tag antibody pulldowns of the sPrC-5-HT_{2A}R, even without agonist. Densitometric analysis indicated that basal levels of ARF1-HA co-immunoprecipitated were increased on average 3.32 ± 1.58 fold over non-specific, as determined with NI IgG. The corresponding value for ARF6-HA was lower, at 0.32 ± 0.05 fold increase over non-specific. Although co-immunoprecipitation of both isoforms of ARF with the receptor appeared to be increased by addition of 5-HT

(mean increase to 1.61 fold and 1.26 fold of control with ARF1-HA and ARF6-HA respectively), only the effect on ARF1-HA was statistically significant(Robertson *et al.*, 2003).

ARF binding to 5-HT$_{2A}$ receptor domains.

To examine the receptor-ARF interaction in more detail, we generated GST fusion protein constructs of the intracellular loop 3 (i3; I^{258}-G^{326}) and carboxy-terminal tail (ct; N^{376}-V^{471}) of the 5-HT$_{2A}$R, and investigated their ability to bind ARF1-HA and ARF6-HA *in vitro*. Figure 4.2 is a schematic representation of the 5-HT$_{2A}$R showing the amino acid sequences used for these GST constructs. GST-fusion protein constructs of the i3 and ct domains of the 5-HT$_{2A}$R, or the STREX exon of the BK channel as a control, were attached to glutathione Sepharose beads and used in *in vitro* interaction assays at equivalent input levels (as estimated by GST immunoreactivity or Coomassie staining (Fig. 4.3a)). The STREX exon is a peptide of unrelated sequence to the 5-HT$_{2A}$ receptor, from the BK (Big Potassium) channel which is a membrane associated protein with an entirely different function, and was used as a negative control. The input levels of ARF1-HA and ARF6-HA or the negative mutant constructs, deficient in GTP binding; T31N-ARF1-HA and T27N-ARF6-HA were also balanced for HA-immunoreactivity (Fig. 4.3a). Figure 4.3b compares ARF1-HA and ARF6-HA interaction with the constructs. ARF1-HA displayed much greater relative binding to the ct domain of the 5-HT$_{2A}$R than to the BK channel construct or the i3 domain of the 5-HT$_{2A}$R. ARF6-HA showed a much lower level of binding to the 5-HT$_{2A}$ct construct and little interaction with the 5-

HT_{2A}i3 or BK constructs. Figure 4.3c shows the binding profiles for the functionally negative mutants of ARF1 and ARF6; T31N-ARF1-HA and T27N-ARF6-HA. The binding of T31N-ARF1-HA to the 5-HT_{2A}ct construct was greatly reduced compared to that of the ARF1-HA wild type, and the low background levels of T31N-ARF1-HA binding to 5-HT_{2A}i3 and BK channel constructs were similar to those seen with wild type ARF1-HA. T27N-ARF6-HA showed a low level of binding to the 5-HT_{2A}ct construct that appeared to be similar to that of the ARF6-HA wild type. The apparently slightly higher levels of T27N-ARF6-HA binding compared to the wild type in the experiment displayed correspond to its somewhat higher input level. GTPγS facilitated the interaction of submaximal levels of ARF1-HA (cytosolic extract additions were reduced to a level where ARF1-HA binding was seen to be less than previous experiments, and a difference could be seen by visualisation of the ARF1-HA by western blot procedures) with the 5-HT_{2A}ct construct and also appeared to strengthen a weak interaction with the 5-HT_{2A}i3 construct, which had been minimal in the absence of added nucleotide (Fig. 4.3d). GTPγS did not facilitate T31N-ARF1-HA binding to the receptor constructs. (Similar results were obtained in 3 different experiments).

Further to this work, a range of concentrations of both ARF1 and ARF6 were added to the GST-5-HT_{2A}i3 and GST-5-HT_{2A}ct constructs to assess the concentration-dependence of binding. Figure 4.4 shows the relative proportion of ARF isoform bound to the constructs at increasing ARF concentrations, expressed as a ratio of densitometric values for HA-immunoreactivity of the bound ARF versus GST immunoreactivity in the construct. Increasing the concentration of ARF1-HA present with each construct caused corresponding increases in the amount of ARF

bound. ARF1-HA bound to the GST-5-HT_{2A}ct construct to a much greater extent than to the GST-5-HT_{2A}i3 construct. ARF6-HA also bound to both the GST-5-HT_{2A}ct and the GST-5-HT_{2A}i3 constructs, but to a much lesser extent than the ARF1-HA bound to either construct, requiring higher levels of added ARF6-HA to obtain detectable binding. It must be noted, however, that there is a major limitation in the GST-fusion protein experiments mentioned above. What has not been measured in these experiments is the accumulation of active GTP-ARF in vitro, and the possible effects of endogenous ARF GEFs in the COS7 cell cytosolic preps to the observed effects.

The GST-fusion protein experiments suggested that the ct domain of the 5-HT_{2A}R provides a binding site for ARFs at which ARF1 shows a higher affinity than ARF6. To address the question of whether the binding of ARF1 to the 5-HT_{2A} receptor constructs was direct, and to try to ascertain the affinity of this binding, a variation of the standard technique had to be implemented, since the COS7 cytosolic extracts containing transfected ARF-HA isoforms introduced other unknown cytosolic proteins into the equation. To ensure a higher purity of ARF was being introduced to columns, an alternative approach using ARF1-V5-His_6 was used (Robertson *et al.*, 2003). The use of the His_6 tag allows for purification of the ARF isoform via the tag's ability to bind to cobalt-derivatised columns. Comparison of the immunoreactivity for ARF1-V5-His_6 bound to the GST-5-HT_{2A}ct construct with known amounts of purified ARF1-V5-His_6, the percentage purity of the purified protein was assessed by staining with high sensitivity Colloidal Coomassie on an SDS-PAGE gel and identifying the correct band by Western blot. A standard curve was then produced from the purified ARF1-V5-His_6, which when co-processed with

experimental cytosolic inputs allowed an estimate of the affinity of interaction of ARF1-V5-His_6 with the GST-5-HT_{2A} receptor domains (Robertson *et al.*, 2003). These experiments showed not only that the ARF1-V5-His_6 bound directly to the 5-HT_{2A} receptor domains, but nonlinear curve fitting of the saturation curve gave a value for affinity (50% saturation) of (1.7 ± 0.4 nM), with 90% occupany of available sites by 4 to 5 nM. This shows ARF-V%-His_6 to be of lower affinity than that for arrestin interaction with the M_3R i3 domain (Wu *et al.*, 1997), but higher than the corresponding interaction of $G\beta\gamma$ (Wu *et al.*, 1998). The 5-HT_{2A} receptor i3 domain shows only low affinity for ARF *in vitro* but may still represent an auxiliary binding site *in vivo*. The interaction of ARF1-HA with the ct or i3 domain of the 5-$HT_{2A}R$ appeared to be facilitated by GTPγS, suggesting that occupancy of its nucleotide recognition site by GTP rather than GDP promotes the interaction. Correspondingly, the GTP-binding-defective mutant ARF1 construct (T31N-ARF1-HA) showed an almost complete lack of specific binding to the ct or i3 domain GST fusion proteins, that was unmodified by GTPγS. The lower level of ARF6-HA binding appeared to be little affected by T27N mutation of ARF6-HA, but this was not investigated further. The means by which agonist may induce increased (BFA-sensitive and GTP status-sensitive) binding of ARF1 to the 5-$HT_{2A}R$ is not clear. Involvement of BIG1/2 is implicated by the BFA sensitivity, but it is not known whether agonist activation of the receptor might facilitate GTP loading of ARF1 by direct protein:protein interaction, by regulation of BIG1/2 or by other means. However, GTP binding operates a conformational switch in ARFs that might contribute to additional protein:protein interactions (Goldberg, 1998). In experiments carried out with BIG1 on the GST constructs of the 5-HT_{2A} receptor

domains to assess the binding capability of BIG1, or if BIG1 had an effect on the ability of ARF1-HA to bind to the receptor domains, insufficient expression of BIG1-HA in COS7 cells meant that neither binding of BIG1-HA to any of the GST-5-HT_{2A} constructs, nor any effects of BIG1-HA on the binding of ARF to the constructs could be detected (data not shown).

The role of the NPxxY motif in ARF1 association with the 5-HT_{2A} receptor.

The differential signalling properties of the wild type 5-HT_{2A}R and the N^{376}D mutant 5-HT_{2A}R (Mitchell *et al.*, 1998; Mitchell *et al.*, 2003) suggest that the NPxxY motif, at the junction of the ct and the 7th transmembrane domain, may participate in some way in the binding of ARF1 to the receptor. To test this theory, signalling experiments and co-immunoprecipitation studies with both the wild type and N^{376}D mutant form of the receptor were carried out in the lab. 5-HT (1 μM) -induced PLD activation by the wild type sPrC-5-HT_{2A}R was significantly reduced by BFA (100 μM) or by co-expression of T31N-ARF1-HA but not T27N-ARF6-HA (Robertson *et al.*, 2003). Corresponding responses of the N^{376}D-sPrC-5-HT_{2A}R examined in the same experiments showed no significant inhibition by either BFA or T31N-ARF1-HA. Co-immunoprecipitation experiments were carried out in COS7 cells co-transfected with ARF1-HA and either the wild type sPrC-5-HT_{2A}R or its N^{376}D mutant form. After stimulation with 5-HT (1 μM, 5 min) or under control conditions, solubilised extracts were immunoprecipitated with the HA-tag antibody. The 5-HT_{2A}Rs associated with the immunoprecipitate was assayed as specific

[^{3}H]ketanserin binding. Low levels of non-specific [^{3}H]ketanserin binding were present in each case and these showed no discernable differences between samples (Robertson *et al.*, 2003). In cells transfected with the wild type sPrC-5-HT_{2A}R, but not those expressing the N376D mutant, significant levels of specific [^{3}H]ketanserin binding became associated with the HA tag-directed immunoprecipitate following 5-HT stimulation. Thus 5-HT stimulation appeared to cause an increased interaction between the sPrC-5-HT_{2A}R and ARF1-HA (Robertson *et al.*, 2003).

Although the tm7 NPxxY motif has been implicated as a critical determinant of ARF co-immunoprecipitation and ARF-dependent signalling in rhodopsin family GPCRs, the precise site of ARF binding to the ct of the 5-HT_{2A}R remains to be elucidated. Mutation of this motif to DPxxY strongly inhibits BFA-sensitive, ARF-mediated activation of PLD (Mitchell *et al.*, 1998) and sPrC-5-HT_{2A}R co-immunoprecipitation with ARF1-HA (Robertson *et al.*, 2003).

Figure 4.5 shows results from GST-fusion protein experiments investigating whether the N^{376}PLVY motif in the 5-HT_{2A} R ct domain may directly form part of the binding site for ARF1-HA. GST-fusion protein constructs of the wild type (N^{376}-V^{471}) 5-HT_{2A}ct, the mutant (N^{376}D-V^{471}) 5-HT_{2A}ct and the truncated (K^{385}-V^{471}) 5-HT_{2A}ct were prepared. Equal inputs of these constructs (and GST alone) were determined by Coomassie Blue staining and by GST immunoreactivity at the predicted molecular mass before interaction assays with ARF1-HA. The ratios of the densitometric values for bound ARF1-HA immunoreactivity to fusion protein input were then calculated on an arbitrary scale relative to that for the wild type construct. Densitometric values for fusion protein input levels and HA-immunoreactivity were calculated utilising ScanAnalysis© software. Conditions were selected so that

densities of both ECL (enhanced chemiluminescence) and colloidal Coomassie staining were in the lower part of the range since the scales of density against concentration tend to become non-linear at higher values. Therefore measurements at low densities tend to give a more accurate representation of protein concentration.

Both individual images and the mean densitometry ratios for band ARF1-HA: construct input showed a clear reduction in binding (to around 50%) by the $N^{376}D$ mutation and a further loss (to around 20%) by deletion of the N^{376}-N^{384} sequence (Fig. 4.5). These data imply that both the NPxxY motif and residues around or before K^{385} play an important role in the interaction between ARF1-HA and the 5-HT_{2A}ct.

To further investigate the binding of ARF1-HA to the 5-HT_{2A}ct, GST-fusion protein constructs of the tail were made that had truncations from the carboxyl terminal end of the tail. GST constructs were made that contained the NPxxY motif and subsequent residues but terminated at L^{406} and Q^{396} (Fig. 4.6a). These truncated constructs were expressed, and exposed to ARF1-HA under the same conditions as previously described. Densitometric ratios comparing the amount of each construct present (as shown by Coomassie staining) to the extent of immunoreactivity to the HA tag of the associated ARF1-HA are shown (Fig. 4.6b). There were no clear differences in the binding of ARF1-HA to either of these two truncated constructs (5-HT_{2A}ctL^{406} and 5-HT_{2A}ctQ^{396}) compared to the full length 5-HT_{2A}ct. The mean binding levels of ARF1-HA binding were slightly lower to the shorter constructs, but there were no statistically significant changes from the full length ct construct. In these constructs, the NPxxY motif was preceded by the amino acids glycine (G) and isoleucine (I). These amino acids (G and I) are not identical to the native amino

acids, alanine (A) and valine (V), and were introduced as to produce a short link between the GST protein and the receptor domain construct, in case the proximity of the GST protein might restrict with the ability of the proteins of interest to access this end of the carboxyl-terminal domain, and in particular the N/DPxxY motif. Both G and I are neutral in charge, as are the native A and V, and no perceptible difference was noted in the ability of the 2A ct constructs to bind to ARF1-HA or ARF6-HA in either the presence or absence of the linker amino acids G and I.

These findings suggest that the majority of the key elements involved in 5-HT_{2A}R: ARF1 interaction, at least under these circumstances, may lie within the N^{376}-N^{384} segment and further that the large remaining distal part of the carboxy-terminal tail, at least beyond the region of Q^{396}/L^{406}, plays little role in ARF binding. Structural modelling based on rhodopsin and secondary structure predictions (PHD predict; http://cubic.bioc.columbia.edu/predictprotein/) suggest that the P^{377} residue is likely to form a pronounced kink in the tm7 helix and that T^{381}-K^{385} may form a flexible hinge to an eighth helical segment that runs in the plane of the membrane until a palmitoylation anchor at C^{397} (Sealfon *et al.*, 1995; Backstrom *et al.*, 2000; Dev *et al.*, 2001). In the case of rhodopsin, activation of the receptor newly exposes to the intracellular surface an epitope that includes residues equivalent to L^{378}-Y^{380} here (Becamel *et al.*, 2001), consistent with the idea that receptor activation may reveal residues involved in ARF association. The predicted fourth intracellular loop of rhodopsin, in particular residues equivalent to N^{384}-Q^{386} here, is involved in interaction with the α and γ subunits of transducin (Ernst *et al.*, 2000; Marin *et al.*, 2000). Interactions between amino acids in the NPxxY motif and the subsequent seven residues are thought to influence heterotrimeric G protein activation by both

rhodopsin and the 5-HT_{2C}R (Thomas *et al.*, 1995; Hunyady *et al.*, 1995). Elements of this surface might also contribute to ARF docking. The interaction of 5-HT_{2A}R with $G\alpha_{q/11}$ however, is also thought to involve the carboxyl portion of the i3 loop (Roth *et al.*, 1998).

Additional functional roles have been proposed for the N/DPxxY motif. The most consistent evidence is for a role linking the tm2 and tm7 helices (Bohm *et al.*, 1997). The NPxxY motif and the Y residue in particular, have also been proposed to constitute an internalisation motif in some but by no means all GPCRs (Laporte *et al.*, 1996; Konvicka *et al.*, 1998; Wilbanks *et al.*, 2001; Barak *et al.*, 2003). Mutation of the N or D residue to A generally causes massive disruption of signalling pathways and of internalisation, whereas reciprocal mutation of N or D appears to have relatively minor effects on heterotrimeric G protein signalling (Sealfon *et al.*, 1995; LeGouill *et al.*, 1997; Bohm *et al.*, 1997; Mitchell *et al.*, 1998). In the case of the 5-HT_{2A} receptor, we confirmed that the NPxxY, rather than the DPxxY mutant motif, was necessary for functional BFA-sensitive and T31N-ARF1-HA-sensitive PLD responses from the receptor, for 5-HT-induced co-immunoprecipitation of the receptor with ARF1-HA (Robertson *et al.*, 2003), and for the major part of *in vitro* binding of ARF1-HA to the ct domain of the receptor (Figs. 4.5; 4.6; 4.7).

There is increasing evidence that particular GPCRs can interact with diverse scaffolding and signalling proteins other than their conventional partners, the heterotrimeric G-proteins (Zhou *et al.*, 1994; Premont and Hall, 2002). Receptor ct segments may dock adapter proteins containing PDZ or other domains, signalling proteins and modulators of signalling functions (Bansal and Gierasch, 1991; Zhou *et al.*, 1994; Oakley *et al.*, 2001). In the 5-HT_2 receptor family, the distal ct residues

are targeted by the PDZ-domain proteins, PSD-95 and MUPP-1 (Laporte *et al.*, 1996; Kitano *et al.*, 2002; Brady and Limbird, 2002); interactions that may modify the signalling function and localisation of the receptors. A novel PDZ domain protein, tamalin, has been shown to bind to both mGluR1/5 receptors and the ARF-GEF, ARNO (Slice *et al.*, 1994). It is conceivable that a similar arrangement might occur in the case of the 5-HT_{2A} receptor; locating an ARF-GEF in the proximity of ARF.

ARF may not be the only small G protein that can interact with GPCRs. It has been shown that Rho A can be co-immunoprecipitated in a complex with NPxxY GPCRs (Mitchell *et al.*, 1998), and there is evidence that both $G\alpha_{13}$ and $G\alpha_q$ may interact with Rho-GEFs to facilitate Rho function (Sagi *et al.*, 2001). Rho has also been shown to be required for 5-HT_{2C} recetor-mediated activation of PLD (McGrew *et al.*, 2002), and the link to this pathway has been shown to have been lost in a naturally occurring RNA edited isoform of the 5-HT_{2c} receptor, which is deficient in 5 amino acids of the 2nd intracellular loop (McGrew *et al.*, 2004). The small GTP-binding protein Rab5 has been observed to bind directly to the AT_{1A} receptor, via the last 10 amino acids of the carboxyl-terminal tail, and has been implicated in the ability of the receptor to control targeting between intracellular compartments by directly regulating components of the intracellular trafficking machinery (Seachrist *et al.*, 2002).

In experiments investigating possible links between ARF and the β_2 adrenergic receptor, ARF6 was not shown to directly bind to the β_2 adrenergic receptor, but one of the GTP-exchange factors (GEFs) for ARF6, ARNO (ADP ribosylation factor nucleotide-binding site opener), was. Activation of the β_2-adrenergic receptor promotes the formation of a complex between GDP-ARF6, ARNO and arrestin 2. It

is proposed that arrestin 2 functions as a scaffold to promote ARNO-dependent ARF6 activation and thereby facilitate β_2-adrenergic receptor endocytosis (Claing *et al.*, 2001). Other small G proteins of unknown identity have also been found to associate with the fMLP receptor (Sagi *et al.*, 2001).

Comparison of binding of ARF1 and ARF6 to 5-HT$_{2A}$ and 5-HT$_{2C}$ receptors

GST constructs of the equivalent ct and i3 domains of the 5-HT$_{2C}$ receptor were used to compare the ability of the 5-HT$_{2C}$ and 5-HT$_{2A}$ receptor domains to bind ARF1-HA and ARF6-HA. For similar inputs, the level of ARF1 binding to the 5-HT$_{2C}$ct was almost twice that for 5-HT$_{2A}$ct (taken to be 1). In these experiments, the binding of ARF1-HA to the N/D mutant and the K^{385} deletion construct of the 5-HT$_{2A}$ct was progressively reduced (Fig. 4.7) as shown previously (Robertson *et al.*, 2003). The difference in the binding of ARF1 to the 2Cct cannot be due to any difference in the N376-N384 region, as both receptors have identical sequence to this point. However there are sequence differences in the rest of the carboxy-terminal tail that may account for the different binding capabilities, however further mutational studies would be required to elucidate the residues or motifs responsible for this difference.

Figure 4.8 shows the binding ratio of ARF6-HA to the ct and i3 constructs of the 2A and 2C receptors. As can be seen here, none of the constructs, with the exception of the DPxxY-2Act, show any marked ability to bind to either the 2A or the 2C constructs when compared to the binding of ARF6-HA to GST alone. It appears that ARF6-HA binds better to the DPxxY form of the 5-HT$_{2A}$ct, with binding of ARF6-

HA to the NPxxY form of the ct being low, and not significantly greater than the ARF6-HA binding to the GST alone, while the removal of the whole N/DPxxY motif reduces the binding of ARF6-HA back to the levels of the wild type (NPxxY) construct. The binding of ARF6-HA to the DPxxY form of the ct domain was significantly increased over the basal binding of ARF6-HA to GST alone.

Binding of PLD1 and PLD2 to the ct and i3 domains of the 5-HT_{2A} and 5-HT_{2C} receptors

The potential for PLD to interact directly with members of the family R GPCRs was first suggested when the amino terminal domain of PLD2 was found to interact with the carboxy-terminal tail of the μ-opioid receptor by yeast two-hybrid screening, and indeed the μ-opioid receptor was also seen to constitutively interact with PLD2, but not PLD1, in co-immunoprecipitation experiments (Koch *et al.*, 2003). In support of this principle, physical association of both PLD1 and PLD2 with the muscarinic M_3 receptor has been shown by co-immunoprecipitation assays in other work in this laboratory. In addition the PLD isoforms have been shown to bind to the third intracellular loop and the carboxy-terminal tail of the M_3 receptor by means of GST pulldown assays. Upon stimulation, there was a significant but transient decrease in the association of PLD1 but not PLD2 with the M_3 receptor, as shown by co-immunoprecipitation (Collins, D. and Johnson, M.S. unpublished).

To investigate whether PLD bound to the 5-HT_{2A} receptor domains in vitro, binding of PLD to GST-fusion protein constructs of the 5-HT_{2A} receptor ct and i3 domains were compared to binding of PLD to the $N^{376}D$ mutant 2Act; the K^{385} deletion

mutant 2Act; and the 5-HT_{2C}ct domain. PLD1-HA and PLD2-HA were over-expressed in COS7 cells, and cellular extracts were made under the same conditions as described previously for the ARF-HA constructs, with the addition of the detergents CHAPS (1% (w/v)) and deoxycholate (1% (w/v)), to solubilise the otherwise membrane-associated PLD-HA, and the addition of 20% (v/v) glycerol to stabilise the PLD in the soluble form. These solubilised PLD-containing extracts were allowed to associate with the GST constructs of the i3 and ct domains of the 2A and 2C receptors, which had been immobilised by binding to glutathione beads. Captured proteins were then solubilised in Laemmli buffer and separated by SDS-PAGE.

Figure 4.9 shows a Western blot, immunoblotted for HA to detect the tagged PLD1 and PLD2. The upper half of figure 4.9 shows the binding of PLD1-HA to the receptor domain GST-constructs, as well as to the GST alone control. PLD1-HA bound to all constructs, although the binding to GST alone was much less. The binding of PLD1-HA to the GST-5-$HT_{2A}D^{376}$ct was much increased compared to that of the GST-5-$HT_{2A}N^{376}$ct, whereas the deletion of the region encompassing the N/DPxxY motif in the GST-5-$HT_{2A}K^{385}$ct construct returned the level of binding of PLD1-HA to that of the binding to the GST-5-HT_{2A}ct wild type. PLD2-HA bound to the GST-5-HT_{2A}ct, and to the $N^{376}D$ mutant of the tail, but not to the K^{385} shortened tail construct, or to the 5-HT_{2A}i3 domain (Fig. 4.9 lower panel). The binding of PLD2-HA to the GST-5-HT_{2C}ct was greatly increased compared to that of the PLD2-HA to the GST-5-HT_{2A}ct construct. PLD2-HA binding appeared similar to both the GST-5-$HT_{2A}N^{376}$ct and the GST-5-$HT_{2A}N^{376}$D constructs, but was less to the GST-5-$HT_{2A}K^{385}$ truncated construct. This suggests that the 376-385 sequence may play a

role in the binding of PLD2 but not PLD1 to the 5-HT_{2A}ct domain, but that PLD2 binding is not enhanced by the presence of a D residue at position 376.

The blots shown in figure 4.9 are representative of the results gained from the series of experiments into the binding of PLD isoforms to the GST-5-HT receptor domains. However with the number of constructs examined it is impossible to have the construct inputs 100% balanced in every experiment. To allow for the quantification of the binding patterns of PLD1 and PLD2 to the 5-HT_{2A} and related domains, densitometric ratios of construct present to the immunoreactivity of the HA tag on the bound PLD1 and PLD2 were calculated (as described previously for the ARF isoforms) and these ratios were normalised to that for binding to the GST-5-HT_{2A}ct construct.

Figure 4.10 shows the binding of PLD1-HA to the 5-HT receptor constructs from several experiments (n = 4-5), after correction for construct levels. The binding of PLD1-HA to the 5-$HT_{2A}N^{376}$Dct and to the 5-HT_{2C}ct constructs was significantly greater than that of PLD1 to the 5-HT_{2A}ct and GST over this range of experiments (the total construct input for GST-5-HT_{2C}ct in the experiment shown in Fig 4.9 was low). Removal of the whole NPxxY motif in the form of the K^{385} deletion mutant reduced the binding of PLD1-HA to the tail below that of the DPxxY form of the tail, but binding of PLD1-HA to the K^{385} construct was not reduced below that of the 5-HT_{2A}ct domain, suggesting that the N^{376}-K^{385} segment is not in itself crucial for binding of PLD1 to the receptor, and that the main binding site for PLD1 seems to be due to residues distal to K^{385}. However, the binding of PLD1 to the 2Act is increased in the presence of D^{376}, suggesting that the proximal part of the 2Act may still have a role to play in the binding of PLD1. This is backed up by the difference in binding

of PLD1 to the 2Act and the 2Cct. The differences in the 2Act and the 2Cct do not lie in the proximal part of the domain, but further downstream, and the two carboxy-terminal domains are identical from N^{376}-K^{385}. The fact that PLD1 binds to a much greater extent to the 5-HT_{2C}ct again suggests the contribution of a binding site further down the tail.

In the case of PLD2 (Fig. 4.11) the binding to the 5-HT_{2A} receptor domain constructs generally showed less of an increment above GST alone controls than did PLD1. The mean level of binding to the $2AN^{376}$ct, $2AD^{376}$ct and the 2Ai3 was greater than that to GST alone, but with the n values that could be obtained in the present study, these differences did not reach statistical significance. However, PLD2 showed clear and statistically significant binding to the 5-HT_{2C}ct domain. As both the 5-HT_{2A}ct and the 5-HT_{2C}ct are identical in primary structure up till amino acid T^{386}, the key residues dictating the increased binding of PLD2 to the 5-HT_{2C} receptor ct must be located further downstream to allow for such differences to be detected under these conditions. Although basic secondary structural predictions can be made (PHD predict; http://cubic.bioc.columbia.edu/predictprotein/), the majority of the constructs are not predicted as helical, or any other formal structure, so the domains being investigated are highly flexible and any 3D structure under the experimental conditions used is completely unknown.

PLD2 and not PLD1b has previously been shown to bind to the μ-opioid receptor in HEK293 cells (Koch *et al.*, 2003). These cells also showed BFA-sensitive activation of PLD2, and indeed ARF-immunoreactivity was co-immunoprecipitated with the μ-opioid receptor and PLD2. It has been suggested that ARF binds to the μ-opioid receptor through PLD2, or at least that PLD2 binding to the receptor causes a

conformational change that allows ARF to then bind. It was interesting to note that upon agonist stimulation, the amount of PLD2 that co-immunoprecipitated with the receptor decreases (as was seen with PLD1 with the M_3 muscarinic receptor, unpublished results Collins, D., Johnson M.S.), and yet the amount of ARF pulled down increased, suggesting that the link between ARF and PLD2 binding may not be as straightforward as the proposed mechanism of ARF simply binding through PLD2 (Koch *et al.*, 2003). In the case of the 5-HT_{2A} receptor, ARF1-HA (but not ARF6-HA) was seen to bind to the carboxy-terminal domain. The ARF1 was presented to the GST construct of the domain in the form of cytosolic extract, not purified ARF. However there is unlikely to be any significant content of PLD in the extracts as PLD is a membrane-associated protein, and to extract PLD-HA to introduce it to the GST constructs, an extra stage of membrane solubilisation was required. This is backed up by the fact that purified ARF1-V5-His_6 also binds to the 5-HT_{2A} receptor domains (Robertson *et al.*, 2003). The profile of PLD1 binding to the 5-HT_{2A} receptor with higher apparent affinity than PLD2, does not follow the same pattern of binding as that of ARF1, in that both the D^{376}2Act and K^{385}2Act constructs bind PLD1 better than the N^{376}2Act, and ARF1 to a lesser extent than the N^{376}2Act. Furthermore PLD2 binding is not reduced in the D^{376}N2Act which shows significantly less ARF1 binding than the N^{376}2Act construct. These facts together suggest that there may be no direct link between ARF1 and PLD binding to the 5-HT_{2A} receptor.

Investigation of a potential role of Gβγ in modulating PLD signalling by the 5-HT$_{2A}$ receptor

Since we demonstrated previously that the ability of the M_3 muscarinic receptor to activate PLD via ARF1 is facilitated in the presence of Gβγ subunits, the potential role of Gβγ on ARF1-dependent PLD activation by the 5-HT$_{2A}$ receptor was investigated. The effects of Gβγ upon activation of both the PLD and PLC signalling pathways activated by the M_3 muscarinic receptor and the 5-HT$_{2A}$ receptor were investigated by co-transfection of the Gβγ-sequestering agent GRK2ct (GRK2$_{495-689}$) with the receptors in COS7 cells. Co-transfection of the empty plasmid pcDNA3 was used as a control. As shown in figure 4.12, the PLD response to agonist stimulation of the M_3 receptor was decreased to about 50% in the presence of the GRK2$_{495-689}$ construct, whereas this had no effect upon the PLC response, as detailed in Chapter 3. However, when cells containing the sPrC-5-HT$_{2A}$ receptor were stimulated with agonist, the presence of the Gβγ sequestering agent had no discernable effect on the ability of the 5-HT$_{2A}$ receptor to activate either PLC or PLD. This suggests that the molecular mechanism of ARF-dependent PLD activation differs in the case of the 5-HT$_{2A}$ receptor from that of the muscarinic M_3 receptor. This positive effect of Gβγ on the M_3 receptor PLD activation response does not seem to be mirrored with the 5-HT$_{2A}$ receptor. So not only do the two receptors differ in their ability to activate different isoforms of ARF, but also in the ways in which they apparently link to ARF1.

Arrestin binding to 5-HT$_{2A}$ receptor domains, and the effect of arrestin on ARF binding

It has previously been shown that members of the arrestin family can bind to various intracellular domains of the Type I family of GPCRs. High affinity binding of arrestins to GPCRs generally requires both receptor activation and phosphorylation by GRKs or other kinases (Gurevich *et al.*, 1995). However arrestins can also bind to non-phosphorylated sites upon receptor activation (Wu *et al.*, 1997; DeGraff *et al.*, 2002; Mukherjee *et al.*, 2002). There is a lack of evidence for agonist induced phosphorylation accompanying the desensitisation of some GPCRs, such as the Luteinising hormone (LH/HCG) receptor, and the 5-HT$_{2A}$ receptor (Lamm and Hunzicker-Dunn, 1994; Vouret-Craviari *et al.*, 1995; Gray and Roth, 2001), and indeed the i3 domain of the 5-HT$_{2A}$ receptor has been previously shown to effectively bind arrestins under non-phosphorylated conditions (Gelber *et al.*, 1999; Johnson *et al.*, 2003).

The binding of arrestin 2 and arrestin 3 to both the 5-HT$_{2A}$i3 and ct was investigated. Figure 4.13 shows the binding of both arrestin 2 and arrestin 3 was markedly greater than that to GST alone for both the 5-HT$_{2A}$i3 and 5-HT$_{2A}$ct domain constructs.

The potential ability of arrestin to modulate the binding of ARF1 to the i3 and ct domains of the 5-HT$_{2A}$ receptor was investigated using the GST-fusion protein constructs of the receptor domains. Firstly, the effects of arrestin 2 on the binding of ARF1 to the GST-5-HT$_{2A}$i3 and ct domains was examined. The low level of binding of ARF1-HA to the 5-HT$_{2A}$i3 domain was unaffected by the presence of arrestin 2 (Fig 4.14a, right hand panel). However, the ability of ARF1-HA to bind to the 5-

HT_{2A}ct domain was markedly increased by the presence of arrestin 2 under these conditions (Fig 4.14a left hand panel). The converse question was also investigated, i.e. whether binding of arrestin 2 to the 5-HT_{2A}ct domain was altered in the presence of ARF1-HA. The binding of arrestin 2 showed no change in the presence of ARF1-HA under the conditions tested (Fig. 4.14b).

There have been studies into the functional interactions between arrestin 2 and ARF6 in the regulation of endocytosis of the β_2-adrenergic receptor. Arrestin 2 has been found in complex with ARNO, a GEF for ARF6. Upon agonist stimulation of the β_2-adrenergic receptor, arrestin 2 also interacts with the GDP-bound form of ARF6, becoming an interface for the GTP activation of ARF6 by ARNO, therefore agonist stimulation of the β_2-adrenergic receptor drives the formation of a complex including arrestin 2, ARNO and ARF6, leading to the activation of ARF6, and allowing for the endocytotic process (Claing *et al.*, 2001). It is possible that a similar relationship between arrestin, ARF and an ARF-GEF may be occurring in the case of the 5-HT_{2A} receptor, involving here ARF1 and arrestin 2, and allowing for the association of GDP- ARF1 and ARF-GEF upon agonist activation of the receptor. However, there is no evidence that the activation of ARF1 by the 5-HT_{2A} receptor has a role in the endocytosis or desensitisation of the 5-HT_{2A} receptor, as is seen in the case of the β_2-adrenergic receptor with ARF6. The possible inclusion of any ARF-GEFs for ARF1, e.g. BIG1, in the interaction complex was not investigated in this study.

In conclusion, these experiments provide intracellular signalling and *in vitro* domain interaction evidence for ARF association with the 5-HT_{2A} receptor, corresponding to

its functional activation of PLD. Furthermore, ARF1 rather than ARF6 appears to participate in this mechanism, through a GTP-dependent interaction with predominantly the ct domain of the receptor. The ARF-dependent PLD activation by the 5-HT_{2A} receptor is not diminished by sequestration of free Gβγ subunits, as is the case in the M_3 receptor. However modulation of ARF1 binding to the 5-HT_{2A}ct can be seen in the presence of arrestin 2. There is evidence for a direct interaction of PLD1 and PLD2 with the carboxy-terminal domain of the 5-HT_{2A} and 5-HT_{2C} receptors, but binding of PLD seems to be through a mechanism that is distinct from that for ARF. The binding of ARF to the 5-HT_{2A} receptor does not seem to require the presence of PLD, however further work is required to examine any potential modulatory effects of the presence of PLD on ARF binding to the 5-HT_{2A} receptor.

Figures

Figure 4.1

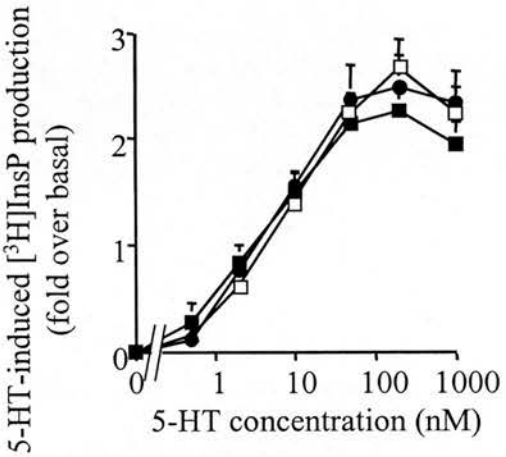
Effects of mutant ARF1-HA and ARF6-HA constructs on signalling responses of the sPrC-5-HT_{2A} receptor.

COS7 cells were co-transfected with the sPrC-5-HT_{2A}R together with either empty vector (●), T31N-ARF1-HA (■) or T27N-ARF6-HA (□). Values are means ± SEM, n = 6-8. a) Shows the concentration-dependence of 5-HT-induced PLC activation; there was no discernable effect of the negative mutant ARFs on this response. b) Shows the concentration-dependence of 5-HT-induced PLD activation. The addition of T27N-ARF6-HA had no significant effect on the ability of the 5-HT_{2A}R to activate PLD, whereas the presence of T31N-ARF1-HA significantly attenuated the PLD response to 5-HT concentrations of 10 nM and above (*$p<0.05$ by Wilcoxon test). c) Shows the brefeldin A (BFA)-sensitivity of the 5-HT (1 μM)-induced 5-HT_{2A}R PLD response; a concentration-dependent inhibition that was statistically significant (*$p< 0.05$, Wilcoxon test) for BFA concentrations of 50 μM and above in empty vector and T27N-ARF6-HA samples. Co-transfection of T27N-ARF6-HA had no discernable effect on BFA sensitivity compared to empty vector, whereas the remaining PLD response in the presence of T31N-ARF1-HA was no longer significantly inhibited by BFA.

Figure 4.1

Effects of mutant ARF1-HA and ARF6-HA constructs on signalling responses of the sPrC-5-HT_{2A} receptor

a



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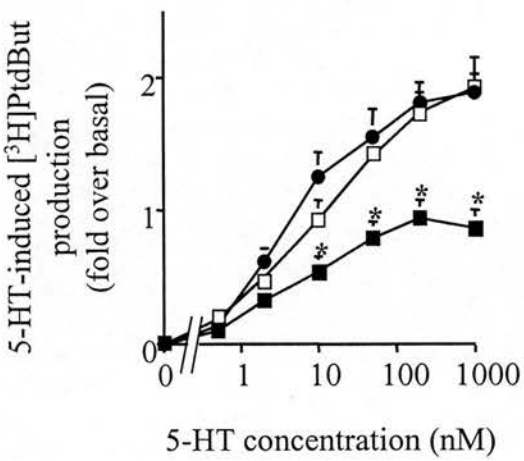

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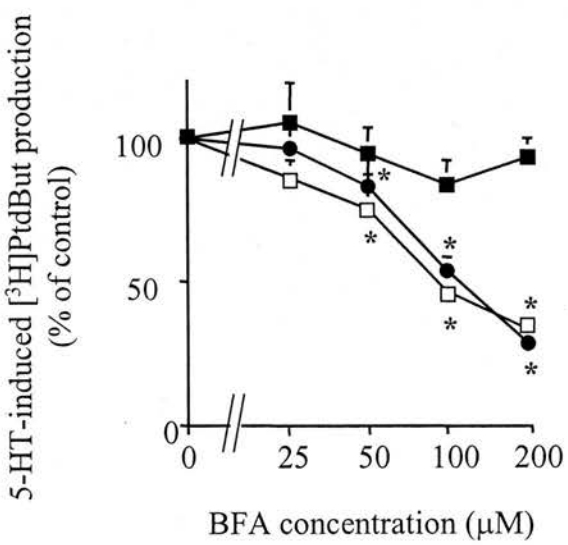


Figure 4.2

Amino acid sequence of the 5-HT_{2A} receptor carboxy-terminal tail and third intracellular loop.

GST-fusion protein constructs were made of the 5-HT_{2A} N^{376}-V^{471} carboxy-terminal tail (ct); the $N^{376}D$ mutant version of the ct domain; the truncated (K^{385}-V^{471}; N^{376}-Q^{396}; N^{376}-L^{406}) versions of the tail; as well as the I^{258}-G^{326} third intracellular loop (i3) domain of the 5-HT_{2A} receptor.

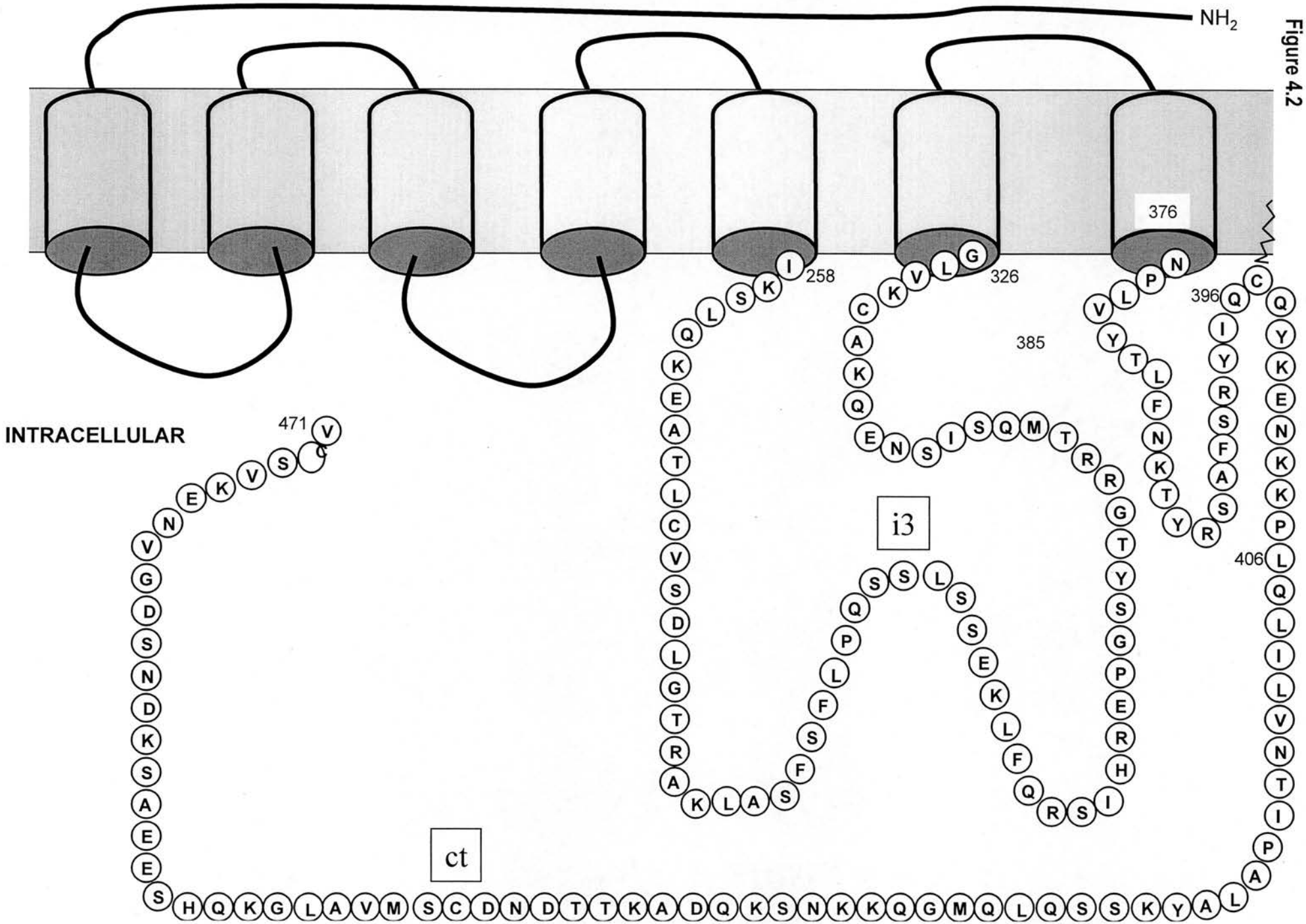


Figure 4.2

Figure 4.3

Interactions of ARF isoforms with GST fusion proteins of domains from the 5-HT_{2A} receptor.

GST-5-HT_{2A}i3, GST-5-HT_{2A}ct and (control) GST-BK channel (STREX exon) constructs were incubated with cellular extracts enriched in particular HA-tagged ARF isoforms. a) Input levels of fusion protein constructs and ARF isoforms were balanced in terms of GST immunoreactivity and HA-immunoreactivity respectively. The fusion protein-construct input levels are shown for GST-5-HT_{2A}i3 (GST-2Ai3), GST-5-HT_{2A}ct (GST-2Act) and GST-BK running at apparent molecular masses of approximately 36, 40 and 34 kDa respectively. Unconjugated GST ran at approximately 29 kDa (not shown). The ARF input levels are shown for ARF1-HA, ARF6-HA, T31N-ARF1-HA and T27N-ARF6-HA. b) and c) show association of the indicated ARF form with GST-5-HT_{2A}i3, GST-5-HT_{2A}ct and GST-BK_{STREX} constructs respectively. ARF1-HA bound selectively to the GST-5-HT_{2A}ct construct, with little binding to the GST-5-HT_{2A}i3, or GST-BK constructs. ARF6-HA showed a similar profile but bound to a much lesser extent than ARF1-HA. The T31N mutation in ARF1-HA severely reduced the ability of the protein to bind to the GST-5-HT_{2A}ct construct, but the equivalent mutation in ARF6-HA had no discernable effect. In d) GST-5-HT_{2A}i3 and GST-5-HT_{2A}ct constructs were incubated with cellular extracts enriched in the indicated HA-tagged ARF isoforms in the presence or absence of GTPγS (100 μM). GTPγS increased the binding of wild type ARF1-HA to both GST-5-HT_{2A}i3 and GST-5-HT_{2A}ct constructs, but did not alter the lack of binding seen with T31N-ARF1-HA. All blots shown are representative of at least 3 experiments.

Figure 4.3

Interactions of ARF isoforms with GST fusion proteins of domains from the 5-HT_{2A} receptor.

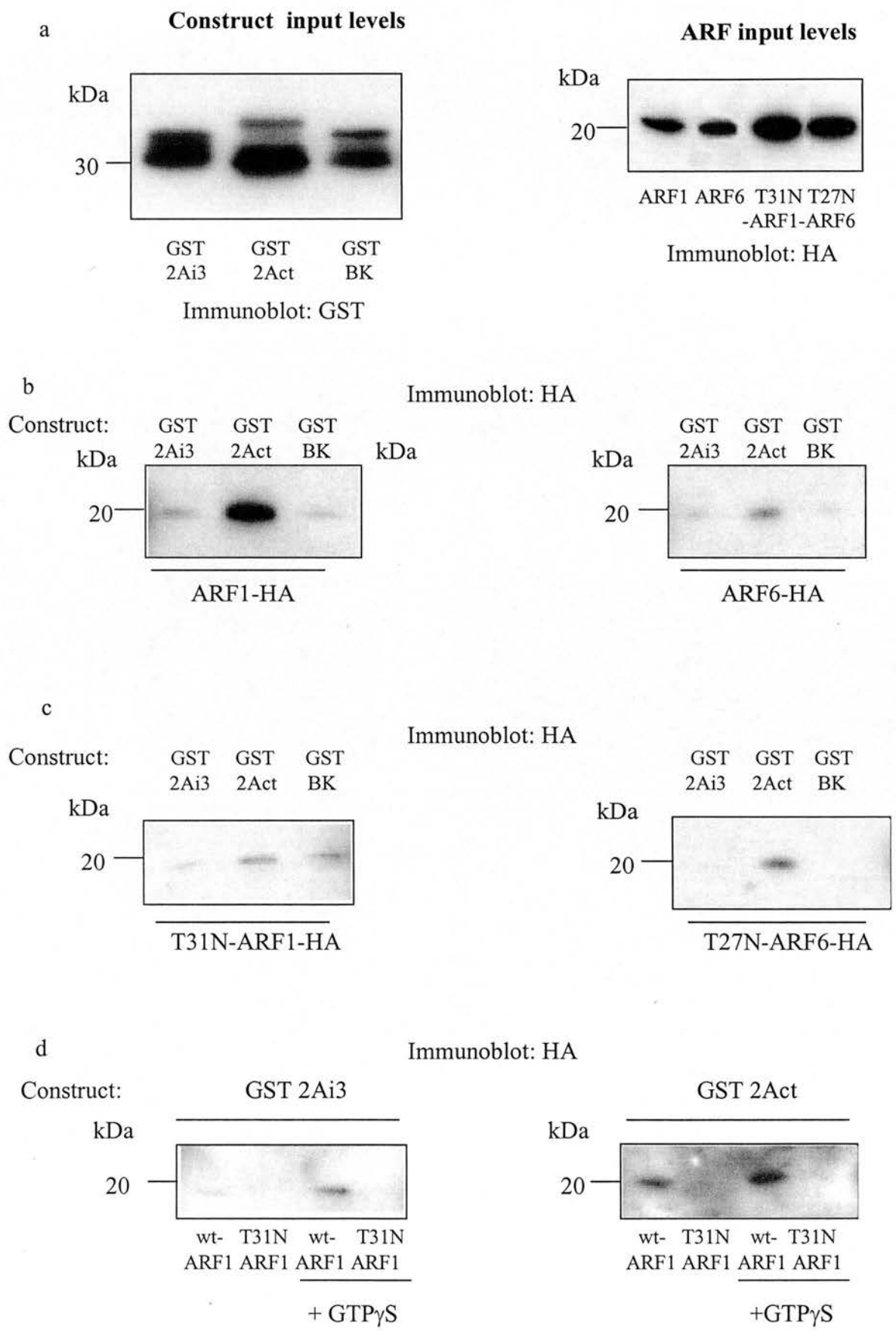


Figure 4.4

Concentration-dependence of ARF1-HA and ARF6-HA binding to the GST fusion proteins of domains from the 5-HT_{2A} receptor.

GST-5-HT_{2A}i3 and GST-5-HT_{2A}ct constructs were exposed to increasing amounts of cellular extracts containing ARF1-HA and ARF6-HA. The content of HA-immunoreactive ARF per μl of the ARF1-HA and ARF6-HA extracts was balanced to contain equivalent amounts of the ARF isoforms. Appropriate volume compensation was made with extract from non-transfected COS7 cells. a) Shows the binding of ARF1-HA and ARF6-HA to the GST-5-HT_{2A}i3 construct. ARF1-HA bound to the construct in a concentration-dependent manner, as did ARF6 but to a much lesser extent. b) Shows the binding of ARF1-HA and ARF6-HA to the GST-5-HT_{2A}ct construct. ARF1-HA bound in a concentration-dependent manner, whereas the binding of ARF6-HA was minimal and only detectable at the highest levels of ARF6-HA input.

Figure 4.4

Concentration-dependence of ARF1-HA and ARF6-HA binding to the GST fusion proteins of domains from the 5-HT_{2A} receptor

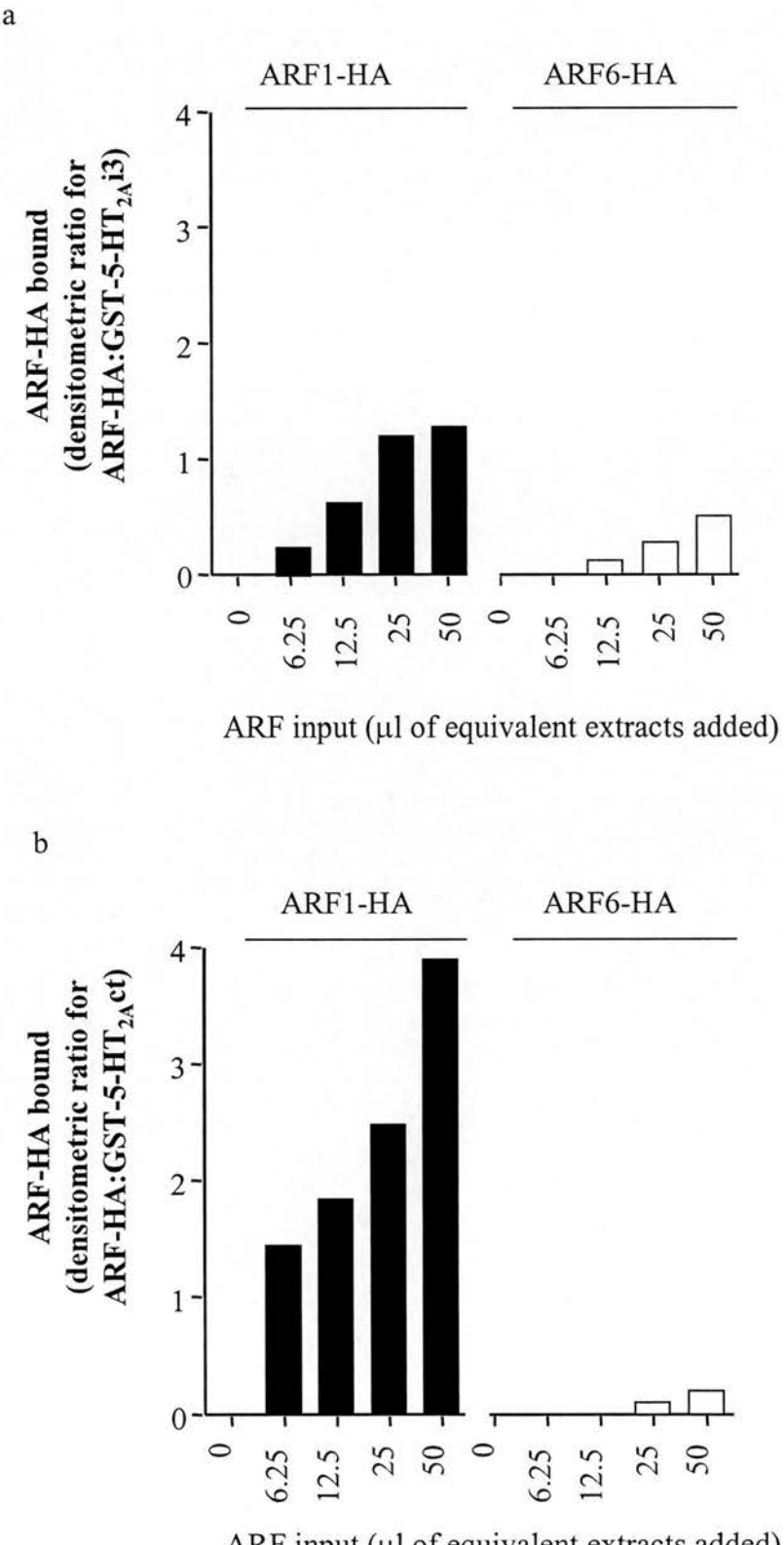


Figure 4.5

Effects of changes in the proximal part of the 5-HT_{2A} receptor ct domain on ARF1-HA binding.

Matched levels of GST-fusion proteins incorporating the (N^{376}-V^{471}) wild type 5-HT_{2A} receptor ct domain, the corresponding $N^{376}D$ mutant or a truncated K^{385}-V^{471} sequence, as well as GST alone were attached to glutathione-Sepharose beads and incubated with equivalent levels of ARF1-HA. Immunoreactivity for bound ARF1-HA was quantified by densitometry and the ratio to the level of input for each GST-fusion protein construct was calculated. These ratios were then normalised to that found for the wild type ct construct. Values are means ± SEM, n=3 (for 2Act 385-471) -5 (for all other constructs). A typical example of ECL film images for HA-immunoreactivity bound to these constructs, and GST-immunoreactivity is shown below. The mutation of the N^{376}-D at the membrane end of the 5-HT_{2A}ct construct leads to a 50% reduction in the construct's ability to bind ARF1-HA *in vitro*. The removal of the 8 amino acids at this end of the 5-HT_{2A}ct construct further reduces the construct's ability significantly to bind ARF1-HA, compared to the 5-$HT_{2A}ct_{376-471}$ construct (*$p < 0.01$, Wilcoxon test).

Figure 4.5

Effects of changes in the proximal part of the 5-HT_{2A} receptor ct domain on ARF1-HA binding

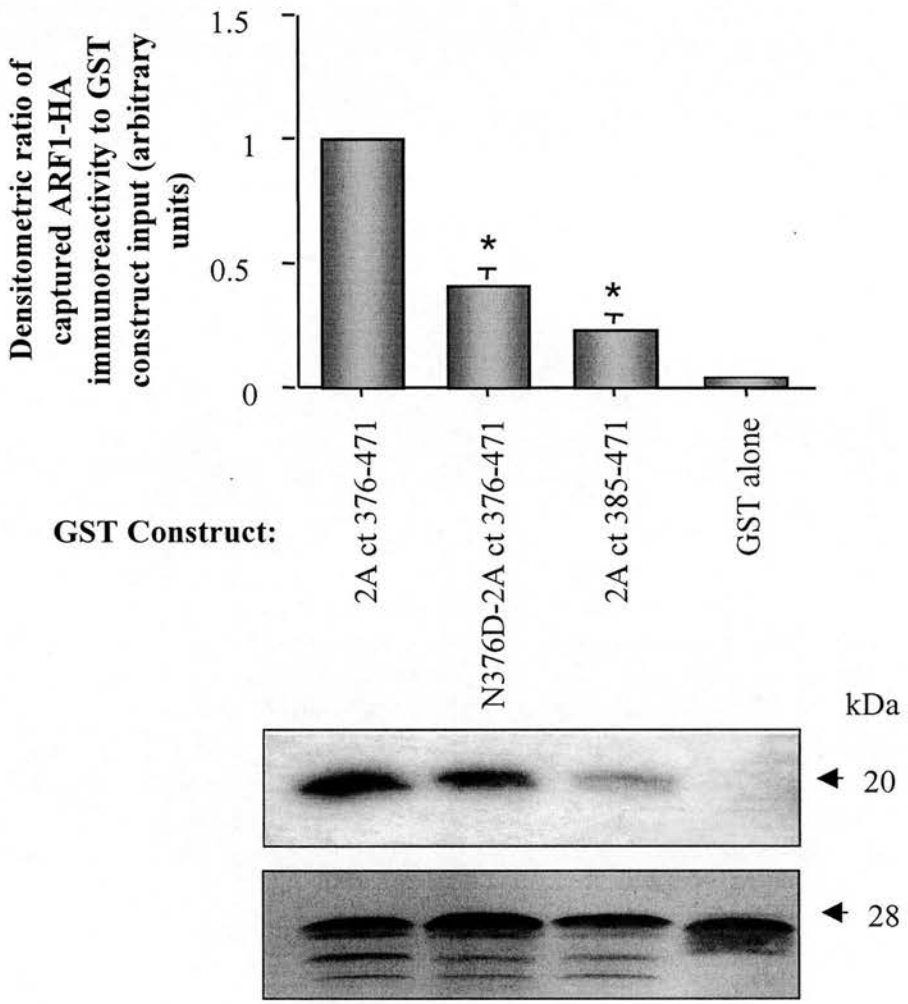


Figure 4.6

Effects of changes in the distal part of the 5-HT_{2A} receptor ct domain on ARF1-HA binding.

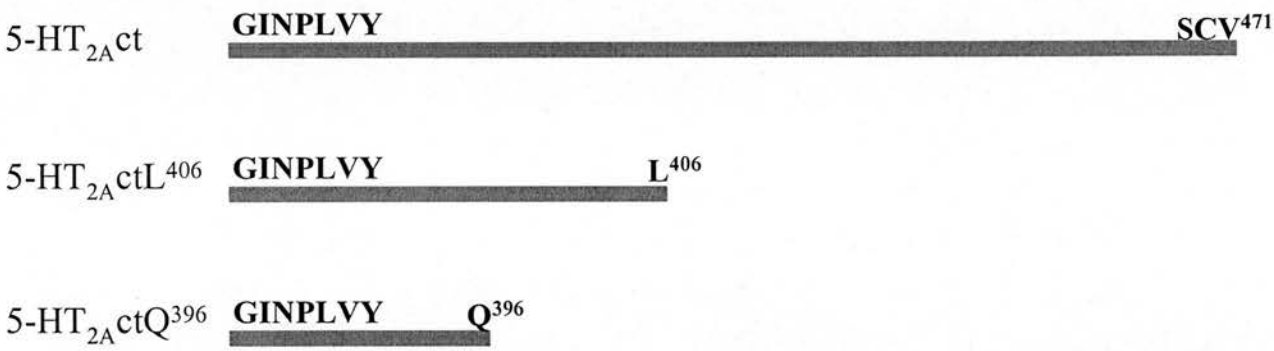
a) Shows a map of the GST constructs that were made of the 5-HT_{2A} carboxy-terminal tail to investigate deletions of various parts of the tail: $GIN^{376}PLVY\text{-}V^{471}$ (Full length tail), $GIN^{376}PLVY\text{-}L^{406}$ and $GIN^{376}PLVY\text{-}Q^{396}$ (i4 loop).

b) Shows matched levels of GST constructs of the 5-HT_{2A}ct domain ($GIN^{376}PLVY\text{-}V^{471}$; $GIN^{376}PLVY\text{-}L^{406}$; $GIN^{376}PLVY\text{-}Q^{396}$) and GST alone when attached to glutathione-Sepharose beads, and incubated with ARF1-HA-enriched COS7 cell cytosolic extract. Immunoreactivity for bound ARF1-HA was quantified by densitometry and the ratio to the level of input for each GST-fusion protein construct was calculated. These ratios were normalised to that found for the 5-HT_{2A}ct construct. Values are means ± SEM, n=4-6.

Figure 4.6

Effects of changes in the distal part of the 5-HT_{2A} receptor ct domain on ARF1-HA binding

a **Distally truncated 5-HT_{2A}ct GST constructs**



b **Mean binding of ARF1-HA to distally truncated 5-HT_{2A}ct domain GST constructs**

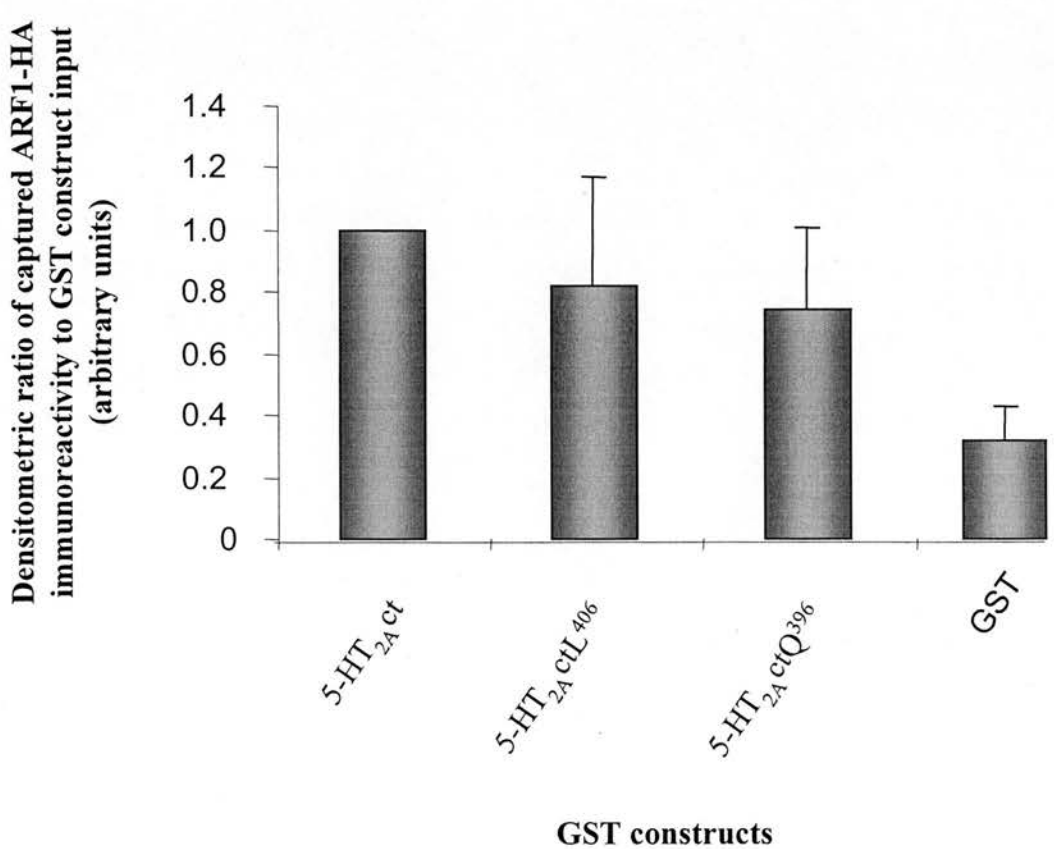


Figure 4.7

Comparison of ARF1-HA binding to various GST fusion protein constructs of 5-HT_{2A} and 5-HT_{2C} receptor domains.

Matched levels of the constructs: GST-5-$HT_{2A}ct_{376\text{-}471}$ (N^{376}2Act); GST-5-$HT_{2A}ctN^{376}D_{376\text{-}471}$ (D^{376}2Act); GST-5-$HT_{2A}ct_{385\text{-}471}$ (K^{385}2Act); GST-5-$HT_{2A}i3_{258\text{-}326}$ (2ai3); GST-5-$HT_{2C}ct_{364\text{-}458}$ (2Cct); and GST alone were attached to glutathione-Sepharose beads and incubated with equivalent levels of ARF1-HA. Immunoreactivity for bound ARF1-HA was quantified by densitometry and the ratio to the level of input for each GST-fusion protein construct was calculated. These ratios were then normalised to that found for the wild type 5-HT_{2A} ct construct. Values are means ± SEM, n=3-8. The 5-HT_{2C}ct construct seemed to display a greater degree of binding to ARF1-HA *in vitro* than the 5-HT_{2A}ct construct, however it cannot be said to be significant, due to a low n number. The binding of ARF1-HA to the 5-HT_{2A}ct construct was significantly higher than that to GST alone (*$p < 0.01$, Wilcoxon test).

Figure 4.7

Comparison of ARF1-HA binding to various GST fusion protein constructs of 5-HT_{2A} and 5-HT_{2C} receptor domains

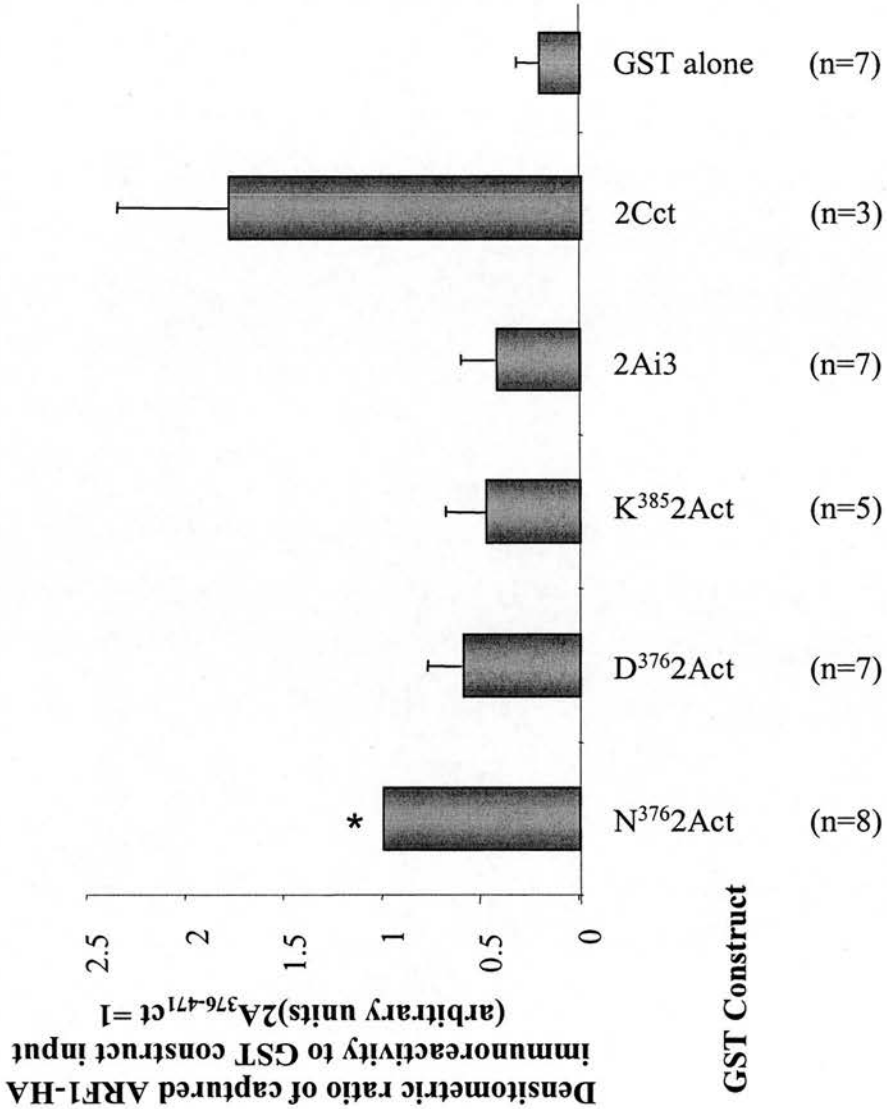


Figure 4.8

Comparison of ARF6-HA binding to various GST fusion protein constructs of 5-HT_{2A} and 5-HT_{2C} receptor domains.

Matched levels of the constructs: GST-5-$HT_{2A}ct_{376\text{-}471}$ (N^{376}2Act); GST-5-$HT_{2A}ctN^{376}D_{376\text{-}471}$ (D^{376}2Act); GST-5-$HT_{2A}ct_{385\text{-}471}$ (K^{385}2Act); GST-5-$HT_{2A}i3_{258\text{-}326}$ (2ai3); GST-5-$HT_{2C}ct_{364\text{-}458}$ (2Cct); and GST alone were attached to glutathione-Sepharose beads and incubated with equivalent levels of ARF6-HA. Immunoreactivity for bound ARF6-HA was quantified by densitometry and the ratio to the level of input for each GST-fusion protein construct was calculated. These ratios were then normalised to that found for the wild type 5-HT_{2A}ct construct. Values are means ± SEM, n=4-5. ARF6-HA showed no significant increase in binding to the 5-$HT_{2A}i3_{258\text{-}326}$ or 5-$HT_{2c}ct_{376\text{-}471}$ constructs, compared to the 5-HT_{2A}ct construct, and this itself was not discernibly different from the binding of ARF6-HA to GST alone. The deletion of the first 9 amino acids (N^{376}-N^{384}) from the 5-HT_{2A}ct construct had no apparent effect on the ability of the ARF6-HA to bind the construct which was minimal anyway. However, the mutation of N^{376}-D had the effect of increasing the ability of ARF6-HA to bind to the construct to over twice the binding of ARF6-HA to the N^{376} construct (2.343 ± 0.82 fold), and this level of binding was significantly greater than the binding of ARF6-HA to GST alone (*$p < 0.05$, Wilcoxon test).

Figure 4.8

Comparison of ARF6-HA binding to various GST fusion protein constructs of 5-HT_{2A} and 5-HT_{2C} receptor domains

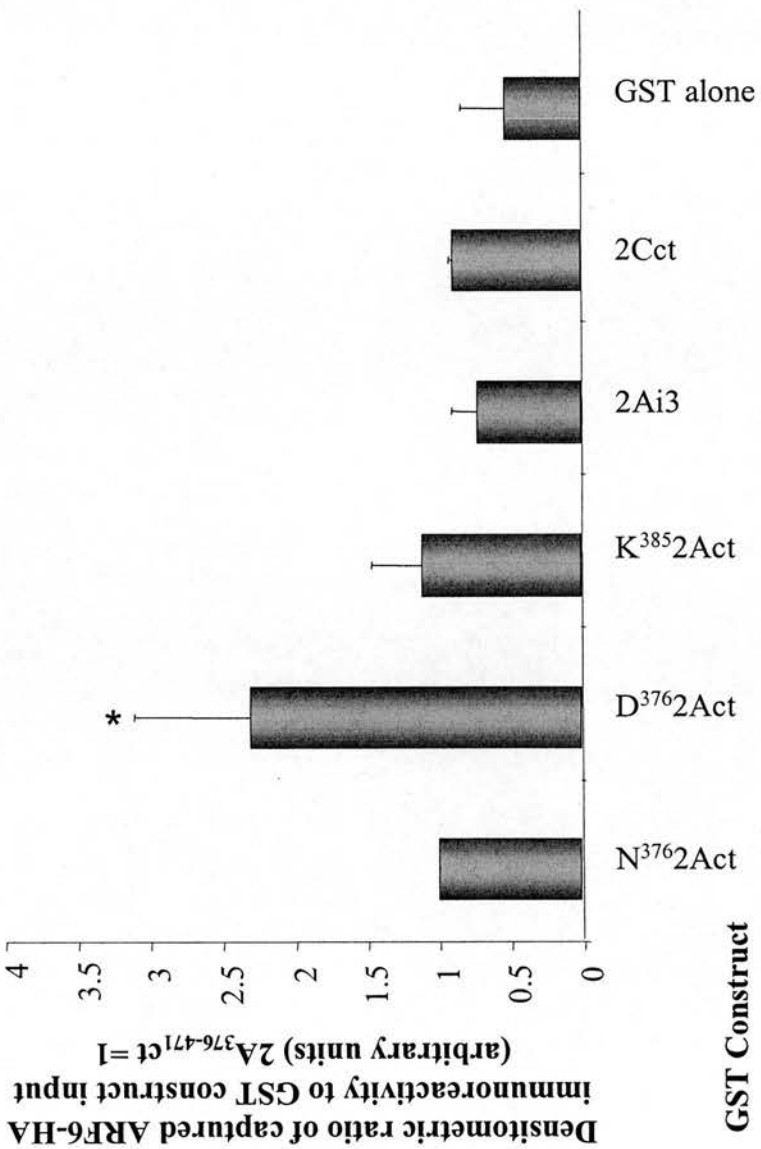


Figure 4.9

Binding of PLD1-HA and PLD2-HA to GST fusion protein constructs of 5-HT_{2A} and 5-HT_{2C} receptor domains.

Matched levels of the constructs: GST-5-$HT_{2A}ct_{376\text{-}471}$ (N^{376}2Act); GST-5-$HT_{2A}ctN^{376}D_{376\text{-}471}$ (D^{376}2Act); GST-5-$HT_{2A}ct_{385\text{-}471}$ (K^{385}2Act); GST-5-$HT_{2A}i3_{258\text{-}326}$ (2ai3); GST-5-$HT_{2C}ct_{364\text{-}458}$ (2Cct); and GST alone, were attached to glutathione Sepharose beads, and exposed to equal amounts of PLD1-HA-containing COS7 cell extract as previously described. As seen in the upper panel, PLD1-HA bound to all constructs, to a greater extent than to GST alone, however the binding of the PLD1-HA to the $N^{376}D$ mutant version of the 5-HT_{2A}ct construct was greater than that of the other constructs (Fig. 4.9 upper panel). The binding of PLD2-HA to the same constructs is also shown (Fig 4.9 lower panel). As can be seen here, the binding to the 5-HT_{2A}i3 construct was no greater than that to the GST alone, however, PLD2-HA showed detectable binding to both N^{376} and D^{376} forms of the 5-HT_{2A}ct, as well as to the 5-HT_{2C}ct. GST construct input levels were balanced as much as possible, although small fluctuations in construct levels account for the differences between band densities in the example blot and the mean densitometric ratios in the subsequent figures.

Figure 4.9

Binding of PLD1-HA and PLD2-HA to GST fusion protein constructs of 5-HT_{2A} and 5-HT_{2C} receptor domains

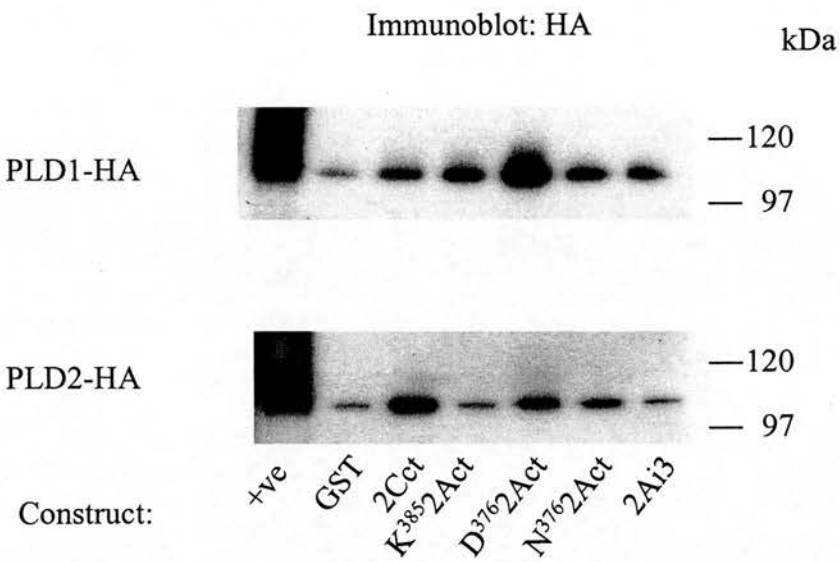


Figure 4.10

Quantification of PLD1-HA binding to GST fusion protein constructs of constructs 5-HT_{2A} and 5-HT_{2C} receptor domain.

Matched levels of the constructs: GST-5-$HT_{2A}ct_{376\text{-}471}$ (N^{376}2Act); GST-5-$HT_{2A}ctN^{376}D_{376\text{-}471}$ (D^{376}2Act); GST-5-$HT_{2A}ct_{385\text{-}471}$ (K^{385}2Act); GST-5-$HT_{2A}i3_{258\text{-}326}$ (2ai3); GST-5-$HT_{2C}ct_{364\text{-}458}$ (2Cct); and GST alone were attached to glutathione-Sepharose beads and incubated with equivalent levels of PLD1-HA. Immunoreactivity for bound PLD1-HA was quantified by densitometry and the ratio to the level of input for each GST-fusion protein construct was calculated. These ratios were then normalised to that found for the wild type 5-HT_{2A} receptor ct construct. Values are means ± SEM, n=4-5. A modest but significantly greater level of PLD1-HA binding to the 5-$HT_{2A}N^{376}$ct construct was observed compared to GST alone (*$p<0.05$ Wilcoxon test), however mutation of the N^{376}-D, and the deletion of the 9 amino acids N^{376}-N^{384} allowed for a significant increase in the binding of PLD1-HA to the 5-$HT_{2A}D^{376}$ct constructs compared to the binding of PLD1-HA to the N^{376}2Act alone (+$p<0.05$ Wilcoxon test). PLD1-HA bound significantly better to the 5-HT_{2C}ct when compared to the binding of PLD1-HA to GST alone, or to the 5-$HT_{2A}N^{376}$ct (* or + respectively $p<0.05$ Wilcoxon test).

Figure 4.10

Quantification of PLD1-HA binding to GST fusion protein constructs of constructs 5-HT_{2A} and 5-HT_{2C} receptor domain

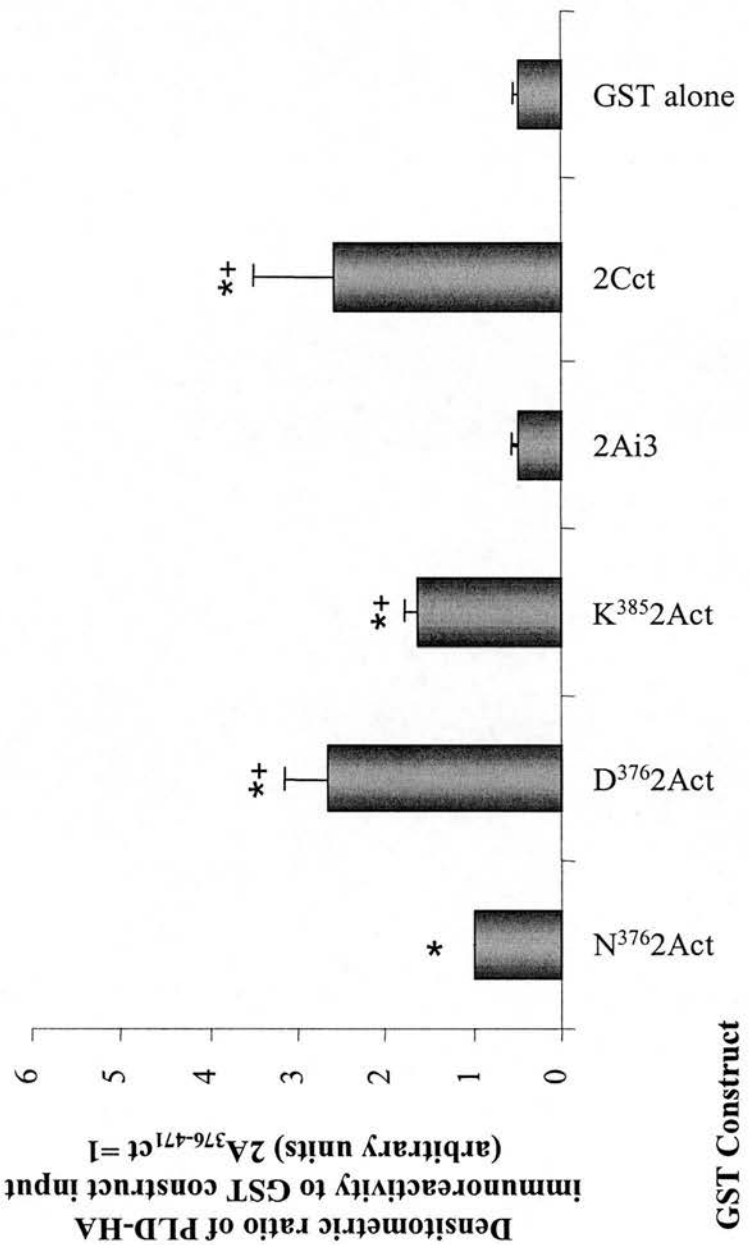


Figure 4.11

Quantification of PLD2-HA binding to GST fusion protein constructs of constructs 5-HT_{2A} and 5-HT_{2C} receptor domain.

Matched levels of the constructs: GST-5-$HT_{2A}ct_{376\text{-}471}$ (N^{376}2Act); GST-5-$HT_{2A}ctN^{376}D_{376\text{-}471}$ (D^{376}2Act); GST-5-$HT_{2A}ct_{385\text{-}471}$ (K^{385}2Act); GST-5-$HT_{2A}i3_{258\text{-}326}$ (2ai3); GST-5-$HT_{2C}ct_{364\text{-}458}$ (2Cct); and GST alone were attached to glutathione-Sepharose beads and incubated with equivalent levels of PLD2-HA. Immunoreactivity for bound PLD1-HA was quantified by densitometry and the ratio to the level of input for each GST-fusion protein construct was calculated. These ratios were then normalised to that found for the wild type 5-HT_{2A} receptor ct construct. Values are means ± SEM, n=3-4. There was binding of PLD2-HA to the 5-$HT_{2A}N^{376}$ct construct, which seemed greater than that to GST alone, however, the overall level of binding of PLD2-HA to the constructs seemed to be less than that of PLD1-HA and the apparent binding of PLD2-HA to the 5-$HT_{2A}N^{376}$ct construct was not statistically significant compared to GST alone. Binding of PLD2-HA to the 5-HT_{2A}ct was not discernibly affected by either mutation of N^{376}-D, or by deletion of amino acids N^{376}-N^{384}. PLD2-HA bound to a significantly greater extent to the 5-HT_{2C}ct construct than to either GST alone or the 5-$HT_{2A}N^{376}$ receptor constructs (* or + respectively $p<0.05$ Wilcoxon test).

Figure 4.11

Quantification of PLD2-HA binding to GST fusion protein constructs of constructs 5-HT_{2A} and 5-HT_{2C} receptor domain

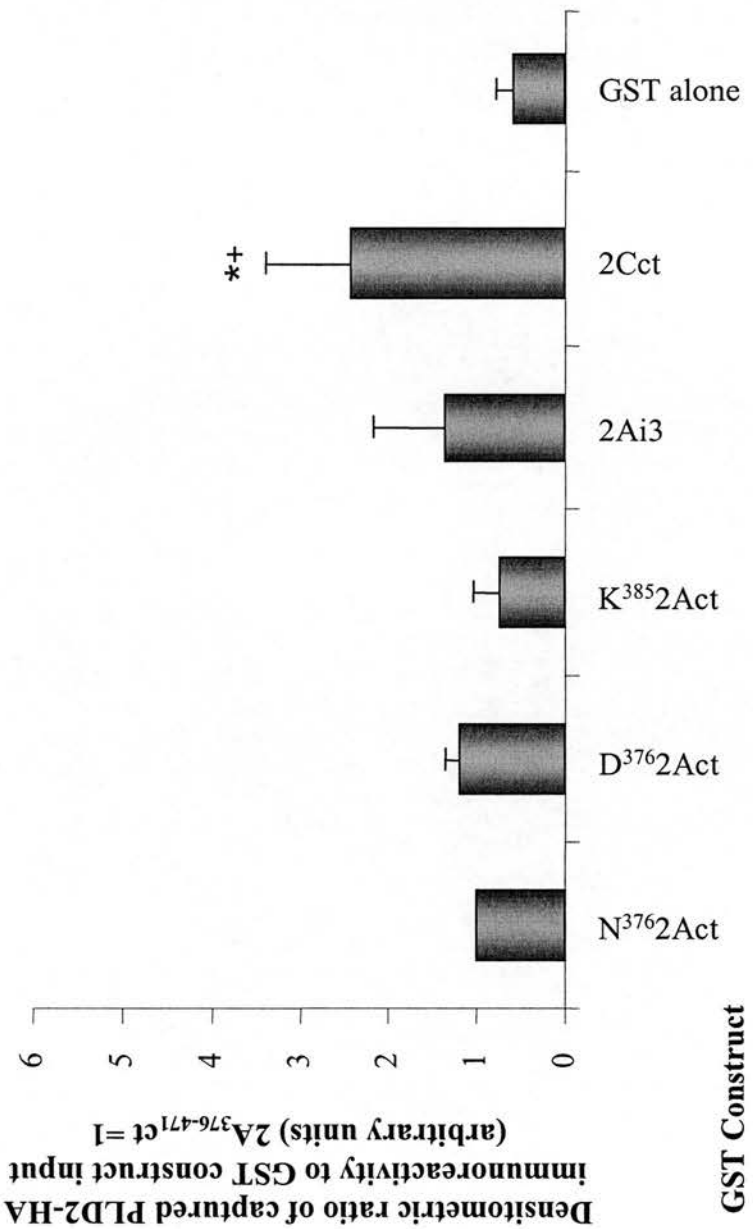


Figure 4.12

Effects of co-transfection of the Gβγ sequestering agent GRKct ($GRK2_{495-659}$) on PLD and PLC signalling by the muscarinic M_3 and 5-HT_{2A} receptors.

The Gβγ subunit-sequestering agent GRK2ct, or control vector pcDNA3 were co-transfected into COS7 cells with either the sPrC-5-HT_{2A} receptor or the sFM_3 receptor. The signalling via PLC and PLD of both receptors was then measured after stimulation by the appropriate agonist (1μM 5-HT, or 200μM CCh). Responses in the presence of the GRK2ct construct were compared with control responses. Values are means ± SEM (n=6). Co-transfection of the GRKct construct significantly reduced the PLD response of the M_3 receptor to 200μM carbachol, but not that of the 5-HT_{2A} receptor to 1μM 5-HT ($p<0.05$, Wilcoxon test). PLC responses of the receptor were unaffected by GRK2ct.

Figure 4.12

Effects of co-transfection of of the Gβγ-sequestering agent GRKct ($GRK2_{495-689}$) on PLD and PLC signalling by the muscarinic M_3 and 5-HT_{2A} receptors.

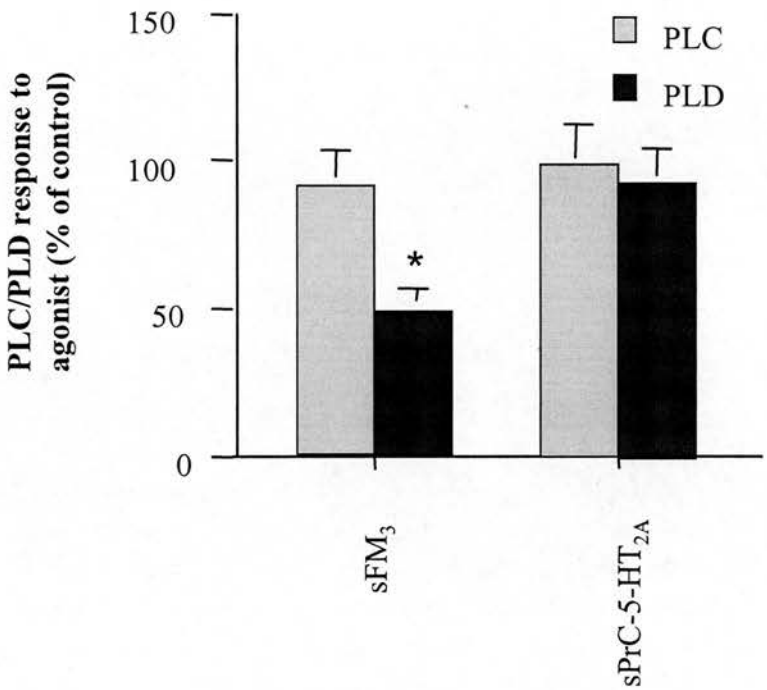


Figure 4.13

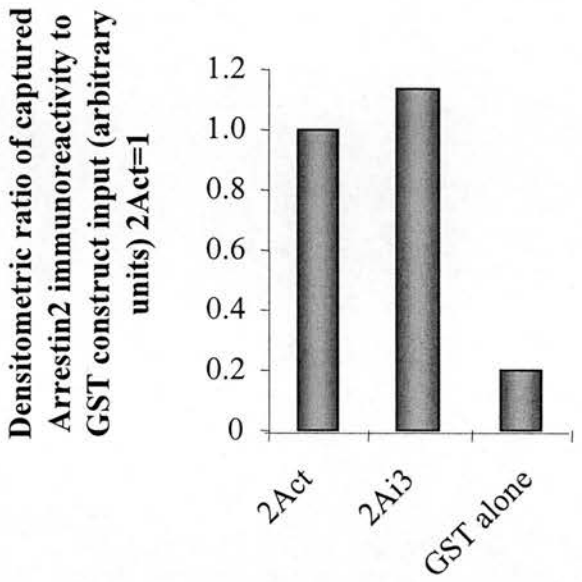
Quantification of arrestin 2 and arrestin 3 binding to GST fusion protein constructs of 5-HT_{2A} and 5-HT_{2C} receptor domains.

Matched levels of the constructs: GST-5-$HT_{2A}ct_{376-471}$ (2act); GST-5- $HT_{2A}i3_{258-326}$; (2Ai3); and GST alone were attached to glutathione-Sepharose beads and incubated with equivalent levels of arrestins. Immunoreactivity for bound arrestin was quantified by densitometry and the ratio to the level of input for each GST-fusion protein construct was calculated. These ratios were then normalised to that found for the 5-HT_{2A}ct construct. Arrestin 2 bound strongly to both the 5-HT_{2A}ct and the 5-HT_{2A}i3 constructs but not to GST alone as shown in figure 4.13a. Values are means ± SEM for n=2 experiments. Arrestin 3 binding displayed a very similar pattern, with strong binding to both the 5-HT_{2A}ct and the 5-HT_{2A}i3 constructs as can be seen in figure 4.13b. Values are means, showing individual data for n=2 experiments.

Figure 4.13

a

Quantification of arrestin 2 binding to GST fusion protein constructs of 5-HT_{2A} and 5-HT_{2C} receptor domains



b

Quantification of arrestin 3 binding to GST fusion protein constructs of 5-HT_{2A} and 5-HT_{2C} receptor domains

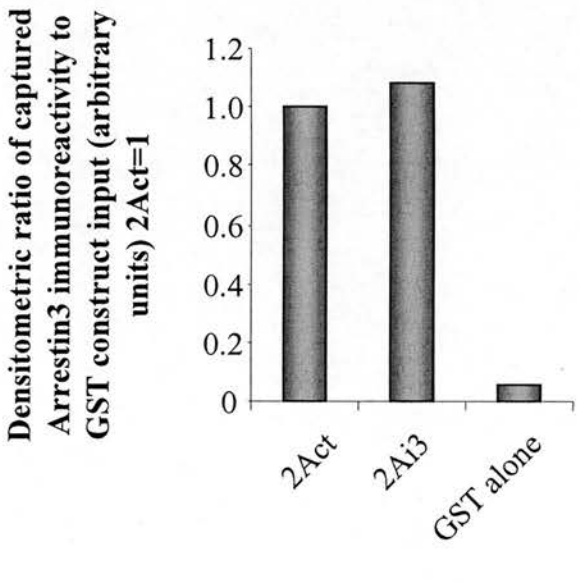


Figure 4.14

Investigation of interactions between ARF1-HA and arrestin 2 in association with the 5-HT_{2A} receptor domain GST-fusion proteins.

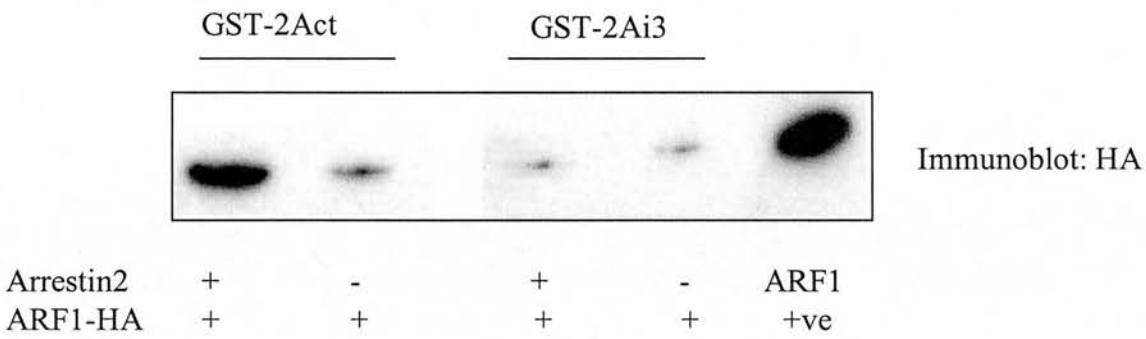
a) The constructs GST-5-$HT_{2A}ct_{376-471}$ (2Act) and GST-5-$HT_{2A}i3_{258-326}$ (2Ai3) were incubated with ARF1-HA in cytosolic extracts and additionally further extracts, which were enriched with arrestin 2, or non-enriched cytosolic extract. The addition of the arrestin 2 increased the ability of ARF1-HA to bind to the 5-HT_{2A}ct construct, but had no effect on the binding of ARF1-HA to the 5-HT_{2A}i3 construct. b) The construct GST-5-$HT_{2A}ct_{376-471}$ (2Act) was attached to glutathione-Sepharose beads and incubated with arrestin 2-containing cytosolic extract. Additional samples of extract containing ARF1-HA (or control) were added to samples. Binding of the arrestin2 to the construct was not detectably affected by the addition of ARF1-HA. The blots show results from a typical experiment repeated several times.

Figure 4.14

Investigation of interactions between ARF1-HA and arrestin 2 in association with the 5-HT_{2A} receptor domain GST-fusion proteins

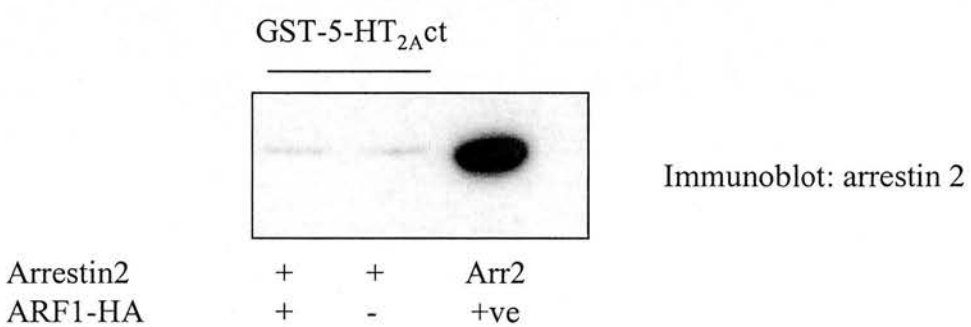
a

Effects of arrestin 2 on binding of ARF1-HA to 5-HT_{2A} receptor GST-fusion protein constructs



b

Lack of effect of ARF1-HA on the binding of arrestin 2 to the 5-HT_{2A}ct-GST fusion protein



Chapter 5:

Interactions of a novel protein partner with domains of the 5-HT_{2A} receptor

Introduction

Various GPCRs have been shown to have a considerable number of binding partners, (not just the heterotrimeric G-proteins) and the bulk of GPCR interacting proteins (GIPs), of which more than 40 have been found to date, often interact with the carboxyl-terminal domain of the GPCRs (Bockaert *et al.*, 2003). The exact areas or motifs on the carboxyl-terminal domain to which the GIPs bind are known to vary considerably. A minority of GPCR C-termini have a consensus PDZ target motif. The last 3 or 4 amino acids at the carboxyl end of the tail constitute the minimal sequence required to PDZ domain proteins to associate, with the 3-4 amino acids upstream of this determining the exact nature of the proteins interacting (Sheng and Sala, 2001). Binding partners to the carboxyl terminal tail of the 5-HT_2 receptors have been recently investigated. The multi-PDZ domain protein MUPP1 was shown to bind to the 5-HT_{2A}, 5-HT_{2B} and 5-HT_{2C} receptors and, in the case of the 5-HT_{2C} receptor, this has been proved to be via the PDZ target motif at the extreme carboxyl terminus of the receptor (Becamel *et al.*, 2001). Both the 5-HT_{2A} and 5-HT_{2B} receptors share this PDZ target domain. The homologous, but not identical, PDZ target domain of the 5-HT_{2C} receptor has also been shown to bind to PSD-95 (Becamel *et al.*, 2002; Becamel *et al.*, 2004) (we have independently confirmed the interaction of PSD-95 with the 5-HT_{2A} receptor, unpublished findings).

The N/DPxxY motif at the proximal end of the carboxy-terminal tail of many family R GPCRs may allow for selection of ARF1/6 dependent signalling pathways, (although not necessarily through direct binding of ARF to these residues). The

adjacent region of the tail upstream of a palmitoylated cysteine membrane anchor (which has been described as a putative fourth intracellular loop) is thought to contribute additionally to ARF docking (Robertson *et al.*, 2003). Alpha-helical structures within the fourth intracellular loop of some GPCRs have also been shown to bind to various other partners including periplakin and actin binding proteins (Feng *et al.*, 2003), and the binding site for JAK2 to the AT_1 receptor has also been shown to be a YIPP motif in a putative eighth helical domain (amino acids 319-322) within the receptor carboxyl terminal tail (although the AT_1 receptor does not contain the consensus Palmitoylation site that would constrain this region into a fourth intracellular loop) (Ali *et al.*, 1997).

JAK2 has also been shown to associate with the 5-HT_{2A} receptor, via co-immunoprecipitation with the receptor and also associates with its downstream target STAT3 (Signal Transducers and Activators of Transcription). The exact region of JAK2 binding within the 5-HT_{2A} receptor is unknown, (with no obvious YIPP-like motif being present) although the carboxyl tail is a good candidate (GuilletDeniau *et al.*, 1997).

Furthermore, through proteomic analysis, a range of proteins have been found to bind to the 5-HT_{2C} receptor carboxy-terminal tail, through a variety of motifs. These include Veli-3, Dlgh3 (MPP3), calmodulin, and dynamin I (Becamel *et al.*, 2002). A ternary complex that involves Veli-3, CASK and Mint1 that has been found to bind to the 5-HT_{2C} receptor, however the 5-HT_{2A} receptor does not associate with a similar complex (Becamel *et al.*, 2004), so some marked differences in protein:protein interactions exist, even between these two highly related receptors.

The technique of Matrix Assisted Laser Desorption/ Ionisation Time of Flight Mass Spectrometry (MALDI-TOF MS) is now a frequently used approach for the identification of candidate protein:protein interactions. Any captured proteins are digested with specific proteases, to produce accurate peptide molecular weight 'fingerprints' of proteins. These can then be screened against a database of analogous peptides from known proteins to gain a variety of end results, such as detecting mutations and polymorphisms, characterising post-translational modifications, or (as in this case) the identification of unknown proteins (Pappin *et al.*, 1993).

It was decided use this approach to search for novel protein binding partners for both the third intracellular loop and the carboxy-terminal tail of the 5-HT_{2A} receptor, since these domains are implicated in the signalling and cellular deployment of the receptor.

Results

Identification of a novel binding partner for the 5-HT_{2A} receptor carboxy terminal tail: S100B.

GST-fusion proteins of the 5-HT_{2A} receptor i3 and ct domains and GST alone were incubated with rat brain soluble extract. Captured proteins were separated by SDS-PAGE and bands of interest (Fig 5.1) were excised for analysis by MALDI-TOF MS. Of the protein bands tested (since they were apparent in the SDS-PAGE lanes from the 5-HT_{2A} receptor constructs, but not GST alone), bands number 2, 3, 4 and 6 had high probability matches for GST, suggesting that they are in fact breakdown

products of the GST constructs (Table 5.1). Band number 1 was identified with high probability as an *E. coli* protein, Elongation factor Tu (EF-Tu) (Table 5.1). It is likely that this is a contaminant from the original expression of the GST-5-HT_{2A} receptor domains in the *E. coli* strain BL-21. Band 5 emerged from the database search as the most promising result. The highest match, with 5 out of 45 peptides matched, coverage of 40% and a MOWSE score of $7.986e^{+04}$, was a protein named S100B (table 5.1). The database search engine matched the protein as the human version of S100B. As the original cytosol was from rat brain, a search was done on the homology of human and rat S100B. The amino acid composition of both proteins is shown below, with the human version of the protein as the top line, and the rat S100B sequence below:

0 10 20 30 40 50

SELEKAMVAL IDVFHQYSGR EGDKHKLKKS ELKELINNEL SHFLEEIKEQ

SELEKAMVAL IDVFHQYSGR EGDKHKLKKS ELKELINNEL SHFLEEIKEQ

60 70 80 90

EVVDKVMETL DNDGDGECDF QEFMAFVAMV TTACHEFFEH E

EVVDKVMETL DEDGDGECDF QEFMAFVSMV TTACHEFFEH E

As can be seen, there is a difference of only 1 amino acid between the two species at position 62, which would not be likely have any significant difference on their match

results and indicate that the GIP identified was indeed rat S100B (sequences from Swissprot Protein database http://us.expasy.org/sprot/)

An antibody to S100B was used to confirm if this was indeed the unknown protein isolated as band 5. Figure 5.3 indicates that not only was the unknown partner correctly identified as S100B, but that it also binds specifically to the GST- 5-HT_{2A}ct construct, and not to either the GST 5-HT_{2A}i3 or to GST alone.

S100B binds to the carboxyl-terminal tail of the 5-HT_{2A} receptor in a calcium dependent manner.

The S100 group of proteins are members of the S100/calmodulin/troponin C super family of EF-hand calcium-binding proteins. There are 2 main subgroups of S100 proteins: S100A, S100B (Donato, 1991; Donato, 1999), which can form homo- and heterodimers. S100B is found predominantly in the central nervous system. The S100 proteins contain two EF-hand calcium binding sites. S100B has been shown to bind to several different proteins, including p53 (Baudier *et al.*, 1992; Rustandi *et al.*, 1998) and glial fibrillary acidic protein (GFAP) (Bianchi *et al.*, 1994) in a calcium-dependent manner. To determine the effect of calcium on the binding of S100B to the 5-HT_{2A}ct, S100B binding was examined under control conditions, in the presence of 1mM Ca^{2+}, or with the calcium-chelating agent BAPTA, at a concentration of 2mM. It was observed that both S100B at 12kDa and a 24kDa immunoreactive species (quite possibly a dimeric form(s) of S100B) bound under basal conditions to the GST-5-HT_{2A}ct, but not the GST-5-HT_{2A}i3 construct. The addition of BAPTA to chelate any Ca^{2+} present in the brain extract markedly decreased the ability of the

S100B to bind to the GST-5-HT_{2A}ct, whereas the addition of 1mM Ca^{2+} increased the ability of both the S100B monomer and (putative) dimer to bind to the GST-5-HT_{2A}ct construct. In the presence of additional calcium, a small amount of binding of S100B was observed to the GST-5-HT_{2A}i3, but to a much lesser degree than to the GST-5-HT_{2A}ct. No additional magnesium was present in these experiments, and the level of magnesium present in the experiments was unknown.

Discussion

The S100 proteins, of which up to 19 variants have been described, belong to the superfamily of S100/calmodulin/troponin C calcium-binding proteins. A highly conserved group of calcium binding proteins, ranging in size form 10-12 kDa, the S100 family of proteins are differentially expressed in a large number of cell types. The family is organised into 2 main groups: S100A, of which there are 14 isoforms (S100A1-13, plus S100A8-like); S100B which consists of S100B alone; and another 4 proteins that do not fit into either of the previous sub groups (S100P, Profilaggrin, Trychohyalin and Repetin which are larger proteins that exhibit an S100 motif along their primary sequence) (Donato, 1999; Donato, 2003). The most common member of this family is S100B, which was first described as a major constituent of glia (Moore, 1965), but is now known to be more widely expressed in a range of tissues (Donato, 1991). S100 is present in the nervous system where it is substantially found as homodimers of the S100A or S100B isoforms and also as S100A/S100B heterodimers. $S100A1_2$, $S100B_2$ homodimers and S100A1/S100B dimers are especially common (Donato, 1999). The B isoform is most represented in the brain

(~85% of the total S100)(Donato, 1986), where it is found intracellularly in the cytoplasm of glial cells. Isoforms of the S100A group are present in the nervous system as about 5% of the total S100 pool (Isobe *et al.*, 1984), and they are also present in muscle and kidney. S100 is also found extracellularly and although the mechanism of and reasons for its export from the cell are uncertain, a role in neurotrophic and mitogenic activity has been suggested (see Fano *et al.*, 1995 for a review). There is also a direct correlation between the extracellular levels of S100 and damage to the brain tissues, as documented in the cases of Alzheimer's disease (AD) (Peskind *et al.*, 2001), dementia caused by Down's syndrome (DS) (Mrak *et al.*, 1997), progressive neurodegeneration in AIDS (Soderlund *et al.*, 2004), autopsy-diagnosed Creutzfeld-Jacob disease (CJD)(Nooijen *et al.*, 1997), and schizophrenia (Lara *et al.*, 2001). For this reason, S100B is used as a sensitive marker of brain damage.

The intracellular location of S100 proteins is mostly the cytoplasm, where these proteins are thought to have many different roles, which are likely to be calcium-dependent manner. S100B for example has been shown to bind to a range of targets, including: p53, where it protects p53 from thermal denaturation and degradation; fructose-1-6-bisphosphate aldolase and phosphoglucomutase where S100B been suggested to have a role in the regulation of energy metabolism; membrane-bound guanylate cyclase, where S100B may have a function in cell cycle regulation; microtubules, where S100B may inhibit assembly via sequestration of tubulin and stimulation of Ca^{2+} sensitivity of performed microtubules. S100B has also been shown to bind to a large number of intracellular targets, where no clear function has

yet been suggested, these include Annexin II, GAP-43 (neuromodulin) and neurogranin (see Donato, 2003 for a review).

Dimerisation seems to be important for the biological activities of the S100 proteins. The S100 monomers dimerise by means of the monomers interacting in an antiparallel fashion. Upon Ca^{2+} binding, a hinge region on each monomer swings out, forming a cleft, which is though to be important in the binding of S100 to target molecules. (Donato, 1999; McClintock *et al.*, 2002; Donato, 2003). By this mechanism, the S100 dimer functionally crosslinks two homologous or heterologous target proteins (Donato, 2003)

There is no previous evidence to suggest that S100 can bind to any of the GPCRs, although a small amount of S100 (5-7% of the total amount) has been shown to be membrane associated (Rusca and Calissano, 1970). It has been shown that S100AB can activate the membrane associated adenylate cyclase (AC) of cerebral cortex membranes, in the presence of GTP. What is more intriguing is the fact that the effect of S100 on AC was not detectable in the presence of an antibody to the pertussis toxin sensitive G-protein G_i (Fulle *et al.*, 1992), suggesting a possible role of S100 in the activation of AC by G_i or at least modulation of this process.

Another calcium- binding protein calmodulin (CaM), which belongs to the same superfamily of calcium binding proteins as S100 has recently been shown to co-immunoprecipitate with the 5-HT_{2A} receptor in an agonist-dependent manner. Furthermore, the binding sites for calmodulin were shown to be on the second intracellular loop (i2) and the carboxy-terminal tail domains. The interaction is thought to involve putative CaM binding sites at amino acid positions L^{382} and F^{392} on the ct domain, which overlaps with a putative PKC substrate site that is readily

phosphorylated by PKC *in vitro*. Since the PKC-mediated phosphorylation and CaM binding on the ct were mutually exclusive, it has been suggested that CaM binding to the 5-HT_{2A}ct has a role in regulation of 5-HT_{2A} receptor phosphorylation and desensitisation (Turner and Raymond, 2005).

Recent work suggests another possibly relevant role of S100 in that S100A10 (formerly known as p11 or calpactin I light chain) has been shown to participate in the trafficking of the membrane ion channel Na(V)1.8 and acid-sensing ion channels (ASICs) to the plasma membrane (Okuse *et al.*, 2002; Donier *et al.*, 2005).

In conclusion, through the means of protein capture by binding to GST constructs of 5-HT_{2A} receptor domains, and identification via Matrix Assisted Laser Desorption/ Ionisation Time of Flight Mass Spectrometry (MALDI-TOF MS), the novel binding partner, S100B was found to bind to the 5-HT_{2A}ct, but not the 5-HT_{2A}i3. This binding to the carboxy-terminal tail domain was found to be increased in the presence or Ca^{2+} and decreased to an undetectable level in the absence of Ca^{2+}. The possible functional significance of this interaction is not yet clear and sufficient time was not available within the project to investigate the question further. Nevertheless, potential roles in 5-HT_{2A} receptor trafficking, interactions with other proteins and modulation of signalling are all reasonable possibilities that should be further investigated.

Figures

Figure 5.1

Proteins captured from rat brain cytosol by GST-fusion proteins of 5-HT_{2A} receptor domains.

GST constructs of the 5-HT_{2A} receptor 3rd intracellular loop (i3) and carboxyl tail (ct) and GST alone were exposed to cytosolic extract of homogenised rat brain which had been pre-incubated with GST to pre-clear non-specific binding. Proteins that bound to the domain constructs were eluted from the constructs first by high salt solution (1M NaCl), and then by x2 Laemmli buffer. Eluates were then run on a 12.5% SDS-PAGE gel and transferred to PVDF membranes. Figure 5.1 shows the resulting membranes after staining with colloidal Coomassie stain. Lane 1-3 show the proteins removed from the glutathione beads by x2 Laemmli buffer, lanes 4-6 show the proteins eluted by high salt. Proteins eluted from the GST alone are shown in lanes 1 and 4. Lanes 2 and 5 show the proteins removed from the GST-5-HT_{2A}i3 construct, and those removed from the GST-5-HT_{2A}ct construct are shown in lanes 3 and 6.

Figure 5.1

Proteins captured from rat brain cytosol by GST-fusion proteins of 5-HT_{2A} receptor domains

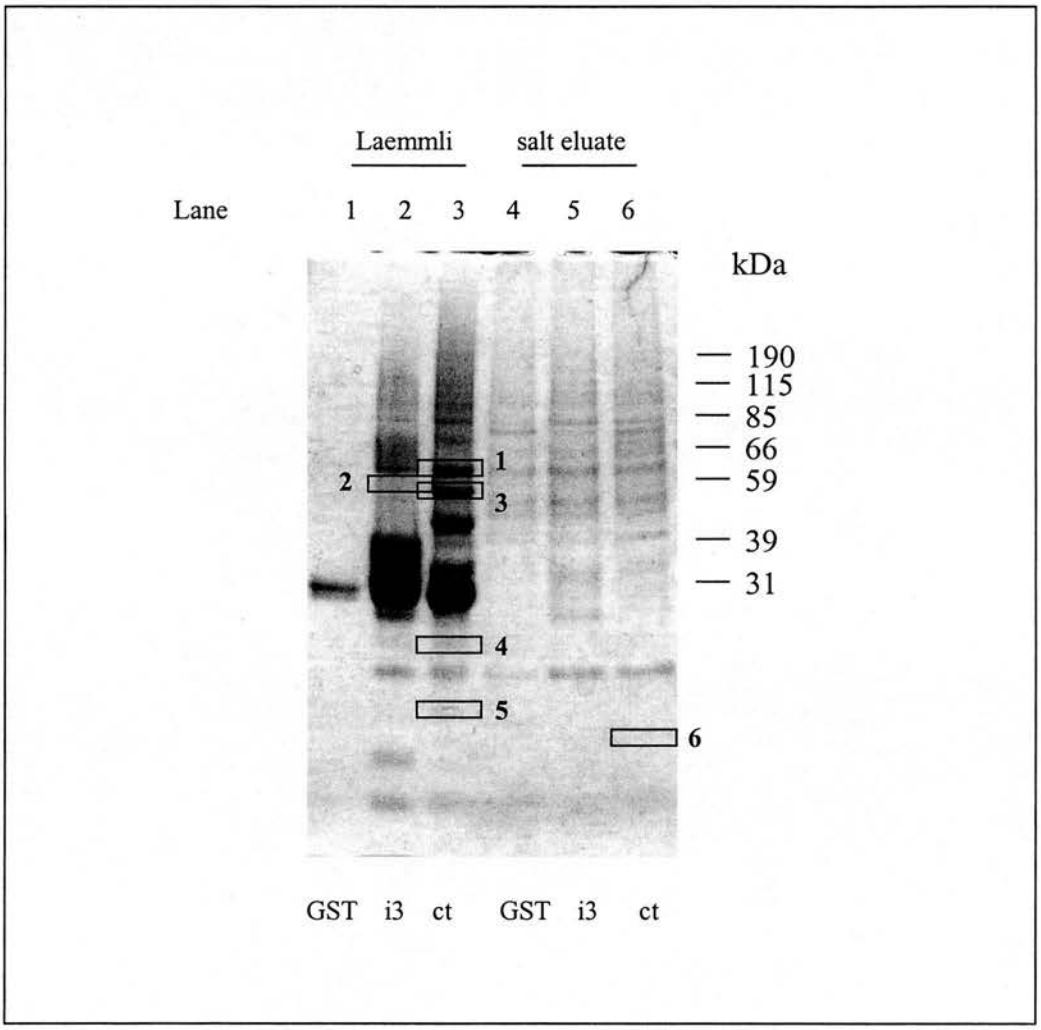


Figure 5.2

MALDI-TOF MS of tryptic digest of protein band 5 resolved on SDS-PAGE gel.

The data here were collected in positive ion and reflector mode. Experimentally determined mass/charge are labelled on the peptides. Band 5 from Fig 5.1 was matched as S100B, with 40% peptide coverage, and 5/45 peptide masses matched.

Figure 5.2

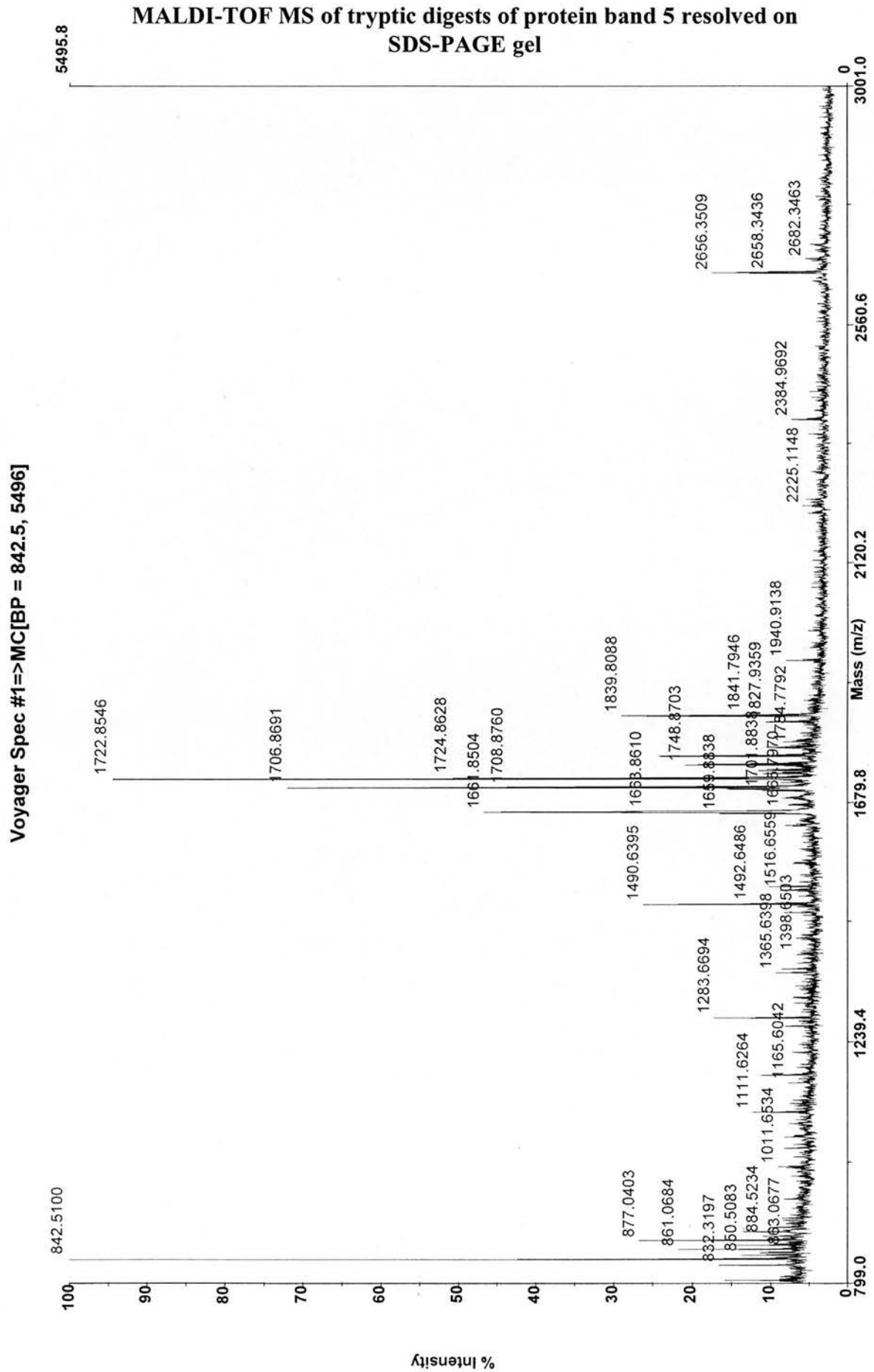

MALDI-TOF MS of tryptic digests of protein band 5 resolved on SDS-PAGE gel
Voyager Spec #1=>MC[BP = 842.5, 5496]
% Intensity
Mass (m/z)
5495.8
842.5100
1722.8546
1706.8691
1724.8628
1839.8088
1748.8703
1841.7946
1940.9138
1490.6395
1492.6486
1283.6694
2656.3509
2658.3436
2682.3463
2384.9692
2225.1148
877.0403
861.0684
832.3197
850.5083
884.5234
863.0677
1011.6534
1111.6264
1165.6042
1365.6398
1516.6559
1398.6503
100
90
80
70
60
50
40
30
20
10
0
799.0
1239.4
1679.8
2120.2
2560.6
3001.0

Figure 5.3

S100B binds specifically to the GST-5-HT_{2A}ct construct.

GST constructs of the 5-HT_{2A}i3 and 5-HT_{2A}ct domains as well as a GST alone control were exposed to rat brain cytosol, which had been pre-absorbed for any interactions to GST alone. Protein bound to the construct was solubilised by incubation in x2 Laemmli buffer, and run on a 12.5% gel, transferred to PVDF membrane, and immunoblotted with HRP-linked S100B antibody. S100B bound strongly to the 5-HT_{2A}ct construct, but not to either the GST-5-HT_{2A}i3 construct or GST alone. Figure is representative of 3 separate experiments. The lower panle shows the GST-construct levels present in this experiment, as visualised by GST-immunoreactivity.

Figure 5.3

S100B binds specifically to the GST-5-HT_{2A}ct construct

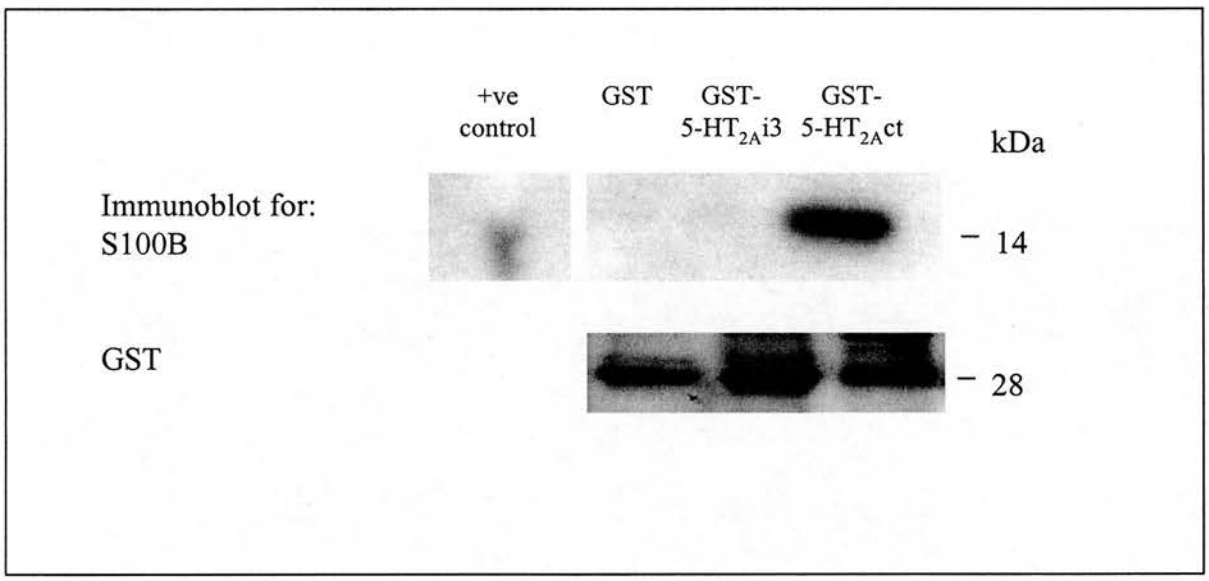


Figure 5.4

S100B binding to the 5-HT_{2A}ct construct is calcium-dependent.

The binding of S100B to the GST constructs of the 5-HT_{2A} i3 and ct domains was carried out as previously (Fig 5.3), and in the presence of 1mM Ca^{2+}, and 2mM BAPTA, a calcium chelating agent. The 3 right hand lanes of Figure 5.4 show the ability of S100B to bind to the GST-5-HT_{2A}ct construct under the various conditions, and the left hand lanes show the binding of S100B to the GST-5-HT_{2A}i3 construct. Equivalent levels of each construct were present in all lanes.

Figure 5.4

S100B binding to the GST-5-HT_{2A}ct is calcium-dependent

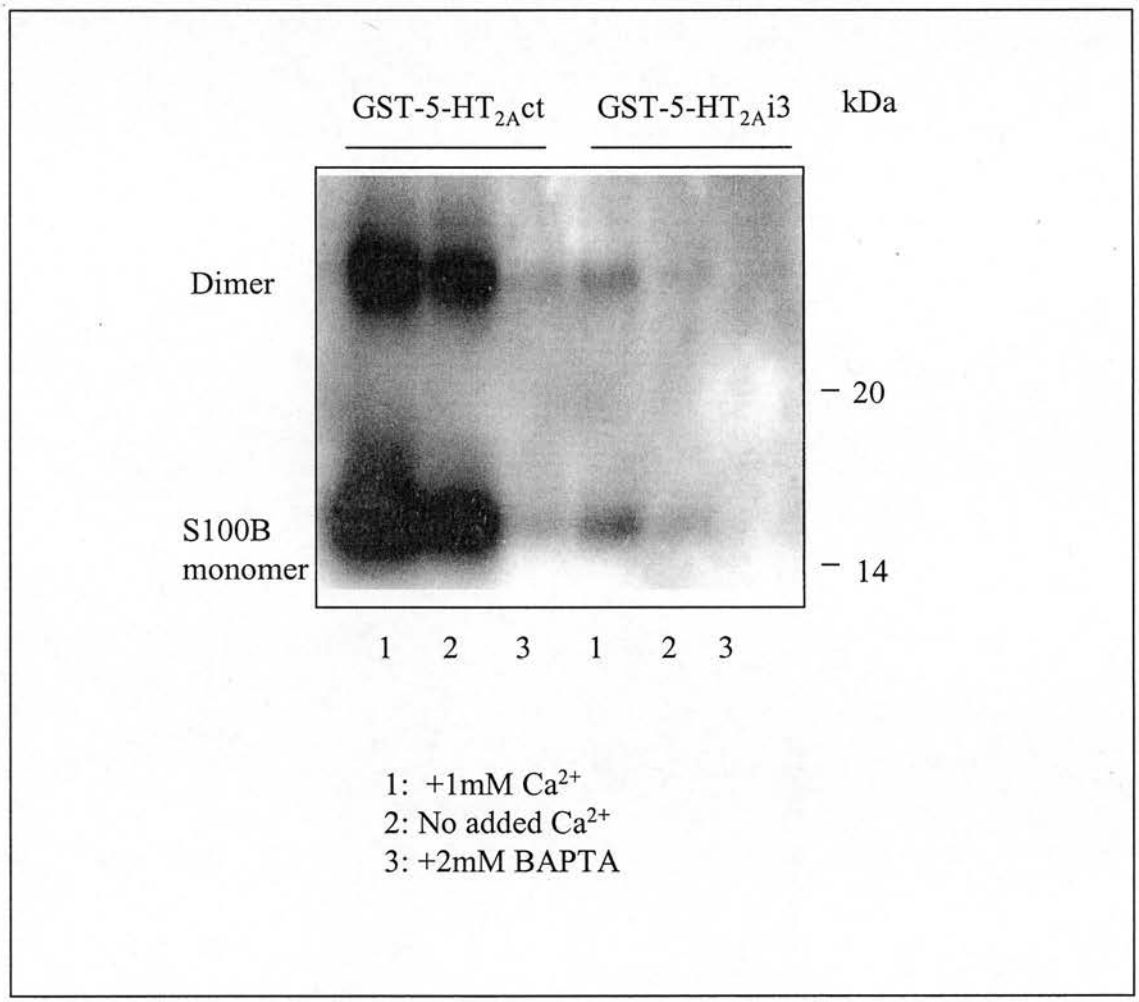


Table 5.1

Potential identities of protein bands bound to GST constructs of 5-HT_{2A} domains, as indicated by Protein Prospector software surveying the NCBI and Swissprot databases.

Results from MALDI-Time of flight (TOF) mass spectroscopy (MS) analysis are shown. The criteria used to identify a good match include the extent of sequence coverage, the number of peptides matched, the probability (MOWSE) score and the molecular mass of the protein identified. This table shows the top search results using Protein Prospector software, in the NCBI or Swissprot databases, for the protein bands extracted from the gel shown previously (Fig 5.1). Band No corresponds with the band numbers shown in Fig 5.1. From this table it can be seen that bands 2, 3, 4 and 6 are most likely fragments of the GST protein. Band number 1 seems to be the *E.coli* protein Elongation factor Tu (EF-Tu). Band number 5 seems to have the strongest correlation of all, with 5/45 peptide masses matched, 40% coverage, and a high MOWSE score of $7.986e^{+04}$ and was identified as S100B.

Table 5.1

Potential identities of protein bands bound to GST constructs of 5-HT_{2A} domains, as indicated by Protein Prospector software surveying the NCBI and Swissprot databases

Band No	MOWSE score	Masses matched	%Coverage	Protein MW (Da)	Protein name
1	1.37e+10	14/24	42	43313.8	Elongation factor Tu (EF-Tu) E.coli
1	8.37e+17	27/35	66	43182.6	Elongation Factor Tu (EF-Tu-A) E.coli
2	6.33e+9	17/43	56	25498.8	Glutathione S-Transferase
2	2.94e+7	14/25	49	25498.8	Glutathione S-Transferase
3	1.94e+5 3.36e+4	7/23 6/23	42 50	15980 15329	Heamoglobin beta chain (Rat) Heamoglobin alpha chain (Rat)
3	1.31e+5	10/43	39	25498.8	Glutathione S-Transferase
3	1.85e+3	4/83		86645.5	Serine/Threonine protein phosphatase with EF-hands-2
4	1.30e+6	8/27	51	15980	Haemoglobin beta chain (Rat)
4	3.2e+5	7/25	51	15979.5	Haemoglobin beta chain (Rat)
4	1.99e+4	7/37	13	89122.9	Homoserine Dehydrogenase (E.coli)
4	3.02e+7	11/52	40	25498.8	Glutathione S-Transferase
5	**7.986e+04**	**5/45**	**40**	**10744**	**S-100 protein, beta chain** (human)
5	1.28e+5	5/45	35	10430	30S ribosomal protein S19 (E.coli)
5	5.72e+3	6/61	13	65464.0	T-box transcription factor (mouse)
6	6.23e+7	14/82	55	25498.8	Glutathione S-Transferase
6	2.97e+5 2.36e+5 1.63e+5	10/113 13/113 12/113	17 38 42	83323.9 42268.0 41737.0	ATP dependent Helicase (mouse) Glutamine synthase (rat) Actin (human)
6	9.51e3	7/53	24	41019.2	Gamma actin (mouse)

Chapter 6:

Conclusions and Discussion

The recognition and characterisation of the various intracellular signalling pathways activated by the family R GPCRs is very important when attempting to understand the physiological mechanisms of intercellular and intracellular communication. The family R GPCRs represent the largest family of GPCRs, which is in turn the largest super-family of cell surface receptors in nature, containing receptors for a wide range of ligands. The variation available at the receptor level is further amplified by the fact that these cell surface receptors can each activate multiple intracellular pathways. Most GPCRs signal through heterotrimeric G-proteins, to activate the specific intracellular signalling pathway evoked by that G-protein (Figure 6.1 a), but there is growing evidence that GPCRs can activate multiple intracellular pathways, and are not linked to a single intracellular pathway as was once thought (Figure 6.1b shows the complex interaction of signalling proteins elucidated by this study). The new challenge is to decode how the receptors are selecting to activate the pathways they have available to them.

One method the receptors may use to gain selective access to signalling pathways is selection at the level of the ligand. There is evidence that some receptors can activate different pathways within the cell when activated by different agonists (Offermann *et al.*, 1994; Robb *et al.*, 1994; Kenakin, 1997; Berg *et al.*, 1998; Pommier *et al.*, 1999). This suggests that the receptor can exist in different active conformations, and these conformations can interact with and selectively activate different signalling pathways. One example of this is the 5-HT_{2A} receptor can select for either PLA_2 or PLC pathways in NIH3T3 cells upon activation by structurally distinct ligands (Kurrasch-Orbaugh *et al.*, 2003b).

There is also evidence that there may be some means of selecting between pathways by masking the ability of the receptor to couple to one or another pathway, for example, in the 5-HT_{2A} receptor, calmodulin has been shown to bind to the second intracellular loop, thereby dampening the ability of the receptor to bind to and activate the trimeric G-proteins (Turner and Raymond, 2005). The same sites bound by calmodulin on the 5-HT_{2A} receptor are also sites of PKC-phosphorylation, and by binding to the receptor at these sites in an agonist-dependent manner calmodulin could partially protect the receptor from PKC-mediated internalisation and desensitisation, perhaps allowing the receptor to continue signalling via an alternative second messenger system than that induced by the trimeric G-proteins (Turner and Raymond, 2005). This consensus sequence for calmodulin binding which may attenuate both trimeric G-protein association and PKC-phosphorylation has also been seen in the most of the 5-HT receptors, $M_{1\text{-}5}$ muscarinic receptors, and the $\alpha_{2A\text{-}D}$ and $\beta_{1\text{-}3}$-adrenergic receptors (Turner and Raymond, 2005). Whether this attenuation of trimeric G-protein interaction still allows for interaction of other signalling partners, for example the small G-proteins like ARF and RhoA, has still to be elucidated.

Another factor to be taken into account is the presence of various signalling partners within cell types. For a receptor to be able to activate any of the pathways mentioned previously, the components of that pathway have to be present in the cell studied, and they also have to be available for the receptor to associate with, either expressed in the cell type, or indeed within the same cellular compartment as the receptor.

A current field of enquiry in the study of GPCRs that is of considerable interest, is the discovery that GPCRs seem to need to be in the form of either homodimers, or

heterodimers to function properly (see Milligan *et al.*, 2003; Milligan, 2004 for a review). Furthermore whether this interaction is as a homodimer or a heterodimer seems to affect the signalling abilities of the receptors involved. The role of receptor dimerisation and selection of signalling pathways is an area of much present and future research. It is known that dimers appear to activate a single heterotrimeric G-protein (Fotiadis *et al.*, 2003; Liang *et al.*, 2003; Fotiadis *et al.*, 2004), and indeed this is also true for the activation of the ERK pathway by the M_3 receptor (Novi *et al.*, 2004). However, what of the other signalling pathways, for example PLA_2 and PLD, do they also become activated by dimers in the same manner, i.e. one GPCR dimer pair activates a single pathway-activating protein (PLD, PLC or PLA_2, in this case the triggering of PLD signalling)? Furthermore, can pathways activated by the small G proteins also be activated by the same GPCR dimer that is also linked to one heterotrimeric G-protein in either a concurrent or sequential manner? Thus there remain many unknowns in the field of selectivity and stoichiometry of transduction by GPCRs.

For many years the activation of PLD by GPCRs was seen as downstream of the activation of PLC, via PKC activation, however, in recent years many studies have shown that indeed PLD can be activated by a more direct route, namely via the small G-proteins ARF and RhoA. It has been shown in chapter 3 of this study, by the use of dominant negative ARF mutants, that both ARF1 and ARF6 are major contributors to the activation of PLD by the M_3 receptor in 1321N1 cells, and in transfected COS7 cells, whilst the PLC response of the M_3 receptor remained unaffected. Similar observationshave been made for the angiotensin II and ET-1

receptors in A10 cells (Shome *et al.*, 2000). It has further been shown in that there is a direct interaction between ARF1 or ARF6 and the M_3 muscarinic receptor, as both ARF1 and ARF6 can be co-immunoprecipitated with the receptor under basal conditions, with the amount of ARF1 associated with the receptor increasing after carbachol stimulation (Mitchell *et al.*, 2003). The site of this interaction was narrowed down in chapter 3 to the i3 and ct domains of the M_3 receptor, where both isoforms of ARF were seen to bind to both the M_3i3 and the M_3ct GST-fusion protein constructs, with ARF1 binding to the M_3ct domain (which contains the NPxxY motif) to a greater extent than ARF6. The NPxxY motif was shown to have a great influence on the binding of ARF1 and ARF6 to the carboxy-terminal tail domain of the 5-HT_{2A} receptor, which, unlike the M_3 receptor, seems to stimulate PLD activation via ARF1 alone and not ARF6. One interesting point is that the N^{376} to D mutant form of the 5-HT_{2A} receptor, as well as the P_{2U} purinergic receptor (the native sequence of which contains a DPxxY motif) do not activate PLD via ARF1, but instead appear to activate PLD via ARF6 and PKC (Mitchell *et al.*, 2003). This backs up the results shown in chapter 4, where ARF1 binding to the 5-HT_{2A}ct domain is decreased in the presence of the N^{376}D mutation, whereas the binding of ARF6 is increased over that of ARF1.

It seems that the NPxxY motif is not the whole story though, since although the M_3 and 5-HT_{2A} receptors both contain the NPxxY motif, they seem to bind the ARF isoforms via different mechanisms, with the M_3 utilising both the i3 and ct domains, whereas the 5-HT_{2A} receptor seems to prefer the ct domain alone, with any contribution by the 2Ai3 being much less than that of the M_3i3. The M_3 muscarinic receptor is also able to utilise ARF6 to activate PLD upon agonist stimulation, which

seems to be through the M_3i3 domain. The differences between these two receptors are not limited to their ability to activate PLD via ARF6. ARF-dependent PLD activation by the M_3 muscarinic receptor is not only sensitive to co-transfection of dominant negative ARF1 and ARF6 constructs, and the ARF-GEF inhibitor brefeldin A (BFA), but also to co-transfection with the Gβγ-sequestering agent $GRK2_{495-689}$. This peptide seems to block the activation of PLD via ARF1 in the case of the M_3 receptor, but not in the case of the 5-HT_{2A} receptor, suggesting that the means of activation of the ARF isoforms by these two receptors differ not only in the isoforms of ARF which they can activate, but also seemingly in the means of activation. The M_3 receptor seems to utilise Gβγ in its activation of ARF in some way that the 5-HT_{2A} receptor does not. The difference may be in the binding site for ARF in the receptors. The M_3 muscarinic receptor has an unusually long i3 domain (R^{253}-Q^{491}) at 239 amino acids in length, and this is utilised in the binding of both ARF1 and ARF6 to the receptor. The i3 domain of the 5-HT_{2A} receptor is not as large (I^{258}-G^{326}) at a third of the size (68 amino acids), and so it might be predicted that there is at least the potential for a number of additional interaction sites in the M_3i3 Some of these have already been characterised (Tobin *et al.*, 1997; Wu *et al.*, 1998; Wu *et al.*, 2000), but the exact nature of the interaction between Gβγ, ARF and the M_3i3 remains unknown.

In the case of the 5-HT_{2A} receptor, the interaction between ARF1 and the carboxy-terminal tail of the receptor seems to be highly influenced by the N/DPxxY domain, and not by the presence of Gβγ. However, the presence of arrestin 2 binding to the ct domain increases the ability of ARF1 to interact with the ct, leading to further differentiation between the mechanism of action of the M_3 and 5-HT_{2A} receptor in

ARF-dependent activation of PLD. The interaction of arrestin 2 with ARF6 upon activation of the β_2-adrenergic receptor is well documented, whereby recruitment and activation of ARF6, which is involved in the endocytosis of the receptor, is attained (Claing *et al.*, 2001). However it is unsure if the interaction of ARF1, arrestin 2 and the 5-HT_{2A} receptor delivers the same effect as that in the case of ARF6 and the β_2-adrenergic receptor. To further investigate this interaction requires assays of the signalling events upon 5-HT_{2A} receptor stimulation in the presence of arrestins. The use of dominant negative and positive mutants of arrestin need some caution in interpretation, as arrestins have varied roles within the cell, so ensuring selective blockade of arrestin function in this context alone could prove to be difficult. What is known, is that arrestin's usual role in a cell is to desensitise receptors by binding to phosphorylated sites on intracellular receptor domains (usually the i3 and ct domains) and leading to uncoupling of the heterotrimeric G-protein signalling and endocytosis of the receptor. In the example of binding to the 5-HT_{2A} receptor domain GST-fusion proteins *in vitro*, no phosphorylation of the receptor domains was in place and yet the arrestins still bound. Although the affinity of this interaction was not directly investigated and it may be lower than that to phosphorylated target sites, it seems possible that this binding may play a significant role *in vivo*.

This mechanism for ARF interaction with the 5-HT_{2A} receptor is again at odds with that of the M_3 receptor. Arrestin interactions with the M_3 muscarinic receptor are thought to lead to the desensitisation of the receptor (reviewed in Hosey *et al.*, 1995), whereas there is no direct evidence of this being the case for the 5-HT_{2A} receptor and indeed desensitisation of the 5-HT_{2A} receptor may well be via an arrestin-independent route (Gray *et al.*, 2001).

The role of the association of PLD with the 5-HT_2 family of GPCRs requires further investigation. It seems the ARF and PLD are binding to different motifs within the carboxy-terminal domain of the 5-HT_2 receptors, so they may interact independently with the receptor, although indirect interactions might modify each other's abilities to bind to this domain. Furthermore, it is impossible to tell from these experiments whether PLD and ARF binding occur concurrently in the cell, and whether or not this interaction is dependent upon activation of the receptor. So more experiments for example investigation of the components of the ARF/PLD:receptor complex and their dynamics could be studied by co-immunoprecipitation. It may be that both the ARF and PLD are part of the same larger complex that interacts with the 5-HT receptor, and that they interact with each other, with either the receptor itself, or some other protein (possibly arrestin) acting as a scaffold for this interaction.

Further experimentation into the ability of receptors to signal through the various pathways available, and how the pathway selection is determined, for example the role of GPCR homo- and heterodimers formation, and the role this plays in the signalling of the receptors, be an obvious next step in the progression of these findings, as is looking at these binding pattern in situ, by use of confocal techniques to discover when and where these proteins interact *in vivo*.

Chapter 5 discusses the ability of S100B monomers and dimers to bind to the 5-HT_{2A} receptor carboxy-terminal tail domain, although under physiological conditions, may well be that the S100B binds as a dimer. This interaction is Ca^{2+} dependent, but further work is required to discover if these proteins indeed interact under physiological conditions, and if any role of this possible interaction can be discerned.

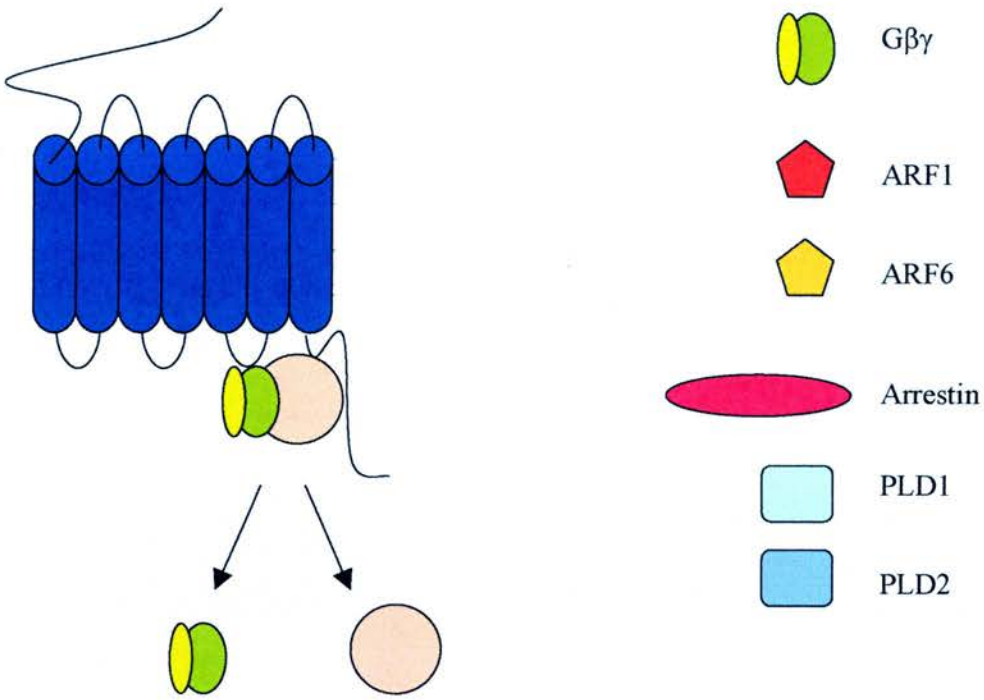
A more exact definition of the binding motif for S100B may lead to a firmer understanding of the physiological importance of this coupling. This function may be similar to that of calmodulin in modulation of 5-HT_{2A} receptor signalling, or it may be in the role of a scaffolding protein, linking the 5-HT_{2A} receptor with other protein partners, or even other receptors. Any role of S100B in signalling of the 5-HT_{2A} receptor could be easily be clarified by use of mutant forms of the S100B in the same signalling assays used in this study. Roles in scaffolding, and/or discovery of the role of S100B *in vivo* could be discovered through confocal experimentation, to see if these proteins are found to co-localise within cells.

The number of signalling pathways available to the superfamily of GPCRs increase the complexity and diversity available to what is already a complex and diverse system. Beyond the genomically defined diversity of GPCR function, splice variations and the context-specific selection of particular signalling pathways further add to the broad spectrum of physiological influences available to this family of cell surface receptors.

Figure 6.1

a

Heterotrimeric G protein signalling

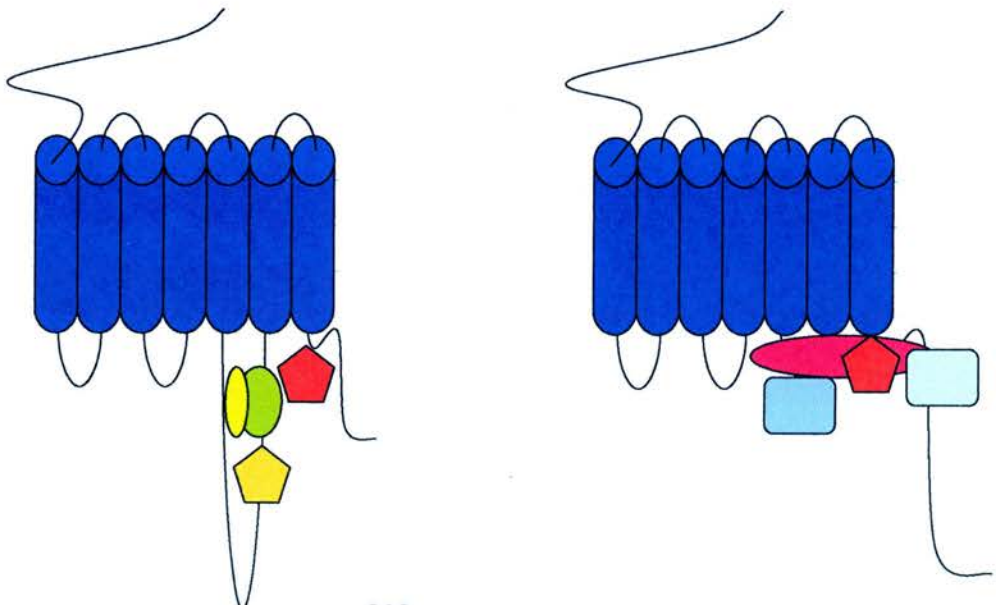


b

Small G protein signalling

M_3 Muscarinic receptor

5-HT_{2A} receptor



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Appendix:

Published Papers

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ADP-ribosylation Factor-dependent Phospholipase D Activation by the M_3 Muscarinic Receptor*

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G protein-coupled receptors can potentially activate phospholipase D (PLD) by a number of routes. We show here that the native M_3 muscarinic receptor in 1321N1 cells and an epitope-tagged M_3 receptor expressed in COS7 cells substantially utilize an ADP-ribosylation factor (ARF)-dependent route of PLD activation. This pathway is activated at the plasma membrane but appears to be largely independent of $G_{q/11}$, phospholipase C, Ca^{2+}, protein kinase C, tyrosine kinases, and phosphatidyl inositol 3-kinase. We report instead that it involves physical association of ARF with the M_3 receptor as demonstrated by co-immunoprecipitation and by *in vitro* interaction with a glutathione *S*-transferase fusion protein of the receptor's third intracellular loop domain. Experiments with mutant constructs of ARF1/6 and PLD1/2 indicate that the M_3 receptor displays a major ARF1-dependent route of PLD1 activation with an additional ARF6-dependent pathway to PLD1 or PLD2. Examples of other G protein-coupled receptors assessed in comparison display alternative pathways of protein kinase C- or ARF6-dependent activation of PLD2.

Many G protein-coupled receptors (GPCRs)[1] can activate phospholipase D (PLD), which catalyzes the hydrolysis of phosphatidylcholine to phosphatidic acid and choline. Both phosphatidates and diacylglycerols (formed by phosphatidate hydrolysis) may act as intracellular messengers. PLD has been implicated as a key regulator of vesicular trafficking, cytoskeletal organization, exocytosis, endocytosis, and further signaling pathways (1–4). Activation of PLD can be brought about by a variety of signaling events (5–8), many of which could potentially contribute to the stimulation of PLD activity by GPCRs. These include the activation of protein kinase C (PKC), protein-tyrosine kinases, phosphatidylinositol 3-kinase (PI 3-kinase), small G proteins of the ARF and Rho families, and possibly the elevation of intracellular Ca^{2+} levels.

This study addresses the mechanism of PLD activation by the M_3 muscarinic receptor expressed endogenously in 1321N1 human astrocytoma cells and heterologously in COS7 cells. The M_3 receptor is a member of the Group I, rhodopsin-related GPCR family that is expressed in the nervous system and peripheral tissues. The best established signaling pathway from the M_3 receptor is the pertussis toxin-insensitive activation of phospholipase C (PLC) via the heterotrimeric G protein $G_{q/11}$, although PLD is also strongly activated. In various cell types, PKC, protein-tyrosine kinases, ARF, and Rho have each been specifically implicated in M_3 receptor-mediated PLD activation (6, 9–12). The data here emphasize the importance of a pathway to PLD that involves direct association between ARF and the M_3 receptor (12).

ARF1 and ARF6 are representative of the main classes of cellular ARFs (Classes I and III) and have distinct subcellular distributions in many cell types. In resting cells, ARF1 is largely cytosolic or Golgi-associated, whereas ARF6 is often localized to the plasma membrane (13–17). Nevertheless ARFs can translocate to Golgi membranes upon GTP loading (13, 18) and to unspecified membranes following formyl-Met-Leu-Phe or M_3 receptor activation (10, 19, 20), so their precise intracellular location following stimulation is not clear.

The isoform of PLD that mediates ARF-dependent responses was thought for several years to be PLD1 because of its activation *in vitro* by ARF (and Rho and PKC) (5, 21). Nevertheless, recent evidence suggests that PLD2, and especially an amino-terminally truncated form of PLD2 can also be activated by ARF (22, 23). Both PLD1 and the truncated form of PLD2 are activated *in vitro* by ARF1 more effectively than by ARF6 (23). In contrast, PLD2 heterologously expressed in cells can be activated to a similar extent by constitutively active ARF1 and ARF6 (7). ARF-dependent PLD activity and GPCR-mediated PLD responses have been described in the plasma membrane compartment (24–26), although the identity of the isoform responsible was not clear. PLD1 is largely associated with Golgi and other intracellular membranes (27–29), but some is also associated with the plasma membrane (30–32), and the enzyme can be recruited to the plasma membrane during exocytosis (26, 33). In contrast, PLD2 is more generally associated with the plasma membrane (27, 34), although it too can be associated with Golgi structures (35).

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[1] The abbreviations used are: GPCR, G protein-coupled receptor; TP, thromboxane A_2; i3, third intracellular loop; tm7, transmembrane domain 7; FLAG, DYKDDDD epitope tag; HA, hemagglutinin; GST, glutathione *S*-transferase; sFM_3, signal sequence-FLAG-tagged M_3 receptor; PLC, phospholipase C; PLD, phospholipase D; PKC, protein kinase C; PI 3-kinase, phosphatidylinositol 3-kinase; ARF, ADP-ribosylation factor; [^{3}H]NMe-QNB, [^{3}H]*N*-methylquinuclidinyl benzilate; [^{3}H]InsP, [^{3}H]inositol phosphate; [^{3}H]PtdBut, [^{3}H]phosphatidylbutanol; PMTx, *P. multocida* toxin; AEBSF, [4-(2-aminoethyl)-benzene]sulfonyl fluoride; BFA, brefeldin A; GEF, GTP exchange factor; PDBu, phorbol 12,13-dibutyrate; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; Bis-Tris, 2-[bis(2-hydroxyethyl)amino]2-(hydroxymethyl)propane-1,3-diol.

The present experiments investigate the mechanisms of M_3 receptor-mediated PLD activation in 1321N1 and COS7 cells in comparison to those utilized by other GPCRs in the same conditions. We address specifically the roles played by ARF1/6 and PLD1/2 as well as the subcellular location of the relevant components and the site at which the PLD activation response occurs. In addition, we provide explicit evidence for agonist-regulated physical association of ARFs with the M_3 receptor and show that this may involve binding to its third intracellular loop (i3) domain.

EXPERIMENTAL PROCEDURES

Materials—Cell culture media were obtained from Invitrogen. Laboratory chemicals were obtained from Merck and were of Analar standard. Pharmacological agents were obtained from Sigma unless otherwise indicated. U73122 and 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF) were from Alexis Biochemicals Ltd. (Nottingham, UK). U46619, *Pasteurella multocida* toxin (PMTx), chelerythrine chloride, myr-PKCα_{19-27}, bisindolylmaleimide I, PP1, genistein, and AG 213 were from CN-Biosciences (UK) Ltd. (Nottingham, UK). Ilimaquinone was from Biomol, Affiniti (Exeter, UK). Aceclidine was from Tocris (Bristol, UK). [^{3}H]NMe-quinuclindinyl benzilate ([^{3}H]NMe-QNB; 84 Ci/mmol), [^{3}H]oxotremorine-M (69 Ci/mmol), [^{3}H]*myo*-inositol (20 Ci/mmol), and [^{3}H]palmitate (40 Ci/mmol) were from PerkinElmer Life Sciences. CGP 41251 (36) was kindly provided by Ciba-Geigy.

Molecular Reagents—In order to prepare the SPFLAGhM3R.pcDNA3 construct, the human M3 receptor was PCR-amplified from first strand cDNA made from RNA extracted from the human neuroblastoma SH-SY5Y cell line using the Stratagene reverse transcriptase-PCR kit. In the first round, a 1.9-kb fragment encoding the FLAG epitope (DYKDDDDA) at the 5′-end was amplified using primer pair FLAGhM3R.fp [5′-GACTACAAAGACGATGACGACGCCATGACCTTGCACAATAAC] and hM3R.rp [5′-ATCATCACCAGAAGTCACCCC], utilizing the Expand High Fidelity PCR System (Roche Applied Science) according to the manufacturer's instructions. In the second round, 0.5 μl of the first round PCR was amplified with the primer 5′-CAGGCATGAAGACGATCATCGCCCTGAGCTACATCTTCTGCCTGGTATTCGCCGACTACAAAGACGATGACG-3′, encoding a modified influenza hemagglutinin signal sequence, and the hM3R.rp. The 1.9-kb fragment was purified by agarose gel electrophoresis and Qiaex II (Qiagen Ltd., Crawley, UK) and then subcloned into the pGEMTEasy cloning vector (Promega Biosciences Inc., Southampton, UK). The reading frame and PCR integrity of the cloned construct were verified by nucleotide sequence analysis. For expression studies, the 1.9-kb insert was released from the pGEMTEasy vector by restriction digestion with *Eco*RI and *Spe*I and subcloned into the *Eco*RI/*Xba*I sites of pcDNA3 (Invitrogen). GST fusion protein constructs of Arg^{252}–Gln^{490} from the M_3 receptor third intracellular domain, M_3i3 (in pGEX-4T-1) (37), and the 58-amino acid STREX exon of the BK channel (in pGEX-5X-1) were kindly provided by Steve Lanier and Mike Shipston, respectively. The expression construct for the N376D mutant 5-HT$_{2A}$ receptor (12), kindly provided by Stuart Sealfon, was subcloned into pcDNA3, incorporating a signal sequence and epitope tag.

Wild type and dominant negative ARF constructs with a C terminus HA epitope tag were kindly provided by Julie Donaldson. The mutant constructs, T31N-ARF1 and T27N-ARF6, are defective in the exchange of GTP for GDP and act as functional dominant negative forms (14). Wild type constructs of PLD1b and PLD2 as well as corresponding catalytically inactive mutants (K898R-PLD1 and K758R-PLD2) and the PIM87 mutant PLD1 (which is selectively defective in activation by PKC) (6, 7) were kindly provided by Mike Frohman.

Cell Culture and Transfection—1321N1 human astrocytoma cells were maintained in Dulbecco's minimal essential medium containing 100 μg/ml penicillin and streptomycin and supplemented with 10% fetal calf serum. COS7 cells were grown in Dulbecco's minimal essential medium containing 10% normal calf serum and 100 μg/ml of penicillin and streptomycin. Prior to transfection, COS7 cells were grown to ~70% confluence and were then transfected using FuGENE-6 (Roche Applied Science) according to the manufacturer's guidelines. Transfected cells were used in experiments 72 h after transfection. In a small number of experiments, HEK 293 cells were similarly transfected. In all experiments involving transfection, equivalent amounts of empty vector were substituted in control samples to compensate for any omitted plasmid.

Ligand Binding Assays—Specific binding of [^{3}H]NMe-QNB was measured in membrane fractions of 1321N1 and sFM$_3$ receptor-transfected COS7 cells. Cells were washed in Hanks' balanced salt solution and then homogenized in ice-cold 50 mM sodium phosphate buffer, pH 7.4 with 2 mM $MgCl_2$, 2 μg/ml aprotinin, and aliquots were taken for protein assay (Coomassie binding method; Pierce). Homogenates were centrifuged at 12,000 × *g* for 30 min at 4 °C, and the pellet was washed twice more. For the binding assay, 1% bovine serum albumin was added. Ligand concentrations were varied from 20 pM to 2 nM, and nonspecific binding was defined by 1 μM NMe-atropine. After 4 h at 25 °C, an excess of ice-cold buffer was added, tubes were centrifuged, and the supernatant was aspirated from the pellet. Data were curve-fitted by nonlinear regression (Fig P, Elsevier-Biosoft, Cambridge, UK). Cell surface specific binding of [^{3}H]oxotremorine-M was measured to 1321N1 and sFM$_3$ receptor-transfected COS7 cells in 12-well plates at 4 °C. Culture medium was replaced with phosphate-buffered saline containing 2 mM $MgCl_2$ and 1% bovine serum albumin and then plates were chilled on ice. Ligand (5 nM), with or without 3 μM NMe-atropine to determine nonspecific binding, was added, and samples were incubated for 16 h at 4 °C to minimize internalization. Incubations were then quenched with excess ice-cold buffer and washed once. Ice-cold "acid strip" solution (0.2 M acetic acid, 0.5 M NaCl) was added for 5 min to release surface-bound ligand. The internalization of specific [^{3}H]oxotremorine-M binding sites into COS7 cells was measured at 37 °C over a time course of 0–50 min following the addition of ligand. Both ligand and NMe-atropine concentrations were as in the experiments carried out at 4 °C. Total and nonspecific binding levels were assessed at each time point. Following 5 min with cold acid strip solution to remove surface-bound ligand, cells were solubilized in 1% SDS, 1 M NaOH and then neutralized, to determine [^{3}H]ligand in both cell surface and internalized compartments.

Signal Transduction Assays—Cellular [^{3}H]inositol phosphate ([^{3}H]InsP) production (PLC activity) was measured in 12-well plates following labeling with 1 μCi/ml [^{3}H]inositol for 18 h in serum-free medium. Agonist responses were measured usually over 30 min in the presence of 10 mM LiCl before cells were lysed in ice-cold 10 mM formic acid, and [^{3}H]inositol phosphates were separated by ion exchange (38). Inhibitory agents and the LiCl were added 30 min and 15 min prior to agonist, respectively. [^{3}H]Phosphatidylbutanol ([^{3}H]PtdBut) production (PLD activity) was measured in 12-well plates following labeling with 1.5 μCi/well [^{3}H]palmitate for 18 h in serum-free medium. It has been shown that the presence of serum causes elevated basal activity of PLD (21). Agonist responses were measured usually over 30 min in the presence of 30 mM butan-1-ol. Assays were terminated, phospholipids were extracted into chloroform/methanol, and [^{3}H]PtdBut was separated by thin layer chromatography (39). Inhibitory agents and the butan-1-ol were added 30 min prior to and immediately before agonist, respectively. In experiments with PMTx (40), agonist incubations were carried out over a total period of 4 h, with replacement of medium containing fresh PMTx and LiCl or butan-1-ol at 2 h. All data from signal transduction and ligand binding experiments are expressed as means ± S.E. from between 4 and 10 separate determinations.

Immunoprecipitation of sFM$_3$ Receptor—In order to immunoprecipitate the sFM$_3$ receptor with any associated proteins, plasmids encoding the sFM$_3$ receptor and either ARF1-HA or ARF6-HA were transiently transfected into COS7 cells. 72 h later, the cells were serum-deprived for 4 h. Cells were then exposed to carbachol (20 μM) or no drug for 15 min and washed once in Hanks' balanced salt solution before being solubilized in immunoprecipitation buffer (phosphate-buffered saline, pH 7.5, 1% CHAPS, 0.75% sodium deoxycholate, 2 μg/ml aprotinin, 4 μg/ml leupeptin, 1 mM AEBSF, 2 μg/ml pepstatin, 1 mM sodium orthovanadate, 1 mM sodium fluoride, 5 mM sodium molybdate, and 50 μg/ml soybean trypsin inhibitor (2 ml/175-cm^2 flask for 1 h on ice). Carbachol was readded where appropriate. Extracts were centrifuged at 12,000 × *g* for 15 min at 4 °C to remove particulate material and precleared with Protein G-Sepharose 4B fast flow (Sigma) (20 μl of 1:1 suspension/ml for 45 min at 4 °C). After centrifugation, the supernatant was removed to tubes containing either mouse monoclonal FLAG antibody (clone M2, 10 μg/ml; Sigma) or nonimmune mouse IgG (10 μg/ml; Sigma) with 40 μl/ml Protein G-Sepharose suspension, before rolling at 4 °C overnight. Beads were collected by centrifugation and washed twice in immunoprecipitation buffer before 40 μl of 2× Laemmli buffer (2% SDS, 5% mercaptoethanol, 20 mM Tris, pH 7.4) was added per ml of original supernatant. SDS-PAGE and electroblotting onto "Immobilon-P" polyvinylidene difluoride membranes (Millipore Ltd., Watford, UK) were carried out using a Phastsystem apparatus (Amersham Biosciences). Western blots were carried out on the samples and original supernatants to detect immunoprecipitated proteins and monitor input levels. The primary antibodies were rabbit polyclonal raised to the third intracellular loop of the M_3 receptor (41) (gift from Andrew Tobin) and rabbit polyclonal against the HA epitope tag (Santa Cruz Biotechnology, Autogen Bioclear Ltd., Calne, UK), followed by preabsorbed sec-

ondary antibodies conjugated to horseradish peroxidase (Chemicon International Ltd., Harrow, UK). Bands were visualized by ECL (Amersham Biosciences) and then measured by quantitative densitometry.

In further experiments, an alternative procedure was used in which the sFM_3 receptor associated with ARF1-HA or ARF6-HA immunoprecipitates was measured by specific [^{3}H]NMe-QNB binding. Cells treated with or without carbachol were solubilized in immunoprecipitation buffer with 10% glycerol, and precleared supernatants were immunoprecipitated with 2 μg/ml 12CA5 mouse monoclonal HA antibody (or nonimmune mouse IgG) for 90 min followed by Protein G-Sepharose for 40 min. This more rapid procedure was designed to minimize the possibility of any nonspecific interactions of the solubilized proteins. Immunoprecipitates were washed in immunoprecipitation buffer with 10% glycerol and then resuspended into [^{3}H]NMe-QNB binding buffer (above) with 10% glycerol and 0.3 mg/ml sonicated phosphatidyl choline prior to ligand binding, as above.

Cell Surface Biotinylation—In some experiments, cell surface proteins were biotinylated using a membrane-impermeant reagent (biotinamidohexanoic acid 3-sulfo-*N*-hydroxysuccinimide ester sodium salt) (Sigma); 1 mM for 2 h at 4 °C). The reaction was quenched with 75 mM glycine (10 min at 4 °C), and cells were washed in phosphate-buffered saline before returning to minimal essential medium and warming to 37 °C. Cells were then stimulated with 20 μM carbachol (10 min) or control before solubilization. Extracts were incubated with monomeric avidin-agarose (1 h at 4 °C) and washed in solubilization buffer before biotinylated proteins were eluted by incubation in 2 mM biotin for 30 min at 4 °C. These supernatants were then subjected to immunoprecipitation with 12CA5 HA antibody (or nonimmune IgG control) and subsequently used in specific [^{3}H]NMe-QNB binding assays, as above.

GST Fusion Protein Interaction Assays—The GST-M_3i3 (Arg^{252}–Gln^{490}) construct in pGEX-4T-1 and the control GST-BK_{STREX} construct in pGEX-5X-1 were expressed in BL21-RIL bacterial cells, which were then grown up in standard 2× YT (yeast extract, tryptone, NaCl) medium with 2% glucose added. When the cells had reached an A_{600} of 0.6–0.8 units/ml, expression of the fusion proteins was induced by the addition of 0.1 mM isopropyl-β-D-thiogalactoside for 3 h at 37 °C. Cells were harvested by centrifugation and then lysed with BugBuster reagent (Novagen, CN-Biosciences) for 10 min and again centrifuged. The supernatant, containing the GST fusion proteins, was added to glutathione-Sepharose beads (Amersham Biosciences). The beads were incubated with the bacterial supernatant for 20 min at room temperature to allow binding of the GST fusion proteins to the beads. The matrix formed was then washed extensively with phosphate-buffered saline and used immediately.

In order to provide cytosolic extracts enriched with various ARF constructs, transfected COS7 cells were homogenized in ice-cold extraction buffer (2 ml/175-cm^2 flask, 2 μg/ml aprotinin, 1 mM AEBSF, 1 mM dithiothreitol, 2 μg/ml pepstatin, 1 mM sodium orthovanadate, 1 mM sodium fluoride, 50 μg/ml soybean trypsin inhibitor in phosphate-buffered saline). The cells were then homogenized (Ystral homogenizer; setting 3, 15 s) before being centrifuged (12,000 × *g* for 20 min at 4 °C). The supernatant was aliquoted and stored at −40 °C. ARF-HA-enriched extracts were incubated with the GST fusion protein affinity matrix in 250 μl of Buffer A (20 mM Tris-HCl, pH 7.5, 0.6 mM EDTA, 1 mM dithiothreitol, 70 mM NaCl, 0.05% Tween 80) for 90 min at 4 °C with rolling. The beads were washed four times in Buffer A, and then the retained proteins were removed from the beads with 2× Laemmli buffer and applied to 20% homogenous Phastgels (Amersham Biosciences) for SDS-PAGE and subsequent Western blotting. Membranes were probed for HA immunoreactivity to monitor captured ARFs and for GST immunoreactivity to assess levels of fusion protein input (GST alone ~29 kDa, GST-M_3i3 ~49 kDa, and GST-BK_{STREX} ~35 kDa). Antibodies were rabbit polyclonal anti-HA and polyclonal anti-GST (Santa Cruz Biotechnology). Horseradish peroxidase-conjugated secondary antibodies and ECL were as used in the immunoprecipitation studies. Input levels of ARF-HA immunoreactivity in extracts were also monitored, and both fusion protein and ARF inputs were carefully balanced to ensure comparability between samples.

Subcellular Fractionation—Homogenates of sFM_3 receptor-transfected COS7 cells (in 175-cm^2 flasks), either control or treated with 200 μM carbachol for 10 min, were prepared and initially centrifuged at 1000 × *g* for 8 min to remove nuclei and unbroken cells. The remaining membranes were fractionated through gradients of Percoll (Amersham Biosciences) under alkaline conditions designed to optimally separate endoplasmic reticulum, Golgi, and plasma membrane fractions (24). Fractions (0.5 ml) were downloaded from the bottom of the gradient by peristaltic pump (1 ml/min), and adjacent fractions were combined into Laemmli buffer for SDS-PAGE on Nu-PAGE 4–12% gradient Bis-Tris gels (Invitrogen) before immunoblotting for organelle marker proteins as well as for PLD1 and ARF1. The antibodies used were goat polyclonal anti-EEA1 (endoplasmic reticulum marker; Santa Cruz Biotechnology), mouse monoclonal anti-GM130 (Golgi marker; Transduction Laboratories, BD Biosciences, Cowley, UK), mouse monoclonal anti-Na^+/K^+ ATPase α_1 subunit (plasma membrane marker; Upstate Biotech Ltd., Milton Keynes, UK), rabbit polyclonal anti-PLD1 (N-terminal region) (BIOSOURCE International Inc., Nivelles, Belgium), and sheep polyclonal anti-ARF1/3 (Upstate Biotech). For [^{3}H]PtdBut production experiments, each 175-cm^2 flask of cells was labeled with 150 μCi of [^{3}H]palmitate in serum-free medium for 16 h prior to the experiment. Subcellular fractions were extracted with chloroform/methanol according to the standard PLD assay procedure, and [^{3}H]PtdBut was similarly separated by thin layer chromatography.

RESULTS

PLD Activation by Native M_3 Receptors in 1321N1 Cells—Ligand binding studies with [^{3}H]NMe-QNB demonstrated specific muscarinic binding sites in 1321N1 cells and sFM_3 receptor-transfected COS7 cells but not in mock-transfected COS7 cells. In 1321N1 cells, the K_D and B_{max} of specific [^{3}H]NMe-QNB binding were 0.26 ± 0.03 nM and 193 ± 27 fmol/mg protein, similar to previous work (42), which showed that the muscarinic receptors present were almost entirely of the M_3 subtype. In sFM_3 receptor-transfected COS7 cells, the K_D and B_{max} of specific [^{3}H]NMe-QNB binding were 0.58 ± 0.04 nM and 2.64 ± 0.47 pmol/mg protein. In pilot experiments with wild-type M_3 receptor cDNA (lacking the signal sequence and FLAG tag), binding showed similar affinity but lower B_{max}, values.

In 1321N1 cells, the M_3 agonist carbachol caused concentration-dependent increases in both [^{3}H]PtdBut and [^{3}H]InsP production (Fig. 1*a*). The EC_{50} values for these PLD and PLC responses were similar, being 10.2 ± 2.0 and 8.1 ± 1.7 μM, respectively. The nicotinic cholinergic agonist 1,1-dimethyl-4-phenyl-piperazinium iodide caused no discernible increase in [^{3}H]PtdBut production through the range 3–100 μM (1.20 ± 0.17-fold of basal control at 100 μM 1,1-dimethyl-4-phenyl-piperazinium iodide, n = 4), indicating that nicotinic receptors made no significant contribution. The muscarinic partial agonist, aceclidine, activated PLD with a lower maximum response, in the order of 30% of that for carbachol (in line with its reported efficacy in PLC activation). 1321N1 cells also express the thromboxane A_2 (TP) receptor, which like the M_3 receptor is coupled to PLC activation via $G_{q/11}$ but contains an alternative motif in transmembrane domain 7 (tm7) that is believed to disrupt ARF-dependent coupling to PLD activation (12). The selective TP receptor agonist U46619 caused concentration-dependent activation of PLD (Fig. 1*a*) but with properties distinct from the M_3 receptor response.

The PLD response to 200 μM carbachol was inhibited in a concentration-dependent manner by brefeldin A (BFA; a selective inhibitor of a subfamily of ARF GTP exchange factors (ARF-GEFs), known as BIG1/2) (43). The corresponding PLC response was unaffected (Fig. 1*b*). PLD responses to a low concentration of carbachol (10 μM) or to aceclidine (500 μM) showed similar BFA sensitivity to that with 200 μM carbachol, having IC_{50} values of 61.4 ± 9.5, 56.8 ± 11.1, and 55.5 ± 13.5 μM, respectively. In contrast, PLD activation by the TP receptor agonist U46619 was unaffected by BFA. The time course of PLD and PLC activation by carbachol in 1321N1 cells is shown in Fig. 1*c*. There was rapid desensitization of the PLD, but not the PLC response, over the times examined. BFA had no effect on the time course of PLC activation but diminished the initial rate and maximal extent of PLD activity, although the profile of desensitization was unaltered. Since PLD responses can occur downstream of PLC activation, we examined effects of the selective PLC inhibitor U73122. Fig. 1*d* shows that U73122

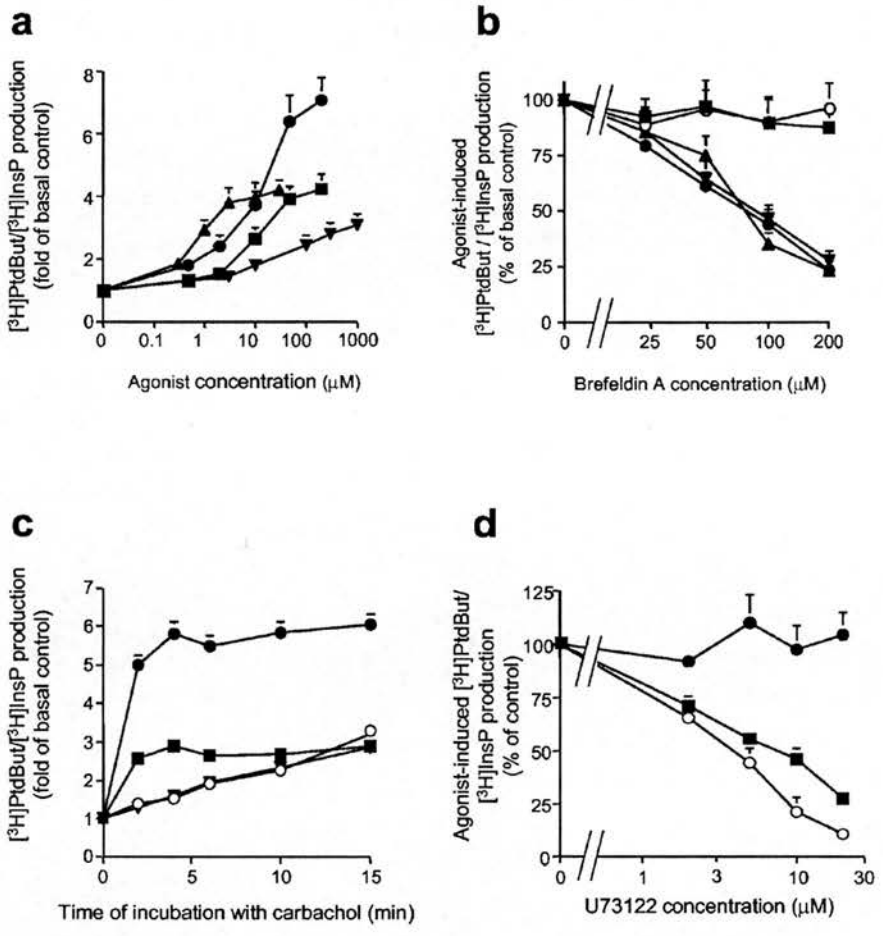

FIG. 1. **Differential involvement of ARF and PLC in the PLD responses of M_3 and TP receptors in 1321N1 cells.** The [^{3}H]PtdBut (PLD) and [^{3}H]InsP (PLC) responses elicited by the M_3 receptor agonist carbachol and the partial agonist aceclidine as well as the TP receptor agonist U46619 were characterized, together with the effects of the PLC inhibitor, U73122, and the ARF-GEF inhibitor, BFA, on these responses. Values are means ± S.E., n = 4–12. *a*, concentration dependence of PLD responses to carbachol (●), aceclidine (▼), and U46619 (▲) as well as the PLC response to carbachol (■). *b*, concentration dependence of BFA effects on PLD responses to 200 μM carbachol (●), 10 μM carbachol (▲), 500 μM aceclidine (▼), and 30 μM U46619 (■) as well as PLC responses to 200 μM carbachol (○). BFA caused statistically significant inhibition of the PLD responses to carbachol and aceclidine at concentrations of 50–200 μM BFA ($p < 0.05$, Wilcoxon test). *c*, time course of PLD and PLC responses to 200 μM carbachol in the presence/absence of 100 μM BFA. ●, control PLD response to carbachol; ■, in the presence of BFA. ○, control PLC response to carbachol; ▼, in the presence of BFA. *d*, concentration dependence of U73122 effects on PLD responses to 200 μM carbachol (●) and 30 μM U46619 (■) as well as on PLC responses to 200 μM carbachol (○). PLD responses to U46619 and PLC responses to carbachol showed statistically significant inhibition by U73122 at concentrations of 2–20 μM ($p < 0.05$, Wilcoxon test).

had no effect on PLD responses of the M_3 receptor despite inhibiting PLC responses with an IC_{50} value of 3.6 ± 1.9 μM. In contrast, PLD activation by the TP receptor agonist U46619 was readily inhibited by U73122 (IC_{50} value of 3.4 ± 1.4 μM). In case $G_{q/11}$ might play a role that was independent of PLC, we used the selective direct activator of $G_{q/11}$, PMTx, which was found to cause concentration-dependent activation of [^{3}H]InsP production (Fig. 2*a*). PMTx also caused [^{3}H]PtdBut production, and the response to a nearly maximally effective concentration (0.7 nM; 2.84 ± 0.36-fold of basal) was found to be inhibited readily by U73122 (IC_{50} value of 3.1 ± 0.6 μM) but not by BFA (Fig. 2*b*). Pertussis toxin (100 ng/ml; 16 h) had no effect on carbachol-induced [^{3}H]PtdBut production (data not shown), indicating that $G_{i/o}$ do not play a role here.

Since PKC and ARF can act synergistically to activate PLD (5), we further investigated a potential role for PKC in M_3 receptor responses. The selective PKC inhibitors CGP 41251, NPC-15437, chelerythrine chloride, and myristoyl-PKCα_{19-27} all had little or no effect on carbachol-induced PLD activation in 1321N1 cells (only chelerythrine chloride at the highest concentration tested, 30 μM, caused a statistically significant inhibition) (Fig. 2*c*). In contrast, CGP 41251 clearly inhibited the PLD response to phorbol 12,13-dibutyrate (PDBu) at the same concentrations. Any involvement of Ca^{2+} elevation in M_3 receptor PLD responses was investigated using the cell-permeable Ca^{2+} chelator 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid acetoxymethyl ester (1–10 μM). This caused only very minor inhibition of carbachol-induced PLD activation (data not shown).

PLD activation by the M_3 receptor may involve protein-tyrosine kinases in HEK 293 cells (9) but not 1321N1 cells (44), so we investigated the effects of the selective inhibitor of Src family tyrosine kinases, PP1 and the broad-spectrum tyrosine kinase inhibitors genistein and AG 213. None of these had any significant effect on carbachol-induced PLD activation in 1321N1 cells (Fig. 2*d*). However, in HEK 293 cells transiently transfected with the sFM_3 receptor construct, we found that carbachol-induced [^{3}H]PtdBut production was inhibited significantly by genistein and AG 213 with IC_{50} values of 6.3 ± 1.3 and 15.8 ± 4.2 μM, respectively (n = 6).

Receptor-mediated PLD activation in some cells is sensitive to PI 3-kinase inhibitors (45), but we found no effect of wortmannin (1 μM) or LY 294002 (50 μM) on the concentration dependence, time course, or BFA sensitivity of carbachol-induced PLD activation in 1321N1 cells (data not shown).

The Role of ARF1 and ARF6 in PLD Activation by the sFM_3 Receptor Expressed in COS7 Cells—In order to elucidate which ARF isoforms were mediating the M_3 receptor response, we carried out complementary experiments in COS7 cells transfected with the sFM_3 receptor. Carbachol caused concentration-dependent activation of PLD and PLC with EC_{50} values of 9.4 ± 2.2 and 1.2 ± 0.1 μM, respectively (Fig. 3, *a* and *b*). As in 1321N1 cells, the PLD response was inhibited by BFA, with an IC_{50} value of 64.1 ± 16.3 μM, but was resistant to the PKC inhibitor CGP 41251 (86.4 ± 12.2% of control at 10 μM, n = 4). Co-transfection of negative mutant ARF1 or ARF6 constructs caused inhibition of carbachol-induced activation of PLD, but not PLC. In cells with the sFM_3 receptor alone, 200 μM carba-

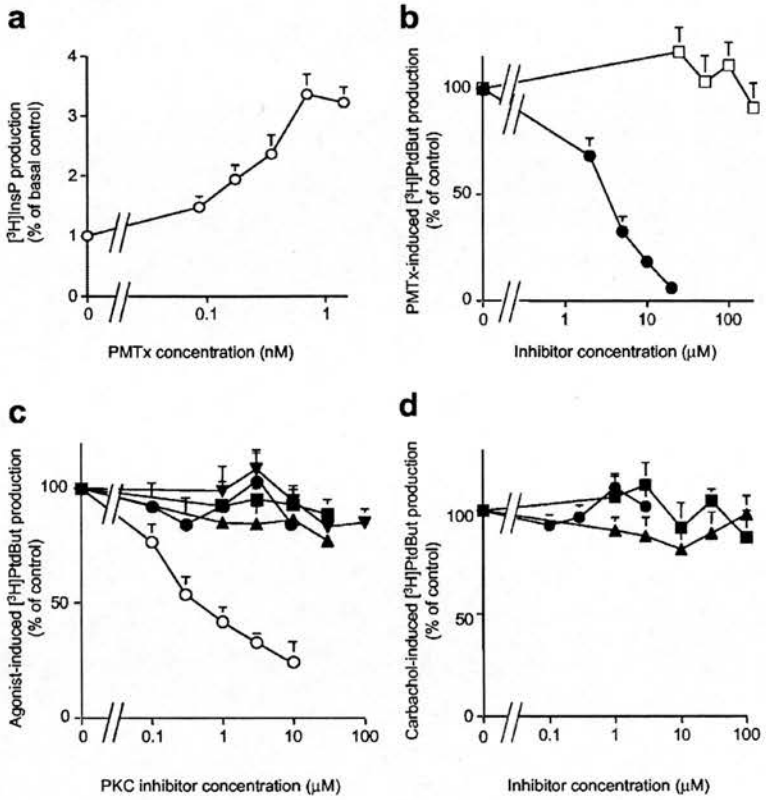

FIG. 2. **Evidence for the lack of major involvement of $G_{q/11}$, PKC, or tyrosine kinases in M_3 receptor PLD responses in 1321N1 cells.** The [^{3}H]PtdBut (PLD) and [^{3}H]InsP (PLC) responses elicited by the $G_{q/11}$ activator PMTx, carbachol, and PDBu were characterized, and their sensitivity to inhibitors of PLC, ARF-GEFs, PKC, and tyrosine kinases was assessed. Values are the means ± S.E., n = 5–10. *a*, concentration dependence of PLC activation in response to PMTx (○). *b*, concentration dependence of the effects of U73122 (●) and BFA (□) on PLD responses to 0.7 nM PMTx. Effects of U73122 were statistically significant at concentrations of 2–20 μM U73122 ($p < 0.05$, Wilcoxon test). *c*, concentration dependence of the effects of PKC inhibitors on the PLD responses to 200 μM carbachol (●, CGP 41251; ■, NPC-15437; ▲, chelerythrine chloride; ▼, myristoyl-PKCα_{19-27}) and to 300 nM PDBu (○, CGP 41251). The only statistically significant effect on PLD responses to carbachol was that of 30 μM chelerythrine chloride, whereas responses to PDBu were inhibited by 0.3–10 μM CGP 41251 ($p < 0.05$, Wilcoxon test). Bisindolylmaleimide I and calphostin C were not used because of known effects on the M_3 receptor and PLD, respectively. *d*, concentration dependence of the effects of tyrosine kinase inhibitors on the PLD responses to 200 μM carbachol (●, PP1; ■, genistein; ▲, AG 213) (none of the effects were statistically significant).

chol caused 5.18 ± 0.50-fold basal [^{3}H]PtdBut production, whereas co-transfection with T31N-ARF1 gave a 2.80 ± 0.21-fold response, co-transfection with T27N-ARF6 gave a 3.26 ± 0.48-fold response, and co-transfection with a combination of the ARF1/6 mutant constructs resulted in a 1.68 ± 0.28-fold response (n = 8). Omissions of constructs were fully substituted by empty vector. The negative mutant ARF values were significantly less than carbachol alone, and the combination showed a further significant reduction. A small residual component of sFM_3 receptor-mediated [^{3}H]PtdBut production remained in the presence of both T31N-ARF1 and T27N-ARF6.

The co-transfection of wild type ARF1 or ARF6 had no significant effect on the activation of PLD by carbachol (Fig. 3*a*). None of the ARF constructs significantly modified basal PLD activity (Fig. 3, *a*, *c*, and *d*; data not shown) or reduced the expression of sFM_3 receptors at the plasma membrane, as assessed by the specific acid-displaceable binding of [^{3}H]oxotremorine-M. For example, specific binding of [^{3}H]oxotremorine-M removed by acid strip represented 372 ± 31 dpm/well for sFM_3 receptor alone, with corresponding values of 348 ± 40 for sFM_3 receptor plus T31N-ARF1 and 328 ± 46 for sFM_3 receptor plus T27N-ARF6 (n = 6). Similarly, 45-min preincubation of 1321N1 cells with 150 μM BFA had no discernible effect on cell surface-specific binding of [^{3}H]oxotremorine-M (data not shown). Fig. 3*c* illustrates the BFA sensitivity of carbachol-induced [^{3}H]PtdBut production with or without the negative mutant ARF1/6 constructs. Controls showed inhibition of responses by BFA with an IC_{50} of 64.1 ± 16.3 μM. The attenuated PLD activation in the presence of T27N-ARF6 remained sensitive to BFA with an IC_{50} of 29.8 ± 17.1 μM. In contrast, the residual responses in the presence of T31N-ARF1 or both T31N-ARF1 and T27N-ARF6 were no longer reduced by BFA. This suggests that the sFM_3 receptor can utilize both ARF1 and ARF6 for activation of PLD, but the BFA sensitivity of the response reflects predominantly ARF1.

In comparison, we examined the PLD response to ATP (acting at native P_{2U} receptors). This was unaffected by BFA but was clearly reduced by the PKC inhibitor CGP 41251 and by transfection of T27N-ARF6 but not T31N-ARF1 (Fig. 3*d*). In contrast to the sFM_3 receptor, the native P_{2U} receptor thus appears to utilize PKC- and ARF6-dependent (but ARF1-independent) pathways for PLD activation. It seems unlikely that the difference between sFM_3 and P_{2U} receptors is due to heterologous expression because the findings with the sFM_3 receptor here mirror those obtained with the native M_3 receptor in 1321N1 cells (Figs. 1*b* and 2*c*) (12). To corroborate this, we transfected COS7 cells with the N376D mutant 5-HT_{2A} receptor, which displays BFA-insensitive responses (in contrast to the wild type 5-HT_{2A} receptor, where BFA is effective) (12). PLD responses of the N376D mutant 5-HT_{2A} receptor were also significantly inhibited by the PKC inhibitors, CGP 41251 and bisindolylmaleimide I, but not by BFA, T31N-ARF1, genistein, or AG 213. Although T27N-ARF6 reduced responses by 20–25%, this did not reach statistical significance (Fig. 3*d* and data not shown).

Physical Association of ARF1/ARF6 with the sFM_3 Receptor—The question of whether ARF1 or ARF6 could participate in some form of direct complex with the receptor was investigated first by co-immunoprecipitation and second by *in vitro* interaction with a GST fusion protein of the M_3 receptor i3 domain. Fig. 4 shows co-immunoprecipitation data from COS7 cells co-transfected with sFM_3 receptor and wild type ARF1-HA or ARF6-HA. Input levels of ARF-HA and the efficiency of sFM_3 receptor pull-down were monitored to ensure balance between samples. In Fig. 4*a*, low levels of ARF1-HA and ARF6-HA immunoreactivity were associated with the sFM_3 receptor in basal conditions, apparently in excess of nonimmune IgG controls. Preincubation of cells with carbachol caused increased association of ARF1-HA but not ARF6-HA with the sFM_3 receptor, as monitored by densitometry of the immunoblots

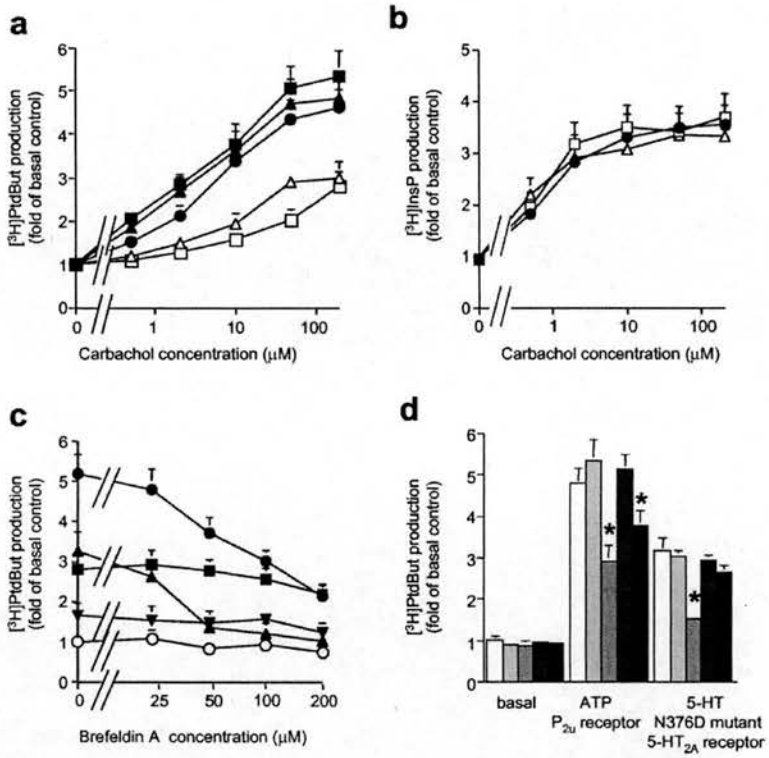

FIG. 3. **Effects of co-transfection with wild type or negative mutant ARF constructs on the PLD and PLC responses of the sFM_3 receptor in COS7 cells.** The [^{3}H]PtdBut (PLD) and [^{3}H]InsP (PLC) responses evoked by carbachol were measured in COS7 cells transfected with the sFM_3 receptor and wild type or dominant negative constructs of either ARF1 or ARF6. Values are means ± S.E., n = 6–10. *a*, concentration dependence of PLD activation evoked by carbachol acting at the sFM_3 receptor in the presence of control vector (●), wild type ARF1 (■), wild type ARF6 (▲), T31N-ARF1 (□), and T27N-ARF6 (△). The negative mutant forms of both ARF1 and ARF6 significantly reduced PLD responses to carbachol at concentrations of 2–200 μM ($p < 0.05$, Wilcoxon test). *b*, shows the concentration dependence of PLC activation evoked by carbachol acting at the sFM_3 receptor in the presence of control vector (●), T31N-ARF1 (□), and T27N-ARF6 (△). *c*, concentration dependence of BFA effects on PLD responses to 200 μM carbachol in the presence of control vector (●), T31N-ARF1 (■), T27N-ARF6 (▲), and T31N-ARF1 plus T27N-ARF6 (▼). ○, effects of BFA on PLD responses of cells transfected with sFM_3 receptor, but no ARF constructs, in the absence of carbachol stimulation. All controls for transfections contained equivalent levels of empty vector. The carbachol-evoked PLC responses of sFM_3 receptor-transfected cells were unaffected by BFA (data not shown). BFA (50–200 μM) caused significant inhibition of the PLD responses to carbachol only in the presence of control vector or of the negative mutant form of ARF6 ($p < 0.05$, Wilcoxon test). *d*, shows the effects of the ARF-GEF inhibitor, BFA (150 μM; *light gray columns*) the PKC inhibitor, CGP 41251 (10 μM; *medium gray columns*), and transfected T31N-ARF1 (*dark gray columns*) or T27N-ARF6 (*black columns*) on basal [^{3}H]PtdBut production or that in the presence of ATP (10 μM), acting at the native P_{2U} receptor or 5-HT (10 μM) acting at the co-transfected N376D mutant 5-HT_{2A} receptor. *, statistically significant differences from corresponding control responses (*white columns*) ($p < 0.05$, Wilcoxon test).

(3.50 ± 1.15- and 1.35 ± 0.40-fold control respectively; means ± S.E., n = 6). Fig. 4*b* shows an alternative, more rapid and quantifiable procedure in which sFM_3 receptor association with ARF1-HA/ARF6-HA immunoprecipitates was measured as specific [^{3}H]NMe-QNB binding. This showed low basal levels of co-immunoprecipitated binding sites but increased association with the receptor for ARF1-HA and (to a lesser extent) ARF6-HA following carbachol stimulation. Fig. 4*c* characterizes the time course of sFM_3 receptor-ARF1-HA association following the addition of carbachol, showing a peak around 5 min and then gradual return to basal levels by 25 min.

The subcellular location of the sFM_3 receptor-ARF1-HA association was investigated by cell surface biotinylation experiments. COS7 cells co-transfected with sFM_3 receptor plus ARF1-HA (or sFM_3 receptor alone) were stimulated with carbachol (or control), surface-biotinylated, and then solubilized. Biotinylated proteins were captured on monomeric avidin beads and eluted before HA immunoprecipitation. In both basal and carbachol-stimulated conditions, 75–90% of the specific [^{3}H]NMe-QNB binding found in direct HA immunoprecipitates was recovered in the biotinylation/avidin recovery procedure. Values for direct HA immunoprecipitates were 202 ± 31 and 384 ± 55 dpm/assay for basal and carbachol-stimulated respectively, whereas corresponding values from biotin/avidin capture were 157 ± 20 and 312 ± 32 dpm/assay (n = 5). All equivalent values for cells transfected with sFM_3 receptor alone did not exceed 55 dpm/assay and were similar.

Fig. 5 shows *in vitro* association of ARF1-HA or ARF6-HA with a GST fusion protein construct of the M_3i3 domain, a control construct, or GST alone. The levels of each GST construct were shown to be similar by Coomassie Blue staining and by GST immunoreactivity. ARFs were supplied as enriched extracts from transfected COS7 cells, and binding was monitored by HA immunoblot. The data (which are representative of at least three separate experiments) demonstrate specific *in vitro* interaction of both ARF1-HA and ARF6-HA with the GST-M_3i3 but not control constructs.

The Role of PLD1 and PLD2 in [^{3}H]PtdBut Production by the sFM_3 Receptor in COS7 Cells—Since both PLD1 and PLD2 can potentially be activated by ARFs, we investigated which PLD isoform was responsible for the ARF-mediated response of the receptor. Immunoblots for PLD isoforms in membranes of 1321N1, COS7, and HEK 293 cells showed that both PLD1 and PLD2 were present in each case (as in most cell types) (46) with a mean ratio of PLD1/PLD2 levels decreasing in the order COS7 > 1321N1 > HEK 293 (data not shown). Catalytically

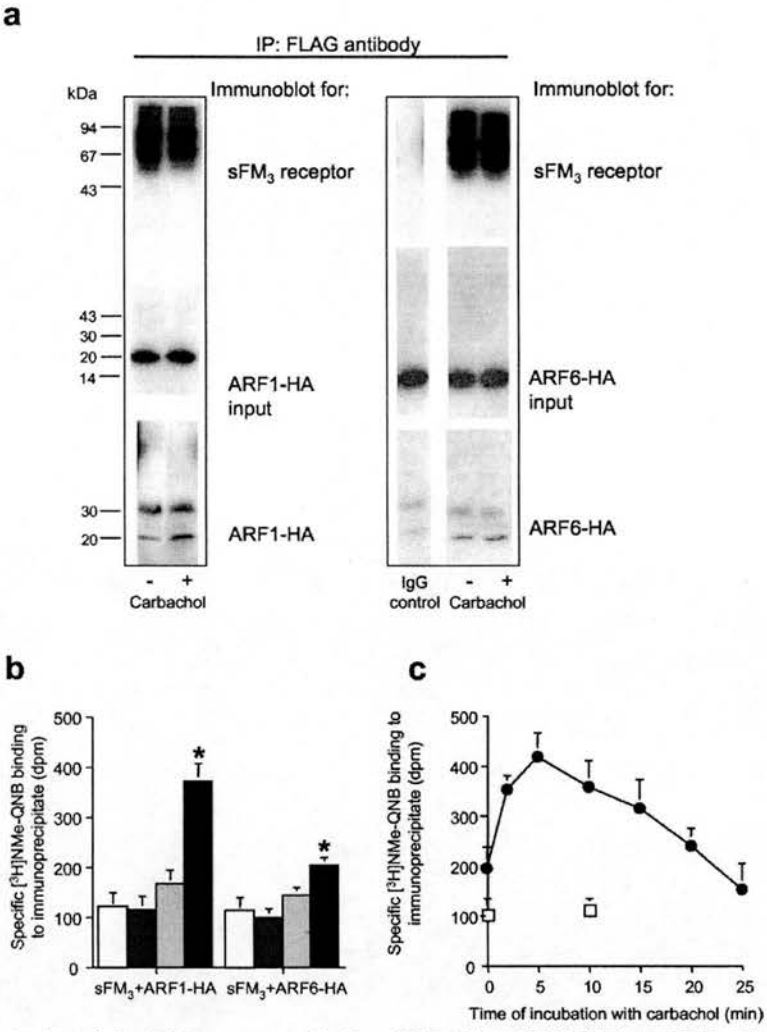

FIG. 4. **Co-immunoprecipitation of the sFM_3 receptor and either ARF1-HA or ARF6-HA from COS7 cells.** *a*, COS7 cells co-transfected with sFM_3 receptor plus ARF1-HA or ARF6-HA were stimulated with carbachol (20 μM, 15 min) or control prior to solubilization. Extracts were immunoprecipitated with FLAG antibody (or nonimmune IgG control) before SDS-PAGE and Western blotting. The *left panel* is from cells co-transfected with sFM_3 receptor and ARF1-HA; the *right panel* is from cells with sFM_3 receptor and ARF6-HA. In the *top sections*, the immunoprecipitate was probed with an antibody against the M_3 receptor i3 sequence. The *middle sections* show the input levels of immunoreactive ARF-HA in original extracts. The *bottom sections* show HA immunoreactivity associated with the immunoprecipitated receptor and indicate a carbachol-induced increase in the association of ARF1-HA but not ARF6-HA. The receptor runs as a broad band centered at about 90 kDa, diffuse because of glycosylation. ARF1-HA and ARF6-HA run at ~20 kDa. A nonspecific band was seen at ~30 kDa in all samples, which is likely to reflect nonspecific cross-reaction with immunoglobulins. These observations were typical of six separate experiments. *b*, results from an alternative procedure in which ARF-HA immunoprecipitates were probed for the presence of sFM_3 receptor by the measurement of specific [^{3}H]NMe-QNB binding. Extracts from control mock immunoprecipitation with nonimmune IgG are shown for unstimulated cells (*white columns*) or following 20 μM carbachol for 5 min (*dark gray columns*). Corresponding anti-HA immunoprecipitates are shown from cells that were unstimulated (*light gray columns*) or carbachol-stimulated (*black columns*). Both ARF1-HA and ARF6-HA showed significantly increased association with specific [^{3}H]NMe-QNB binding sites following carbachol compared with unstimulated or nonimmune IgG controls. Values are means ± S.E., $n = 6$. *, $p < 0.05$, Mann-Whitney *U* test. Input levels of both sFM_3 receptor and ARF1-HA/ARF6-HA in original cell extracts were shown to be matched between samples (data not shown). *c*, time course of association between ARF1-HA and the sFM_3 receptor as reflected by specific [^{3}H]NMe-QNB binding. The ARF1-HA immunoprecipitates, but not nonimmune IgG controls (● and □, respectively), showed a rapid time course of carbachol-induced increases in interaction (peaking at around 5 min and declining again to basal levels within 30 min).

inactive mutants of PLD1 (K898R-PLD1) and PLD2 (K758R-PLD2) were co-transfected with the sFM_3 receptor to assess any disruption of carbachol-induced [^{3}H]PtdBut and [^{3}H]InsP responses (Fig. 6, *a* and *b*). K898R-PLD1 but not K758R-PLD2 significantly reduced carbachol-induced [^{3}H]PtdBut responses (Fig. 6*a*), although both constructs were adequately expressed (data not shown). In contrast, transfection of a PLD1 mutant with selectively reduced responsiveness to activation by PKC but not ARF/Rho (PIM87-PLD1 (6, 7), caused a significant increase in sFM_3 receptor responses, which remained sensitive to BFA with an IC_{50} of 65.4 ± 13.1 μM ($n = 4$). Neither K898R-PLD1 nor PIM87-PLD1 affected basal [^{3}H]PtdBut responses, whereas K758R-PLD2 caused a small, but consistent reduction (in the order of 20–30%) (Fig. 6, *a* and *e*). The catalytically inactive PLD mutants had no discernible effect on PLC responses of the receptor (Fig. 6*b*).

We then asked whether the K898R-PLD1-sensitive or -resistant components of the sFM_3 receptor [^{3}H]PtdBut response corresponded to the sensitivity to BFA, T31N-ARF1, or T27N-ARF6 (Fig. 6, *c* and *d*). Whereas control responses were inhibited by BFA with an IC_{50} of 47.4 ± 6.3 μM, and those in the presence of K758R-PLD2 were still clearly inhibited (IC_{50} of

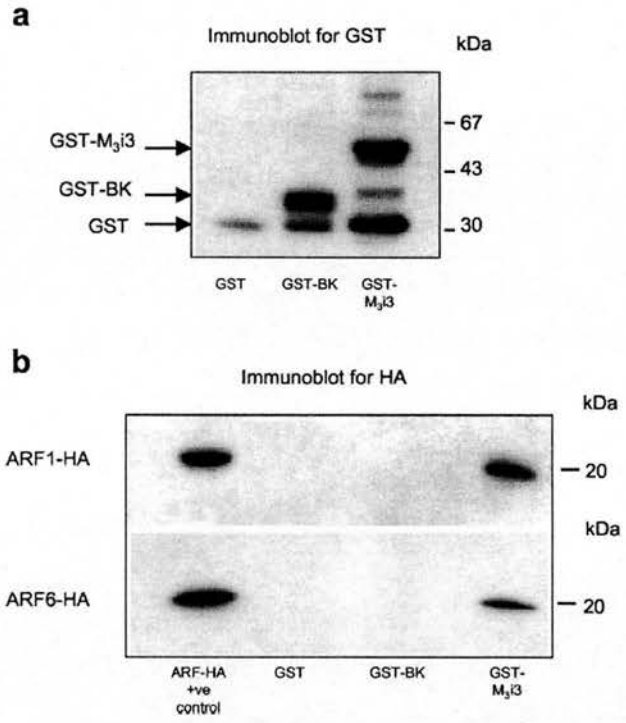

FIG. 5. ***In vitro* association of ARF1-HA or ARF6-HA with GST fusion protein of the M_3 receptor third intracellular loop.** GST fusion proteins were captured on glutathione-Sepharose to form affinity matrices, which were incubated with cytosolic extracts from COS7 cells transfected with ARF1-HA or ARF6-HA. Attached proteins were separated by SDS-PAGE and immunoblotted. Control GST constructs (GST-BK, a segment of the BK potassium channel (~35 kDa), and GST alone (~29 kDa)) were compared with the M_3i3 construct (~49 kDa)). *a*, input of constructs, immunoblotted for GST. *b*, HA immunoblots to detect bound ARF1-HA or ARF6-HA, demonstrating specific binding of each to the M_3i3 construct. The positive control lanes reflect the level of ARF input (with a 12.5-fold dilution factor).

33.1 ± 8.9 μM, n = 10), the residual response in the presence of K898R-PLD1 was unaffected by BFA (Fig. 6*c*). We further examined the effects of T31N-ARF1 and T27N-ARF6 on responses in the presence of K898R-PLD1 or K758R-PLD2 expression. Fig. 6*d* shows that negative mutant ARF1 and ARF6 constructs significantly inhibited both control responses and those in the presence of K758R-PLD2. The residual [^{3}H]PtdBut response in the presence of K898R-PLD1 was no longer sensitive to further inhibition by the negative mutant ARF1 construct but retained a small yet significant inhibitory effect of T27N-ARF6. These data suggest that the receptor uses an ARF1-mediated (BFA-sensitive) pathway to PLD1 and an ARF6-mediated (BFA-insensitive) pathway that can lead to either PLD1 or PLD2.

Fig. 6*e* demonstrates, in contrast, that K758R-PLD2 (but not K898R-PLD1) inhibits [^{3}H]PtdBut responses of the P_{2U} receptor. Matching observations were made with the (similarly BFA-insensitive) N376D mutant 5-HT_{2A} receptor. The responses to 5-HT (10 μM) were 2.67 ± 0.36-, 2.89 ± 0.46-, and 1.28 ± 0.15-fold basal for the N376D-5-HT_{2A} receptor alone and that in the presence of K898R-PLD1 or K758R-PLD2, respectively. The inhibition due to K758R-PLD2 was statistically significant ($p < 0.05$, Mann-Whitney U test, n = 6). Fig. 6*e* also shows that PDBu-induced [^{3}H]PtdBut production was attenuated by both K898R-PLD1 and K758R-PLD2, consistent with evidence that not only PLD1 (5–7) but also PLD2 (47–49) can be targeted by PKC. In addition, effects of wild type PLD1, wild type PLD2, and PIM87-PLD1 expression were compared on basal, PDBu-evoked, sFM_3 receptor, and P_{2U} receptor-mediated responses. Wild type PLD2, but not the other constructs, caused a marked increase in basal [^{3}H]PtdBut levels, matching reports of its constitutive activity (27). Responses to PDBu, carbachol, and ATP were all nonselectively increased. In contrast, wild-type PLD1 increased PDBu-evoked and sFM_3 receptor-mediated, but not P_{2U} receptor-mediated responses. PIM87-PLD1 caused a significant increase in sFM_3 receptor-mediated responses only, consistent with the idea that the role of PLD1 in sFM_3 receptor responses is independent of PKC.

Subcellular Trafficking of Components in M_3 Receptor PLD Activation—Since BFA disrupts the structural integrity of the Golgi apparatus at concentrations less than or equal to those used here (50), we asked whether altered trafficking of proteins needed for the signaling pathway, such as PLD itself, might contribute to the inhibitory effect of BFA. First, it is clear that a number of other GPCRs have PLD responses that are unaffected by BFA (Fig. 1*b*) (12). Second, when we compared the effects of BFA (Fig. 1*b*) with those of two further Golgi-disrupting agents, ilimaquinone and nocodazole (31, 35, 51), on PLD responses mediated by M_3 and TP receptors in 1321N1 cells, neither mimicked the effect of BFA receptor; nor did they affect responses to U46619 (30 μM) or PDBu (300 nM) (Fig. 1*b*; data not shown). [^{3}H]PtdBut responses to 200 μM carbachol were 6.62 ± 0.46- and 5.72 ± 0.64-fold basal with ilimaquinone (25 μM for 30 min) and nocodazole (10 μM for 4 h), respectively, compared with values of 6.67 ± 0.34 and 3.31 ± 0.52 for carbachol alone and carbachol plus 100 μM BFA (n = 6). In the presence of ilimaquinone, the IC_{50} for BFA was 72.1 ± 14.1 μM, similar to that in control conditions (Fig. 1*b*) and further suggesting that the effect of BFA on M_3 receptor PLD responses was distinct from any effects on Golgi structure.

To investigate whether endocytosis of the sFM_3 receptor might be necessary for its PLD responses, we utilized a dominant negative construct of dynamin 1, which reduces the internalization of agonist-occupied M_3 receptors (52, 53). Whereas transfection of K44A-dynamin 1 clearly reduced internalization of specific [^{3}H]oxotremorine-M binding to the sFM_3 receptor in COS7 cells, carbachol-induced [^{3}H]PtdBut production was unaltered, suggesting that endocytosis is not important for receptor PLD response (Fig. 7*a*). The internalization of specific [^{3}H]oxotremorine-M binding was unaffected by BFA (150 μM for 30 min) or by transfection of either T31N-ARF1 or T27N-ARF6 (data not shown). To address more directly the possibility that sFM_3 receptor-mediated PLD activation might occur in endocytosing vesicles, we carried out subcellular fractionation of COS7 cell membranes after carbachol stimulation and analyzed the location of the [^{3}H]PtdBut production. Alkaline Percoll gradients (24) were used to separate plasma membrane, Golgi, and endoplasmic reticulum fractions, characterized by immunoreactivity for Na^+/K^+ ATPase, GM130, and EEA1, respectively (Fig. 7*b*). Carbachol induced a large increase in [^{3}H]PtdBut production in plasma membrane fractions, with a much smaller response being detected in Golgi and endoplasmic reticulum fractions.

The question of whether ARF and PLD proteins undergo translocation to the plasma membrane following stimulation with carbachol was addressed by immunoblots on the Percoll gradient fractions. Under basal conditions, ARF1 was distributed through plasma membrane and Golgi fractions, whereas PLD1 was detectable only in non-plasma membrane fractions (Fig. 7*b*). After carbachol stimulation, ARF1 and PLD1 became concentrated or newly detectable, respectively, in plasma membrane fractions, and this translocation was not prevented by the presence of BFA (Fig. 7*b*). ARF6 and PLD2 were detectable in plasma membrane fractions with or without carbachol (data not shown).

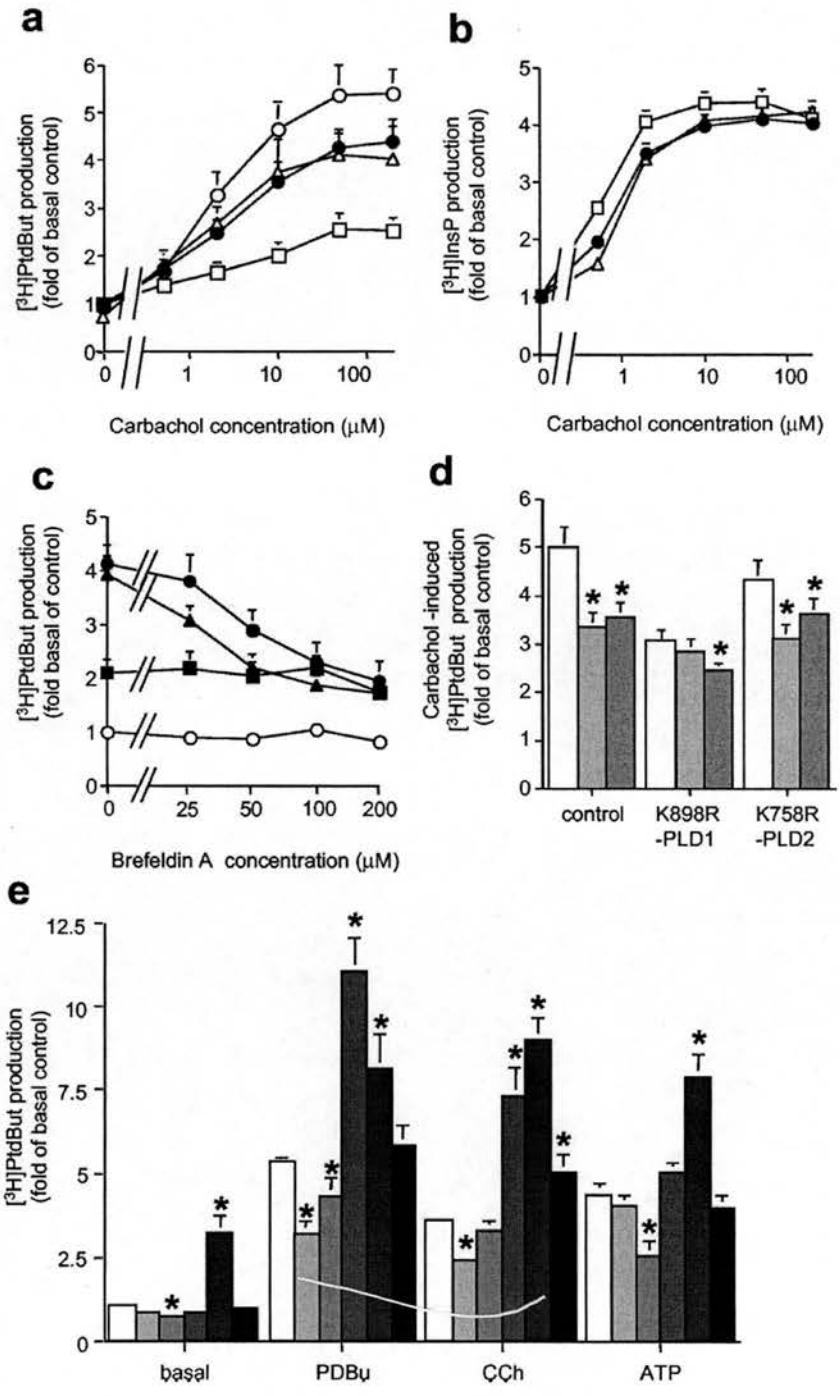

FIG. 6. **Effects of co-transfection with mutant or wild type PLD constructs on the PLD and PLC responses of the sFM_3 receptor in COS7 cells.** The [^{3}H]PtdBut (PLD) and [^{3}H]InsP (PLC) responses evoked by carbachol (and other stimuli) were measured in COS7 cells transfected with the sFM_3 receptor and PLD1/2 constructs. Values are means ± S.E., n = 6–10. *a*, concentration dependence of PLD responses to carbachol in the presence of control vector (●), K898R-PLD1 (catalytically inactive; □), K758R-PLD2 (catalytically inactive; △), and PIM87-PLD1 (PKC activation-deficient; ○). Responses to 10–200 μM carbachol were significantly reduced in the presence of K898R-PLD1, and responses to 50–200 μM carbachol were significantly increased in the presence of PIM87-PLD1 ($p < 0.05$, Wilcoxon test). *b*, concentration dependence of PLC activation by carbachol in the presence of control vector (●), K898R-PLD1 (□), and K758R-PLD2 (△). *c*, concentration dependence of BFA effects on PLD responses to 200 μM carbachol in the presence of control vector (●), K898R-PLD1 (■), and K758R-PLD2 (▲) as well as on basal levels of [^{3}H]PtdBut accumulation (○). BFA (50–200 μM) caused significant inhibition of the PLD responses to carbachol in the presence of control vector or negative mutant PLD2 ($p < 0.05$, Wilcoxon test). *d*, effects of co-transfection with negative mutant ARFs on sFM_3 receptor-mediated PLD responses, either control (empty vector) or the residual responses in the presence of K898R-PLD1 or K758R-PLD2. Cells were additionally transfected with vector (*white columns*), T31N-ARF1 (*light gray columns*), or T27N-ARF6 (*medium gray columns*), and the [^{3}H]PtdBut production induced by 200 μM carbachol was measured. Values are means ± S.E., n = 6. Statistically significant differences from control carbachol-induced responses are indicated with *asterisks* ($p < 0.05$, Wilcoxon test). *e*, comparison of the effects of various PLD1/2 constructs on [^{3}H]PtdBut production mediated by the sFM_3 receptor and the native P_{2U} receptor in COS7 cells as well as basal activity and that induced by PDBu. Basal, PDBu (300 nM)-, carbachol (*CCh*; 200 μM)-, and ATP (10 μM)-evoked responses were assessed in the presence of control empty vector (*white columns*), K898R-PLD1 (*light gray columns*), K758R-PLD2 (*medium gray columns*), wild type PLD1 (*dark gray columns*), wild type PLD2 (*charcoal gray columns*), and PIM87-PLD1 (*black columns*). Values are means ± S.E., n = 6–8. Statistical significance of differences from empty vector controls is indicated by *asterisks* ($p < 0.05$, Wilcoxon test).

DISCUSSION

A BFA-sensitive Route of PLD Activation for the M_3 Receptor but Not Other GPCRs—The M_3 muscarinic receptor shows BFA-sensitive activation of PLD when expressed as a native receptor in 1321N1 cells or heterologously in COS7 cells. Time course experiments showed rapid desensitization of M_3 receptor PLD responses in 1321N1 cells as reported previously (44) and revealed that this was unaltered by BFA. BFA sensitivity of PLD responses was seen at low as well as high agonist concentrations and for a partial agonist, indicating that coupling to this pathway was not restricted to a particular level of agonist occupancy. PLC responses of the M_3 receptor in both cell types were unaffected by BFA as were the PLD responses of control GPCRs, the TP receptor in 1321N1 cells and the P_{2U} receptor and N376D mutant 5-HT_{2A} receptor in COS7 cells. In contrast to the M_3 receptor, which contains an NP*X*X*Y* motif in tm7, each of these contains the alternative DP*X*X*Y* sequence, which is believed to prevent receptor coupling to BFA-sensitive PLD activation (12). PLD responses elicited by PMTx or U46619, but not by carbachol, were inhibited by the PLC inhibitor U73122, suggesting an indirect PLC-dependent route of PLD activation for the TP receptor but not the M_3 receptor.

We considered further whether Ca^{2+} elevation or PKC activity might still play a role in M_3 receptor PLD responses. Ca^{2+}

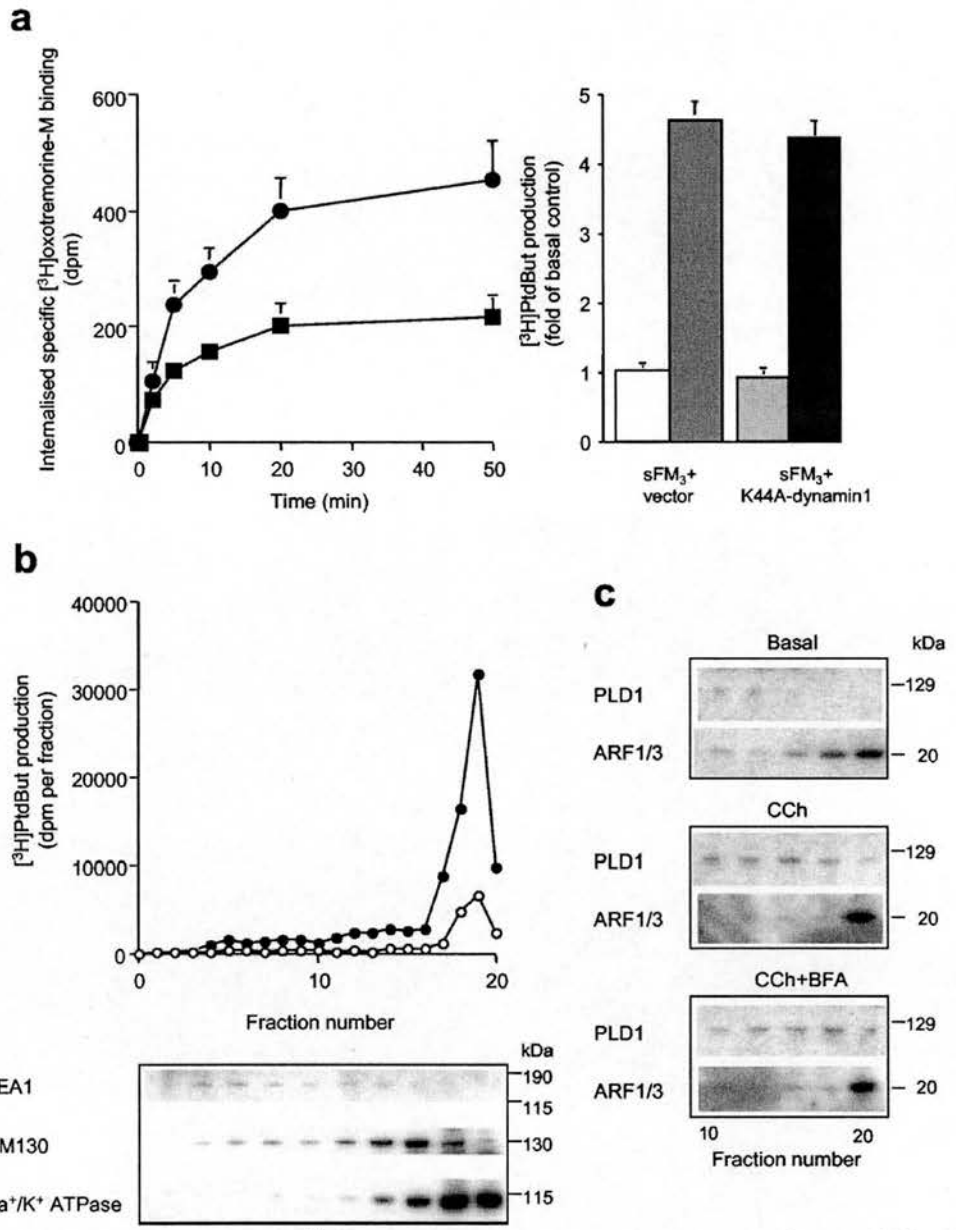

FIG. 7. **Evidence that sFM_3 receptor-mediated [^{3}H]PtdBut production occurs at the plasma membrane of COS7 cells and involves agonist-induced translocation of mediator proteins to that compartment.** *a*, the *left panel* shows the inhibitory effect of transfection with the K44A-dynamin 1 (dominant negative mutant) on internalization of [^{3}H]oxotremorine-M (a hydrophilic agonist ligand) into an acid strip-resistant compartment. ●, cells with sFM_3 receptor alone; ■, cells with sFM_3 receptor and K44A-dynamin 1. The cell surface-specific binding of [^{3}H]oxotremorine-M was unaffected (data not shown). The *right panel* shows [^{3}H]PtdBut production in control cells in basal (*white column*) or carbachol (200 μM)-stimulated conditions (*medium gray column*) as well as in cells co-transfected with K44A-dynamin 1 in basal (*light gray column*) or carbachol-stimulated conditions (*black column*). sFM_3 receptor-mediated PLD activation was unaltered by K44A-dynamin 1. *b*, subcellular distribution of membrane-associated basal (○) or carbachol (200 μM)-stimulated (●) [^{3}H]PtdBut production in sFM_3 receptor-transfected COS7 cells. Membranes were separated on Percoll gradients into fractions, which were characterized by immunoblot for the endoplasmic reticulum, Golgi, and plasma membrane markers (EEA1, GM130, and Na^+/K^+-ATPase, respectively). The response to carbachol was associated predominantly with plasma membrane fractions. *c*, the subcellular distribution of immunoreactivity for native PLD1 and ARF1/3 in membranes of cells under basal conditions or stimulated with carbachol (200 μM) or carbachol plus 150 μM BFA. Although the ARF antibody used cross-reacts with ARF3 as well as ARF1, the latter form is greatly predominant in cells. Under basal conditions, PLD1 was widely distributed, except in plasma membrane fractions, and ARF1/3 was present mainly in Golgi and plasma membrane fractions. Following carbachol stimulation, PLD1 immunoreactivity extended through to the plasma membrane, and ARF1/3 immunoreactivity became concentrated in plasma membrane fractions. BFA treatment did not disrupt carbachol-induced translocation of either PLD1 or ARF1/3.

mobilization does not appear to be an important mediator in 1321N1 (54) or HEK 293 cells (9) but the evidence for a role of PKC in M_3 receptor PLD responses is equivocal. In 1321N1, but not HEK 293 cells, PKC down-regulation is reported to inhibit the M_3 response (9, 55). However, the profound PKC activation involved in the procedure makes interpretation difficult. In apparent contrast, in M_3 receptor-transfected HEK293 cells, co-transfection of the PKC activation-deficient mutant, PIM87-PLD1, yielded smaller PLD responses to carbachol than did excess wild type PLD1 (6), but it was not clear how this com-

pared with responses with native PLD alone. However, in experiments with the related M_1 receptor, PIM87-PLD1 facilitated the response to carbachol, compared with untransfected cells (7), suggesting that PKC-independent pathways to PLD were being utilized, as we found here with the M_3 receptor. Our observations with various PKC inhibitors, designed to block both catalytic and regulatory domains, provide no evidence to suggest a major contribution of PKC to the M_3 receptor PLD response in 1321N1 cells.

BFA inhibited M_3 receptor PLD responses here in 1321N1 and COS7 cells with IC_{50} values of about 50 μM. BFA sensitivity of M_3 receptor PLD responses in HEK 293 cells has been reported previously but with some 2–3-fold lower potency (10), as we confirmed in transiently transfected HEK 293 cells (IC_{50} of 157 ± 23 μM, $n = 4$). The lower potency in HEK 293 cells may reflect greater involvement of an alternative tyrosine kinase-dependent pathway. In A10 smooth muscle cells, PLD responses of angiotensin II and ET-1 receptors were strongly inhibited by BFA (56), whereas formyl-Met-Leu-Phe and ATP receptor responses in differentiated HL60 cells and bradykinin and sphingosine 1-phosphate receptor responses in A549 adenocarcinoma cells were not (57, 58). The extent to which a GPCR demonstrates BFA-sensitive PLD responses in different cell types may well be influenced by the cellular content of mediators for particular pathways. The concentrations of BFA that selectively inhibit M_3 receptor PLD responses here exceed those needed to disrupt the integrity of Golgi membranes (50, 57, 58), but are similar to those that inhibit the ARF-GEFs, BIG1/2 (43). Considering whether disruption of Golgi traffic might play a role here, we confirmed that the cell surface expression of M_3 receptors and their PLC activation were unaffected by BFA (although these measures may not be very sensitive to acute disruption of trafficking). It is possible that PLD responses, but not other responses of GPCRs, may have a selective requirement for protein trafficking and thus may be selectively sensitive to Golgi disruption by BFA. Other GPCRs have clearly BFA-insensitive PLD responses, although theoretically they might generate their PLD responses by different mediators that are unaffected by disruption of the Golgi. However, the selective effect of BFA on M_3 receptor PLD responses was not mimicked by ilimaquinone and nocodazole, which also profoundly disrupt Golgi structure and function. Furthermore, we established that the subcellular location of carbachol-induced [^{3}H]PtdBut production in sFM_3 receptor-containing COS7 cells was predominantly in the plasma membrane fraction and showed directly that whereas the response involved a movement of both PLD1 and ARF1 to this site, the translocation was not prevented by BFA. Similar observations were found using confocal microscopy (data not shown). Therefore, the effects of BFA on PLD responses of particular receptors appear to reflect a specific intervention in signal transduction rather than a general disruption of protein trafficking.

ARF1 and ARF6 Involvement in PLD Activation by the sFM_3 Receptor but Not Other GPCRs—We addressed the role of different subtypes of ARF in sFM_3 receptor PLD activation by transfection of either wild type ARF1 or ARF6 or their dominant negative constructs, T31N-ARF1 and T27N-ARF6 (14). Neither wild type ARF construct significantly affected PLD activation by carbachol, suggesting that the cellular content of endogenous ARFs is probably not a limiting factor. However, dominant-negative ARF1 and ARF6 constructs each inhibited PLD responses without modifying PLC responses. Effects of negative mutant ARF1 and ARF6 in combination were significantly greater than either alone, suggesting that the two ARF isoforms might each play a distinct role. Although negative or positive mutants of ARFs can disrupt Golgi and other vesicular trafficking (14, 59–62), we found that neither the levels of specific cell surface [^{3}H]oxotremorine-M binding sites nor sFM_3 receptor PLC responses were affected by the ARF constructs here. In parallel with our observations, PLD responses of the angiotensin II and ET-1 receptors in A10 cells were inhibited by both T31N-ARF1 and T27N-ARF6 constructs (56). In contrast, we showed that the responses of two DP*XXY*-containing receptors, the P_{2U} receptor and the N376D mutant 5-HT_{2A} receptor, were unaffected by T31N-ARF1 but were clearly inhibited by T27N-ARF6 and PKC inhibitors (P_{2U} receptor) or by PKC inhibitors alone (N376D mutant 5-HT_{2A} receptor). This suggests that ARF6 and PKC may be important in alternative pathways that underlie the BFA-insensitive [^{3}H]PtdBut production seen with some GPCRs. The BFA sensitivity of M_3 receptor PLD responses was clearly preempted in the presence of dominant negative ARF1 but not ARF6, indicating that an ARF1-dependent pathway from the receptor, probably involving BIG1/2, is responsible for the sensitivity to BFA. Correspondingly, it has been shown that BIG1/2 can act as effective, BFA-sensitive ARF-GEFs for ARF1 but not ARF6 (43) and that *in vivo* functional effects of ARF6 are often BFA-insensitive (63, 64). The sFM_3 receptor PLD response in COS7 cells therefore appears to comprise at least two components: an ARF1-dependent BFA-sensitive pathway and an ARF6-dependent, BFA-insensitive pathway.

Physical Association of Both ARF1 and ARF6 with the M_3 Receptor through Its i3 Domain—Low levels of ARF1-HA and ARF6-HA were associated with sFM_3 receptor immunoprecipitates under basal conditions, whereas the amount of associated ARF1-HA but not ARF6-HA was clearly increased when cells were incubated with carbachol. In an alternative, rapid procedure where reduced nonspecific interactions were expected, we found that a small, carbachol-induced increase in ARF6-HA interaction with the receptor could be shown as well as that for ARF1-HA. The time course of carbachol-induced association of ARF1-HA with the receptor was similar to that for the increase in [^{3}H]PtdBut production.

Using GST fusion proteins, we further investigated the interaction of ARF1 and ARF6 with the M_3 i3 domain, which is known to contain sites for interaction with heterotrimeric G proteins, arrestins, G$\beta\gamma$, and the kinases GRK2 and CK1-α (37, 65–67). We showed previously that i3 domain splice variants of the PAC_1 receptor show marked differences in their BFA-sensitive activation of PLD but not other signaling responses (39), suggesting that M_3 i3 might be a good candidate site for ARF docking here. Specific binding of each ARF was demonstrated to the M_3i3 GST fusion protein but not control constructs.

PLD1 Involvement in PLD Activation by the M_3 Receptor but Not Other GPCRs—A catalytically inactive mutant of PLD1, but not PLD2, specifically attenuated carbachol-induced PLD responses in sFM_3 receptor-transfected COS7 cells. A component of the response remained unaffected by either negative mutant PLD1 or PLD2, perhaps due to limited ability of the constructs to access the necessary sites and compete effectively with endogenous PLD. Other studies on the PLD isozyme mediating GPCR responses have given varying results that may partly depend on cell type. In HEK 293 cells, M_3 receptor PLD responses were large in the presence of additional wild type PLD1, but not K898R-PLD1 (6, 7), although it was not clear whether K898R-PLD1 could reduce responses mediated by endogenous PLD. In our experiments, [^{3}H]PtdBut responses of the sFM_3 receptor but not other GPCRs were selectively increased by both wild type PLD1 and PIM87-PLD1 and inhibited by K898R-PLD1, implicating PLD1 as the key effector. Our evidence that PKC inhibitors are ineffective on M_3 receptor PLD responses in 1321N1 or COS7 cells further supports the

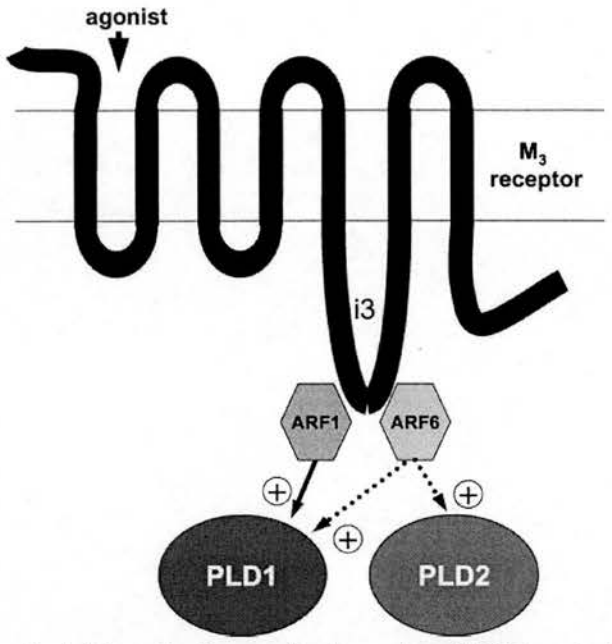

FIG. 8. **Schematic diagram of major routes of ARF-dependent PLD activation used by the M_3 receptor.** Additional routes of PLD activation by the M_3 receptor have been documented and the extent to which each is used is likely to depend on cell type and circumstances. An ARF-independent component may also contribute to the overall PLD response of the M_3 receptor here. Examples of other GPCRs compared under the same conditions appear to use differing pathways of PLD activation.

idea that this connection between the receptor and PLD1 is independent of PKC. In contrast, [^{3}H]PtdBut responses of the P_{2U} receptor and the N376D mutant 5-HT_{2A} receptor were inhibited by K758R-PLD2 but not K898R-PLD1. The addition of wild type PLD2, but not wild type PLD1, facilitated these responses, but effects were nonselective in that basal, M_3 receptor, and PDBu-induced responses were all increased. Other reports also indicate a role of PLD2 in the responses of some GPCRs; in A10 cells, PLD responses to angiotensin II were inhibited by K758R-PLD2 but not K898R-PLD1 constructs (56), and in PC12 cells overexpressing PLD2, bradykinin receptors activate PLD2 through a PKC-dependent mechanism (47). Furthermore, the μ-opioid receptor can elicit a BFA-sensitive PLD response in HEK 293 cells overexpressing PLD2, but not PLD1, that has been proposed to involve PLD2 association with the carboxyl-terminal tail domain of the receptor (68).

Relationship between M_3 Receptor Interaction with a BFA-sensitive, ARF1-dependent Pathway and Its Activation of PLD1—When sFM_3 receptor responses are partially reduced in the presence of K898R-PLD1 (but not K758R-PLD2), any further sensitivity to BFA or T31N-ARF1 is removed. This suggests that the inhibitory effect of BFA seen under normal conditions reflects primarily a pathway involving ARF1 and PLD1. T27N-ARF6 still caused some inhibition of responses in the presence of either K898R-PLD1 or K758R-PLD2, consistent with the idea that the ARF6-mediated component from the receptor may lead to either PLD1 or PLD2. However, we cannot definitively assign a PLD isoform to the ARF6-mediated component, because we are not sure that blockade by negative mutant PLD1 is complete and also negative mutant PLD2 did not significantly reduce control sFM_3 responses.

The Subcellular Localization of sFM_3 Receptor-mediated, ARF1-dependent Activation of PLD1—Since ARF1 (13–18, 69) and PLD1 (27–31) are not normally localized to a large extent at the plasma membrane, either the receptor or these mediators may need to undergo translocation to enable sFM_3 receptor-induced [^{3}H]PtdBut production. One possibility might be that the receptor causes PLD activation in endocytosing vesicles following agonist stimulation. For some GPCRs, such a mechanism involving endocytosis of GPCR and/or transactivated growth factor receptors, is important in their activation of extracellular signal-regulated kinase mitogen-activated protein kinase (70). PLD may be integral to these processes, since it participates in insulin-induced extracellular signal-regulated kinase mitogen-activated protein kinase activation by generating phosphatidic acid in endocytosing vesicles and thereby recruiting the mitogen-activated protein kinase/extracellular signal-regulated kinase kinase kinase, Raf-1 (71, 72). However, our findings seem inconsistent with such a mechanism here. Dominant negative mutant dynamin 1 inhibited endocytosis of agonist-occupied sFM_3 receptors but had no effect on PLD activation, whereas cell surface biotinylation indicated that the great majority of sFM_3 receptors associated with ARF1-HA immunoprecipitates had been present on the cell surface. Furthermore, sFM_3 receptor-mediated [^{3}H]PtdBut production was associated with subcellular fractions containing plasma membrane rather than Golgi or endoplasmic reticulum. In addition, the content of native ARF1 and PLD1 in plasma membrane was clearly and selectively increased following carbachol stimulation. Corresponding results were found in confocal microscopy experiments (data not shown). These observations suggest that agonist-induced translocation of ARF1 and PLD1 to the plasma membrane, into the vicinity of sFM_3 receptors, is important in enabling the receptor to signal via PLD.

In conclusion, the present experiments describe an ARF-dependent activation of PLD by the M_3 muscarinic receptor that appears to be essentially independent of conventional routes of GPCR signaling. Instead, both ARF1 and ARF6 can associate physically with the receptor, as shown by co-immunoprecipitation and GST fusion protein experiments. Dominant negative constructs of ARF1/6 and PLD1/2 showed that the characteristic BFA-sensitive PLD activation shown by the M_3 receptor appears to involve ARF1-mediated activation of PLD1 (Fig. 8). We demonstrated that agonist induces the translocation of ARF1 and PLD1 into the vicinity of the M_3 receptor at the plasma membrane, where the response takes place. An additional ARF6-mediated component may involve PLD1 or PLD2, whereas other factors such as Rho family small G proteins could potentially also be involved. In contrast, examples of DP*XXY*-containing GPCRs, which lack BFA-sensitive PLD responses, utilize PKC (and also in some cases ARF6) to bring about activation of PLD2. The range of GPCR motifs and cellular factors that determine receptor selectivity for these different pathways of PLD activation remains to be determined.

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Selective Interaction of ARF1 with the Carboxy-Terminal Tail Domain of the 5-HT_{2A} Receptor

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ABSTRACT

The 5-hydroxytryptamine 2A receptor (5-HT_{2A}R) is a member of the class I family of rhodopsin-related G protein-coupled receptors. The receptor is known to activate phospholipase C via the heterotrimeric G proteins $G_{q/11}$, but we showed previously that it can also signal through the phospholipase D (PLD) pathway in an ADP-ribosylation factor (ARF)-dependent manner that seems to be independent of $G_{q/11}$ (Mitchell et al., 1998). Both coimmunoprecipitation experiments and the effects of negative mutant ARF constructs on 5-HT_{2A}R-induced PLD activation here suggested that ARF1 may play a greater role than ARF6 in the function of this receptor. Furthermore, we demonstrated using glutathione *S*-transferase (GST)-fusion proteins of receptor domains that ARF1 and ARF6 bind to the third intracellular loop (i3) and the carboxy terminal tail (ct) of the 5-HT_{2A}R. The association of ARF1 with the ct domain of the receptor was stronger than its interaction with i3, or the interactions of ARF6 with either construct. Experiments using ARF mutants that are deficient in GTP loading, and the in vitro addition of GTPγS suggested that GTP loading enhances ARF1 binding to the receptor. The N376PxxY motif in the transmembrane 7 domain of the receptor (rather than a N376DPxxY mutant form) was shown to be essential for ARF-dependent PLD signaling and ARF1 coimmunoprecipitation. In GST-fusion proteins of the 5-HT_{2A}R ct domain, mutation of Asn376 to Asp also markedly reduced ARF1-HA binding, although additional motifs in the Asn376–Asn384 sequence and to a lesser extent elsewhere, seem also to contribute to the interaction.

The 5-hydroxytryptamine (5-HT) receptor superfamily represents a diverse group of receptors encompassing 14 different subtypes. All but one of them (5-HT_3) signal through G-protein-linked pathways. The different families of G protein-coupled receptors (GPCRs) for 5-HT transduce their signals via different heterotrimeric G proteins, with 5-HT_1 (and 5-HT_5) linking to $G_{i/o}$ effectors, 5-HT_2 signaling through $G_{q/11}$ to generate inositol trisphosphate and increase intracellular Ca^{2+} levels, and 5-HT_4, 5-HT_6, and 5-HT_7 all signaling through G_s to increase production of cyclic AMP (Raymond et al., 2001). The 5-HT_{2A}R is of particular interest because it has been implicated in a variety of major psychiatric disorders and in hallucinogenic drug action.

GPCR interactions with heterotrimeric G proteins often (but not exclusively) seem to involve the third intracellular loop (i3) (Wess et al., 1997). The i3 domains of various GPCRs have been shown to provide docking sites for heterotrimeric G protein $\beta\gamma$ subunits (Wu et al., 1998) as well as arrestins (Wu et al., 1997; Gelber et al., 1999), GPCR-kinases (Wu et al., 1998), and indeed ARFs (McCulloch et al., 2001; Ronaldson et al., 2002). In a number of GPCRs, other intracellular loops and ct domains have also been implicated in interactions with heterotrimeric G proteins and arrestins (Wu et al., 1997; Oakley et al., 2001). In the 5-HT_{2A}R, the i3 domain has been shown to be important for coupling to $G_{q/11}$ and the carboxyl terminal segment of the i3 loop in particular may

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ABBREVIATIONS: 5-HT, 5-hydroxytryptamine, serotonin; 5-HT_{2A}R, 5-hydroxytryptamine 2A receptor; GPCR, G protein-coupled receptor; PLD, phospholipase D; ARF, ADP-ribosylation factor; i3, third intracellular loop domain; ct, carboxy-terminal tail domain; tm7, 7th transmembrane domain; sPrC, protein C epitope tag with signal sequence; HA, hemagglutinin epitope tag; BFA, brefeldin A; GST, glutathione *S*-transferase; NI IgG, nonimmune mouse IgG; PLC, phospholipase C; PCR, polymerase chain reaction; PrC, Protein C; bp, base pair(s); DMEM, Dulbecco's modified Eagle's medium; USG, Ultroser G; AEBSF, 4-(2-aminoethyl)-benzenesulfonylfluoride; PAGE, polyacrylamide gel electrophoresis; GTPγS, guanosine 5′-*O*-(3-thio)triphosphate; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonate; SBTI, soybean trypsin inhibitor; ECL, enhanced chemiluminescence; MEM, minimum essential medium; PEG, polyethylene glycol; GEF, GTP-exchange factor; BK, large-conductance potassium channel; PDZ, postsynaptic density 95/disc-large/ZO-1.

play a key role in the interaction (Roth et al., 1998). Arrestin isoforms bind to the i3 loop of the 5-HT_{2A}R as they do to i3 domains of the M_2 and M_3 muscarinic receptors, but show broader specificity in that both nonvisual and visual arrestins are bound (Wu et al., 1997; Gelber et al., 1999).

In addition to $G_{q/11}$-mediated phospholipase C (PLC) activation, the 5-HT_{2A}R can activate several other signaling pathways that may involve alternative direct coupling to the receptor. These include activation of phospholipase A_2 [which may be mediated by a coupling mechanism other than $G_{q/11}$ (Berg et al., 1998)], activation of tyrosine phosphorylation [correlating with evidence for association of the tyrosine kinase Jak2 with the ct domain (Guillet-Deniau et al., 1997)] and ARF-dependent activation of phospholipase D (PLD) (Mitchell et al., 1998).

A specific conserved motif, NPxxY, which is found at the junction of the tm7 and ct domains in a number of rhodopsin family GPCRs, has been implicated as a determinant of ARF-receptor interactions and ARF-mediated signaling, because native receptors with an alternative DPxxY motif, or Asn-to-Asp mutant receptors, show selective defects in this pathway (Mitchell et al., 1998, 2003). However, it has not been clear whether this motif might be accessible as a direct docking site or it instead regulates access to a distinct site.

The two main classes of cellular ARFs are exemplified by ARF1 and ARF6, which are thought to have characteristically distinct subcellular distributions. In many cells, ARF1 is cytosolic or associated with Golgi membranes, whereas ARF6 may be substantially associated with plasma membrane and play a role in regulating endocytosis (D'Souza-Schorey et al., 1995; Peters et al., 1995; Cavenagh et al., 1996). Nevertheless, GTP loading and GPCR activation can cause translocation of ARFs to Golgi and other membranes, and we have shown marked translocation of ARF1 to the plasma membrane after activation of the M_3 muscarinic receptor (Mitchell et al., 2003) and the 5-HT_{2A}R (M. Johnson and R. Mitchell, unpublished observations). Thus both ARF1 and ARF6 are potentially available for interaction with plasma membrane GPCRs after agonist stimulation.

In this study, we have addressed the role of ARF1 and ARF6 in 5-HT_{2A}R-mediated PLD signaling, demonstrated coimmunoprecipitation of ARF1 (and to a lesser extent ARF6) with the receptor, and gone on to characterize the docking of ARFs to GST fusion proteins of receptor i3 and ct domains.

Materials and Methods

Preparation of sPrC-Tagged 5-HT_{2A} Receptor and sPrC-Tagged N376D-5-HT_{2A} Mutant Receptor Constructs. The wild-type human 5-HT_{2A}R cDNA (SCS93) and N376D-5-HT_{2A}R mutant cDNA (SCS103), cloned into pcDNA1-amp (Invitrogen, Paisley, UK), were kindly provided by Stuart Sealfon (Mount Sinai School of Medicine, New York, NY). To create an amino terminal epitope-tagged receptor, SCS93 was PCR-amplified with the synthetic oligonucleotide primers [5′-GAAGATCAGGTAGATCCACGGTTAATC-GATGGTAAGGCCATGGATATTCTTTGTGAAG-3′] encoding the 12-amino acid Protein C tag (PrC) epitope (EDQVDPRLIDGK) and the reverse primer named 5HT_{2A}.rp, [5′-GAATTCTCACACA-CAGCTCACCTTTTCATT-3′] using the Herculase-enhanced DNA polymerase (Stratagene Europe, Amsterdam, NL). The resulting PCR product was checked by agarose gel electrophoresis and extracted with Wizard PCR clean-up resin (Promega, Southampton, UK) before amplifying with the primer [5′-GCCACCATGAAGAC-GATCATCGCCCTGAGCTACATCTTCTGC-CTGGTATTCGCCGAAGATCAGGTAGATCCAC-3′] encoding a modified influenza hemagglutinin signal sequence and 5HT_{2A}.rp. The 1.4-kilobase fragment was purified by agarose gel electrophoresis and Qiaex II (QIAGEN Ltd.,Crawley, UK), then subcloned into TOPO TA cloning vector (Invitrogen). The sPrC-5-HT_{2A} insert was checked by sequencing. For expression studies, the sPrC-tagged wild-type receptor was constructed by subcloning the 200-bp *Eco*RI/*Sal*I fragment from sPrC-5-HT_{2A} cDNA and the 1.5-kilobase *Sal*I/*Xba*I fragment from SCS93 into the *Eco*RI/*Xba*I site of pcDNA3 (Invitrogen). The sPrC-tagged (N376D) mutant receptor was constructed by subcloning the 450-bp *Eco*RI/*Pst*I fragment from sPrC-5-HT_{2A} cDNA and the 1.3-kilobase *Pst*I/*Xba*I fragment from SCS103 into the *Eco*RI/*Xba*I site of pcDNA3 (Invitrogen).

Construction of 5-HT_{2A} Receptor Intracellular Loop 3 and Carboxy Terminal Tail GST-Fusion Proteins. The human 5-HT_{2A}R intracellular loop 3 (Ile258–Gly326) was PCR-amplified from SCS93 with primer pair [5′-GGGTGATCAAGTCACTTCA-GAAAGAAGCTAC-3′] and [5′-CGGAATTCTAGCCCAGCACCTTG-CATGCTTTTGCTCATTGCT-3′], and the resulting 200-bp PCR product was purified, digested with *Bcl*I/*Eco*RI, and subcloned into the *Bam*HI/*Eco*RI sites of pGEX-3X (Amersham Biosciences, Little Chalfont, UK). The 347-bp *Hin*cII fragment encoding the human 5-HT_{2A}R carboxy-terminal tail and including the NPLVY motif (Asn376-Val471) was subcloned into the modified (with Mung bean nuclease; Stratagene, La Jolla, CA) *Bam*HI site of pGEX-2T. The sequences encoding the carboxy-terminal tail amino acids (Lys385–Val471) were PCR-amplified from SCS93 using primer pair Bh2ARCT.KTYRS [5′-CGGGATCCAAAGACCTATAGGTCAGC-CTTTTCACG-3′] and h2ARCT.rp [5′-CAACTCAATTGTCACACA-CAGCTCACCTTTTCATT-3′], and the mutant (N376D-Val471) carboxyl tail was PCR-amplified from SCS103 with primer pair h2ARCT.DPLVY [5′-CGGGATCCAGACCCACTAGTCTACA-CACTGTTCAA-3′] and h2ARCT.rp using *Taq* polymerase (Promega) and the resulting PCR products purified by Wizard cDNA clean-up resin (Promega), digested with *Bam*HI/*Mfe*I and subcloned into the *Bam*HI/*Eco*RI sites of pGEX-2T. The *Bam*HI sites then were modified with Mung bean nuclease. Cloned inserts were checked by sequence analysis.

Transient Transfections of Cells. COS-7 cells were grown to 60% confluence in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) containing 10% normal calf serum and 100 units/ml penicillin and 100 μg/ml streptomycin. Before transfection, medium was changed for DMEM containing 2% Ultroser G (USG; Invitrogen) instead of serum. The cells were then transfected with combinations of the cDNAs for sPrC-5-HT_{2A}R, ARF1-V5-His_6 (Invitrogen), ARF1 and ARF6 tagged with the hemagglutinin epitope (HA) at the carboxyl terminal, or the dominant-negative mutants T31N-ARF1-HA and T27N-ARF6-HA (Peters et al., 1995) (kindly provided by Julie Donaldson, National Institutes of Health). All transfections (normally at a ratio of 4 μg of receptor construct cDNA to 1 μg of ARF construct cDNA per 75-cm^2 flask) were carried out using FuGENE-6 reagent (Roche Diagnostics Ltd, Lewes, UK) according to the manufacturer's guidelines, and appropriate substitutions of empty vectors were made in control samples. Transfected cells were used 72 h after transfection.

Preparation of ARF-Enriched Extracts. COS-7 cells transfected with various ARF-HA constructs were washed with 10 ml of Earle's balanced salt solution (Invitrogen) before being scraped into ice-cold extraction buffer [2 ml/175-cm^2 flask, 2 μg/ml aprotinin, 1 mM 4-(2-aminoethyl)-benzenesulfonylfluoride (AEBSF) (Alexis Biochemicals, San Diego, CA), 1 mM dithiothreitol, 1 mM pepstatin, 1 mM sodium orthovanadate, 1 mM NaF, 50 μg/ml soybean trypsin inhibitor (SBTI) in PBS]. All chemicals were from Sigma Chemical Co. (Poole, Dorset, UK) unless otherwise indicated. The cells were then homogenized [Ystral homogenizer (Scientific Industries Intl. UK Ltd, Loughborough, UK) setting 3, 15 s] before being centrifuged

at 12,000*g* for 20 min at 4°C. The supernatant was aliquoted and stored at −40°C.

Purification of ARF1-V5-His$_6$ on Cobalt Affinity Resin. COS-7 cells were transfected with an expression plasmid encoding ARF1-V5-His$_6$, and enriched cytosol was isolated as described above. The cytosolic extracts over-expressing the ARF1-V5-His$_6$ were then incubated with 'Talon' cobalt affinity resin (BD Biosciences Clontech, Palo Alto, CA) that had been pre-equilibrated with wash buffer (10 mM sodium phosphate, 60 mM NaCl, pH 7.0), for 1 h at 4°C. The resin was washed thoroughly with extraction/wash buffer (additionally containing 5 mM imidazole) before being eluted using elution buffer (additionally containing 300 mM imidazole) according to the manufacturer's instructions. The purified ARF1-V5-His$_6$ was then concentrated using Centriplus centrifugal filters (YM-3; Millipore, Bedford, MA) according to the manufacturer's instructions. The amount of protein present was verified by colorimetric measurement using Coomassie protein reagent (Perbio Science UK Ltd., Tattenhall, Cheshire, UK). The percentage purity of the harvested protein purity was then assessed by staining with high sensitivity Colloidal Coomassie (Simply Blue Safe stain; Invitrogen) on an SDS-PAGE gel, identifying the correct band by Western blotting using mouse monoclonal V5-tag antibody (Invitrogen). Standard curves were produced from the purified ARF1-V5-His$_6$ (linear range from 0.4–7.2 ng ARF1-V5-His$_6$ per well). These were coprocessed with experimental samples of cytosolic inputs and of proteins associating with the receptor in either coimmunoprecipitation or GST-fusion protein experiments, enabling us to assess the amounts of ARF1-V5-His$_6$ present.

Expression of GST Proteins. GST-5-HT$_{2A}$i3 and GST-5-HT$_{2A}$ct constructs, in the pGEX-3X and pGEX-2T vectors, as well as a control GST fusion protein of the STREX insert of the large conductance Ca^{2+}- and voltage-activated K^+ (BK) channel, in the pGEX-5 × 1 vector (kindly supplied by Mike Shipston, University of Edinburgh) were expressed in BL21-RIL bacterial cells, grown in standard 2× yeast extract/tryptone/NaCl medium with 2% glucose. When the cells had reached an A_{600} of 0.6 to 0.8 units/ml, expression of the fusion proteins was induced by the addition of 0.1 mM isopropyl-β-D-thiogalactoside for 3 h at 37°C. Cells were harvested by centrifugation then lysed with BugBuster reagent (Merck Biosciences, Beeston, Nottingham, UK) for 10 min and again centrifuged. The supernatant, containing the GST fusion proteins, was added to glutathione-Sepharose beads (Amersham Biosciences). The beads were incubated with the bacterial supernatant for 20 min at room temperature to allow binding of the GST fusion proteins to the beads. The matrix formed was then washed extensively with PBS and used immediately.

In Vitro Protein Interaction Assays. Cellular extracts enriched in the various HA-tagged ARF constructs were incubated with GST fusion proteins bound to glutathione-Sepharose beads (Amersham Biosciences) in 250 μl of buffer A (20 mM Tris-HCl, pH 7.5, 0.6 mM EDTA, 1 mM dithiothreitol, 70 mM NaCl, and 0.05% Tween 80) for 90 min at 4°C, with rolling. In some experiments, GTPγS (100 μM) was added to the incubations. The beads were washed four times in buffer A and then the retained proteins were removed from the beads with 2× Laemmli buffer (2% SDS, 5% mercaptoethanol, 20 mM Tris-HCl, pH 7.4) before SDS-PAGE and immunoblotting (see below). Input levels of fusion proteins (monitored by GST immunoreactivity) were carefully balanced to ensure comparability between samples. Protein interaction assays using ARF1-V5-His$_6$ were carried out in an identical manner, except that increased amounts of bacterial cytosol containing GST construct was added to the glutathione-Sepharose beads (~2×).

Coimmunoprecipitation of sPrC-5-HT$_{2A}$ Receptor and ARF1/6-HA. Transfected COS-7 cells were incubated in medium alone (no serum or USG) for 4 h before being exposed to 5-HT (10 nM–10 μM) and/or brefeldin A (BFA, 100 μM). Cells were washed once in Hank's balanced salt solution (HBSS; Invitrogen) before the addition of 1 ml/75-cm^2 flask of immunoprecipitation buffer (HEPES 20 mM, pH 7.5, NaCl 150 mM, 1% CHAPS, 0.5% sodium deoxycholate, 2 μg/ml aprotinin, 4 μg/ml leupeptin, 1 mM AEBSF, 2 μg/ml pepstatin, 1 mM sodium orthovanadate, 1 mM sodium fluoride, 5 mM sodium molybdate, and 50 μg/ml SBTI) with 5-HT if it had been used in the initial stimulation. Extraction was carried out on ice for 40 min with occasional agitation. Solubilized cellular extracts were centrifuged at 12,000*g* for 15 min at 4°C to remove particulate material. One ml of supernatant was precleared with 20 μl of protein G-Sepharose 4B fast flow (50% suspension in immunoprecipitation buffer) for 45 min at 4°C. After centrifugation (to pellet the beads), the supernatant was added to a tube containing either mouse monoclonal (Ca^{2+}-dependent) PrC-tag antibody (clone HPC4, 4 μg/ml; Roche) or control, nonimmune mouse IgG, 4 μg/ml; Sigma) with 40 μl/ml protein G-Sepharose suspension and 1 mM $CaCl_2$. Samples were incubated with rolling, at 4°C overnight. In the standard procedure, the beads were pelleted, washed twice in immunoprecipitation buffer before 40 μl of 2× Laemmli buffer containing 5 mM EGTA was added per sample equivalent to 1 ml of original supernatant. In some experiments, the receptor complex was removed from the PrC antibody-protein G-Sepharose beads by incubation in 50 mM Tris and 2 mM EGTA, pH 7.5, for 30 min at room temperature. The supernatant was removed and SDS and mercaptoethanol added equivalent to 2× Laemmli buffer.

Western Blots. Western blots were carried out on samples from immunoprecipitation and GST fusion protein interaction assays. Either 20% or 12.5% precast homogeneous PhastGels (Amersham Biosciences) were used. SDS-PAGE and electroblotting onto polyvinylidene difluoride membranes (Immobilon-P^{SQ}, Millipore, Watford, UK) were performed on a PhastSystem apparatus (Amersham Biosciences). The detection antibodies were rabbit polyclonal anti-HA (Autogen Bioclear, Calne, Wilts, UK), goat polyclonal anti-GST (Amersham Biosciences) and mouse monoclonal anti-PrC (Roche) followed by preabsorbed secondary antibodies conjugated to horseradish peroxidase (Chemicon Intl. Ltd., Harrow, UK). Visualization of antibody bands was by Luminol (New England Biolabs, Hitchin, UK), and blots were exposed to ECL film (Amersham Biosciences). Densitometric analysis of ECL images from Western blots was carried out using the ScanAnalysis program (Biosoft, Cambridge, UK). Immunoprecipitation samples were also run on larger gels on the NuPAGE SureLock mini-cell system (Invitrogen) under reducing conditions, with 10% homogenous Bis-Tris gels and then blotted on the same system according to the manufacturer's instructions.

PLD Assays. Transfected cells in 12-well plates were deprived of USG by transferring to DMEM for 18 h, during which time they were labeled with [^{3}H]palmitate (1.5 μCi/well; PerkinElmer Biosciences, Hounslow, UK). After washing with minimal essential medium containing HEPES (25 mM, pH 7.5) and 0.5% fatty acid-free bovine serum albumin, cells were preincubated for 20 min in similar medium with or without BFA, before addition of butan-1-ol (30 mM) and various concentrations of 5-HT for a further 30 min. The vehicle for BFA (dimethylformamide at 0.2%) was added to control wells and has been shown previously to have no detectable effect on signaling responses. Reactions were terminated by removal of medium and addition of 0.5 ml ice-cold methanol to each well. Phospholipids were extracted and [^{3}H]phosphatidyl butanol was separated on Whatman LK5D thin-layer chromatography plates (Whatman, Maidstone, Kent, UK) as described previously (Mitchell et al., 2003).

PLC Assays. Transfected cells in 12-well plates were deprived of USG by transferring to Earle's balanced salt solution containing 10 mM HEPES, pH 7.5, and 0.18% glucose for 18 h, during which time they were labeled with [^{3}H]inositol (0.75 μCi/well; PerkinElmer Biosciences). The medium was changed for Earle's balanced salt solution containing 10 mM HEPES, 0.18% glucose, and 0.2% bovine serum albumin and washed once. Cells were preincubated for 20 min with 10 mM LiCl (and BFA where required) before being incubated for 30 min with various concentrations of 5-HT with or without BFA. Reactions were terminated by the removal of medium and the addition of 1 ml of ice-cold 10 mM formic acid (Sealfon et al., 1995) and

the cells were left on ice for at least an hour to ensure lysis. Inositol phosphates ([^{3}H]InsP) were separated by anion exchange chromatography as described previously (Mitchell et al., 2003).

[^{3}H]Ketanserin Binding. Assessment of PrC-5-HT_{2A} receptor expression in the transfected COS-7 cells was by homologous displacement of [^{3}H]ketanserin binding (specific activity, 88.0 Ci/mmol; PerkinElmer Life Sciences). After removal of USG from the culture medium for the last 4 h, cell membranes were prepared by harvesting the cells into ice-cold ketanserin binding buffer (50 mM Tris, 5 mM $MgCl_2$, and 1 mM EGTA, pH 7.2) and disrupted with an Ystral homogenizer. Membranes were washed twice in buffer, centrifuging each time at 12,000*g*, 30 min, 4°C. Finally the membranes were suspended in buffer at ~200 μg/ml protein. Membranes were incubated for 60 min at 37°C with 0.8 nM [^{3}H]ketanserin and either no other drug, increasing concentrations of unlabeled ketanserin, or 10 μM mianserin to determine nonspecific binding. At the end of the assay, binding was stopped by addition of ice-cold buffer, the membranes were pelleted by centrifugation (10 min at 12,000*g*) and the supernatant was aspirated. Bound radioactivity was determined by liquid scintillation counting. The homologous displacement curves were fitted to a Hill model using nonlinear curve fitting program, Fig-P (Biosoft, Cambridge, UK). This allowed measurement of K_D and number of binding sites. For experiments to determine receptor binding where cell membranes were solubilized in immunoprecipitation buffer, the lysate was first treated with polyethylene glycol-8000 (PEG; final concentration, 15%) to precipitate solubilized proteins, including PrC-5-HT_{2A} receptor, and washed, again precipitating with PEG before trituration of the pellet in ketanserin binding buffer for the assay. Additionally, PEG was used to terminate the assay, and nonsolubilized membranes included in the experiments as controls were treated in the same way.

When membranes were treated with solubilization buffer, the total amount of specific [^{3}H]ketanserin binding that was recovered from the supernatant plus residual membrane fractions was much less than the input levels of binding in untreated membranes (approximately 15%). It is not clear to what extent this was caused by deleterious effects of detergent/PEG exposure on the ligand binding site or by inefficient capture of solubilized receptor. However, some loss of ligand binding capacity is likely to result during these procedures. It therefore seems likely that the amount of receptor still detectable by ligand binding after extraction represents an underestimate of the amount of receptor protein actually solubilized. Nevertheless, some 97% of the [^{3}H]ketanserin binding that could be recovered was found in the solubilized fraction. When this fraction was immunoprecipitated with PrC-tag antibody, the proportion of solubilized specific binding that became associated with the protein G beads rather than remaining in the supernatant was 70 ± 18%.

[^{3}H]Ketanserin Binding to Coimmunoprecipitates. Cells transfected with wild-type or N376D mutant sPrC-5-HT_{2A} constructs and wild-type ARF1-HA were transferred to USG-free DMEM for 16 h before assay. After washing in PBS at 37°C, and incubation with 5-HT (1 μM, 5 min) in some cases, cells were scraped into ice-cold buffer (PBS, 10% glycerol, 2 μg/ml aprotinin, 4 μg/ml leupeptin, 1 mM AEBSF, 2 μg/ml pepstatin A, 50 μg/ml SBTI, 1 mM sodium orthovanadate, and 1 mM sodium fluoride) and homogenized using an Ystral homogenizer. Nuclei and debris were removed by centrifugation at 1000g for 8 min before the supernatant was centrifuged (12,000*g* for 30 min) to obtain a membrane fragment pellet. The pellet was washed once before trituration in the above buffer containing 1% CHAPS and 0.75% deoxycholate and rolling for 30 min at 4°C. Samples were precleared with protein G-Sepharose and then centrifuged (12,000*g*; 30 min) to obtain the solubilized extracts used for immunoprecipitation. Samples were incubated with 2 μg/ml mouse monoclonal anti-HA tag (clone 12CA5) or nonimmune mouse IgG for 90 min at 4°C and then immune complexes were collected by incubation with protein G-Sepharose for 45 min at 4°C. The pellets from centrifugation at 12,000*g* for 10 min were washed once in solubilizing buffer and then resuspended in [^{3}H]ketanserin binding buffer (see above) that additionally contained 10% glycerol. Ligand binding was assayed as described above. Incubations (250 μl) were stopped by the addition at 4°C of 100 μl of 0.05% bovine γ-globulin in PBS and 1 ml of 30% PEG in PBS. After 15 min on ice, samples were centrifuged at 12,000*g* for 20 min, the supernatant was aspirated, and the tube tips were removed for liquid scintillation counting.

Results

Figure 1 illustrates functional signaling responses of the sPrC-5-HT_{2A}R expressed in COS-7 cells. [^{3}H]Ketanserin binding experiments indicated that the sPrC-5-HT_{2A}R was expressed in COS-7 cell membranes at a mean level of 0.84 ± 0.04 pmol/mg of total protein, with an IC_{50} for homologous displacement of 4.8 ± 0.4 nM. The receptor produced robust PLC and PLD activation responses to 5-HT stimulation that were similar to the untagged receptor (Sealfon et al., 1995; Mitchell et al., 1998) but with slightly greater potency; the EC_{50} values for PLC and PLD responses were 5.0 ± 2.2 and 6.8 ± 1.9 nM, respectively (Fig. 1, A and B). The EC_{50} value for PLC activation by the untagged receptor in similar experiments was found to be 28 ± 2 nM (R. Mitchell and M. Johnson, unpublished observations) and 22 ± 5 nM (Sealfon et al., 1995). The effects of cotransfection of ARF mutants were investigated on 5-HT-induced signaling events mediated by the sPrC-5-HT_{2A}R expressed in COS-7 cells. PLC activation was unaffected by cotransfection of either T31N-ARF1-HA or T27N-ARF6-HA; mutant constructs of the ARF isoforms that have a dysfunctional GTP binding domain (Peters et al., 1995) (Fig. 1A). However, T31N-ARF1-HA, but not T27N-ARF6-HA, significantly inhibited 5-HT_{2A}R-mediated PLD activation (Fig. 1B). Figure 1C shows the inhibitory effect of BFA [a blocker of the BIG1/2 class of ARF GTP-exchange factor (GEF) (Morinaga et al., 1999)] on the PLD response of the 5-HT_{2A} receptor. When only the receptor was expressed, BFA caused a concentration-dependent inhibition of 5-HT–induced PLD activation, with significant inhibition at 50 μM and above. In cells additionally expressing T27N-ARF6-HA, BFA was also inhibitory throughout a similar concentration range. However, in cells expressing T31N-ARF1-HA, the residual 5-HT–induced PLD response became insensitive to BFA. The PLC response of the 5-HT_{2A}R was unaffected by BFA (data not shown).

To investigate whether a physical interaction of either ARF1 or ARF6 with the receptor occurs upon activation, we looked for coimmunoprecipitation of HA-tagged ARF isoforms with the sPrC-5-HT_{2A}R (Fig. 2). When nonimmune mouse IgG (NI IgG) was substituted for the PrC-tag antibody in the immunoprecipitation, minimal levels of either ARF1-HA or ARF6-HA immunoreactivity could be detected in the pulldown assays. Significant levels of ARF1-HA (and to a much lesser extent, ARF6-HA) seemed to be specifically associated with the PrC-tag antibody pulldown assays of the sPrC-5-HT_{2A}R, even under basal conditions (Fig. 2A). Densitometric analysis indicated that basal levels of ARF1-HA coimmunoprecipitated were increased on average 3.32 ± 1.58-fold (n = 5) over nonspecific, as determined with NI IgG. The corresponding value for ARF6-HA was lower, a 0.32 ± 0.05-fold (n = 3) increase over nonspecific. Relative densitometric values of anti-HA tag blots for coimmunoprecipitated ARF1-HA as a percentage of the input of total expressed ARF1-HA were 0.13 ± 0.03% in unstimulated

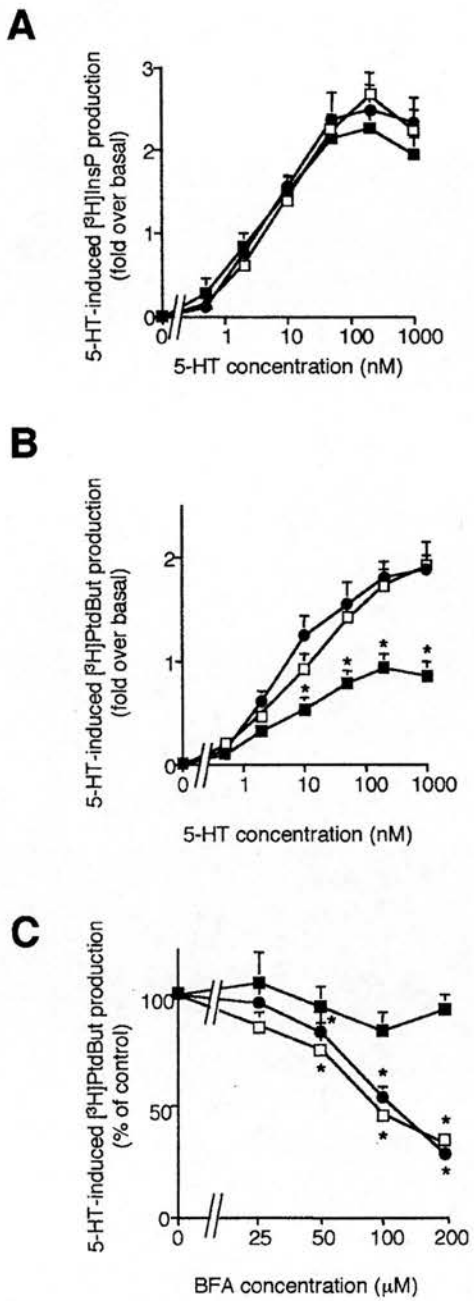

Fig. 1. Effects of mutant ARF1 and ARF6 constructs on signaling responses of the sPrC-5-HT$_{2A}$ receptor. COS-7 cells were cotransfected with the sPrC-5-HT$_{2A}$R together with either empty vector (●), T31N-ARF1-HA (■), or T27N-ARF6-HA (□). Values are means ± S.E.M., n = 6–8. A, concentration-dependence of 5-HT-induced PLC activation; there was no discernible effect of the negative mutant ARFs on this response. B, concentration-dependence of 5-HT-induced PLD activation. The addition of T27N-ARF6-HA had no significant effect on the ability of the 5-HT$_{2A}$R to activate PLD, whereas the presence of T31N-ARF1-HA significantly attenuated the PLD response to 5-HT concentrations of 10 nM and above (*, $p < 0.05$ by Wilcoxon test). C, BFA sensitivity of the 1 μM 5-HT-induced 5-HT$_{2A}$R PLD response and a concentration-dependent inhibition that was statistically significant (*, $p < 0.05$, Wilcoxon test) for BFA concentrations of 50 μM and above in control and T27N-ARF6-HA samples. Cotransfection of T27N-ARF6-HA had no discernible effect on BFA sensitivity compared with control, whereas the PLD response in the presence of T31N-ARF1-HA was no longer significantly inhibited by BFA.

samples compared with 0.21 ± 0.03% after 5-HT (1 μM, 5 min). Equivalent values for ARF6-HA were 0.053 ± 0.010% and 0.067 ± 0.008%, respectively. Although coimmunoprecipitation of both isoforms of ARF with the receptor seemed to be increased by addition of 5-HT (mean increase to 1.61- and 1.26-fold of control, respectively), only the effect on ARF1-HA was statistically significant ($p < 0.05$, Wilcoxon test, $n = 5$ in each case) These values are useful for relative comparisons but do not faithfully reflect the fraction of ARF1/6-HA within an individual cell that is associated with sPrC-5-HT$_{2A}$ receptor, because we know from dual label confocal immunofluorescence experiments (data not shown) that a subpopulation of the COS-7 cells expressing ARF constructs fail to express detectable levels of receptor. When the input level of sPrC-5-HT$_{2A}$R was varied by substituting pcDNA3 for part of the receptor cDNA in the transfection (while keeping the ARF1-HA plasmid concentration constant), less ARF1-HA was coimmunoprecipitated, in proportion to the level of receptor expression as monitored by specific [^{3}H]ketanserin binding (Fig. 2B).

An attempt to quantify the amount of ARF1 specifically coimmunoprecipitating with the receptor was made by cotransfection of an ARF1-V5-His$_6$ construct, the binding of which was then monitored as anti-V5 tag immunoreactivity. Densitometric measurements of ARF1-V5-His$_6$ coimmunoprecipitated with the receptor by PrC tag antibody (or NI IgG control), as well as input levels of ARF1-V5-His$_6$ in cell lysates, were compared with a coprocessed standard curve of anti-V5 tag immunoreactivity prepared using ARF1-V5-His$_6$ protein, purified on a Co^{2+} affinity column. The protein content of the purified ARF1-V5-His$_6$ standard was determined, allowing estimation of the absolute amount of ARF1-V5-His$_6$ that was specifically coimmunoprecipitated in the PrC-tag antibody pulldown assays. Under basal conditions, this was calculated as 11.1 ± 1.0 ng of ARF1-V5-His$_6$ protein from one 175-cm^2 flask. Because essentially all transfected COS-7 cells expressing detectable levels of sPrC-5-HT$_{2A}$R also express ARF constructs, comparison of the amount of coimmunoprecipitated ARF with the B_{max} for [^{3}H]ketanserin binding in equivalent samples allows an estimate of the mean proportion of sPrC-5-HT$_{2A}$Rs that were bound to ARF1-V5-His$_6$. Under basal conditions, this was calculated as 23% of the total; under 5-HT–stimulated conditions, this would be predicted to increase to 37% (1.61-fold of basal association). However, as pointed out above, it is not possible to use this approach to reliably estimate the proportion of expressed ARF construct that is associated with receptor.

The effect of 5-HT on coimmunoprecipitation of ARF1-HA with the sPrC-5-HT$_{2A}$R was investigated further (Fig. 3). Nonimmune mouse IgG controls for 5-HT–stimulated samples gave results similar to those of unstimulated NI IgG controls (data not shown). The time course of 1 μM 5-HT-induced changes in coimmunoprecipitation of ARF1-HA with sPrC-5-HT$_{2A}$R showed a clear increase in association from 2 min, a peak around 5 min, and then a gradual decline (although levels seemed to remain above basal for as long as 2 h) (Fig. 3A). The concentration dependence of the effect of 5-HT (10-min incubation) is shown by the insert in Fig. 3A, reaching a maximum by approximately 1 μM 5-HT. Figure 3B shows that the 5-HT-induced increase in ARF1-HA coimmunoprecipitation with the sPrC-5-HT$_{2A}$R was reduced to basal levels by the ARF-GEF inhibitor, BFA (100 μM) (Fig. 3B). As before, the concentration of ARF1-HA present in the

lysate was very similar between the different conditions (as shown in Fig. 3B, middle), and the amount of receptor immunoprecipitated (Fig. 3B, top) was also even. These observations using immunoprecipitation of the sPrC-5-HT_{2A}R indicate that ARF1, and to a lesser extent ARF6, shows a physical association with the receptor that can be increased in the presence of 5-HT, correlating with the functional studies on PLD activation.

To examine the receptor-ARF interaction in more detail, we generated GST fusion protein constructs of the intracellular loop 3 (i3; Ile258–Gly326) and carboxy-terminal tail (ct; Asn376-Val471) of the 5-HT_{2A}R, and investigated their ability to bind ARF1-HA and ARF6-HA in vitro. Figure 4 is a schematic representation of the 5-HT_{2A}R showing the amino acid sequences used for these GST constructs. GST-fusion protein constructs of the i3 and ct domains of the 5-HT_{2A}R, or the STREX exon of the BK channel as a control, were attached to glutathione Sepharose beads and used in in vitro interaction assays at equivalent input levels [as estimated by GST immunoreactivity (Fig. 5A)]. The input levels of ARF1-HA and ARF6-HA or the negative mutant constructs, deficient in GTP binding, T31N-ARF1-HA and T27N-ARF6-HA, were also balanced for HA-immunoreactivity (Fig. 5A). Figure 5B compares ARF1-HA and ARF6-HA interaction with the constructs. ARF1-HA showed much greater relative binding to the ct domain of the 5-HT_{2A}R than to the BK channel construct or the i3 domain of the 5-HT_{2A}R. ARF6-HA showed a much lower level of binding to the 5-HT_{2A}ct construct and little interaction with the 5-HT_{2A}i3 or BK constructs. Figure 5C shows the binding profiles for the functionally negative mutants of ARF1 and ARF6, T31N-ARF1-HA and T27N-ARF6-HA. The binding of T31N-ARF1-HA to the 5-HT_{2A}ct construct was greatly reduced compared with that of the ARF1-HA wild type, whereas the low background levels of T31N-ARF1-HA binding to 5-HT_{2A}i3 and BK channel constructs were similar to those seen with wild-type ARF1-HA. T27N-ARF6-HA showed a low level of binding to the 5-HT_{2A}ct construct that seemed to be similar to that of the ARF6-HA wild type. The apparently slightly higher levels of T27N-ARF6-HA binding compared with the wild type in the experiment displayed correspond to its somewhat higher input level. GTPγS facilitated the interaction of submaximal levels of ARF1-HA with the 5-HT_{2A}ct construct and also seemed to strengthen a weak interaction with the 5-HT_{2A}i3 construct, which had been minimal in the absence of added nucleotide (Fig. 5D). GTPγS did not facilitate T31N-ARF1-HA binding to the receptor constructs. Similar results were obtained in three different experiments.

Further to this work, a range of concentrations of both ARF1 and ARF6 were added to the GST-5-HT_{2A}i3 and GST-5-HT_{2A}ct constructs to assess the concentration-dependence of binding. Figure 6 shows the relative proportion of ARF isoform bound to the constructs at increasing ARF concentrations, expressed as a ratio of densitometric values for HA-immunoreactivity of the bound ARF versus GST immu-

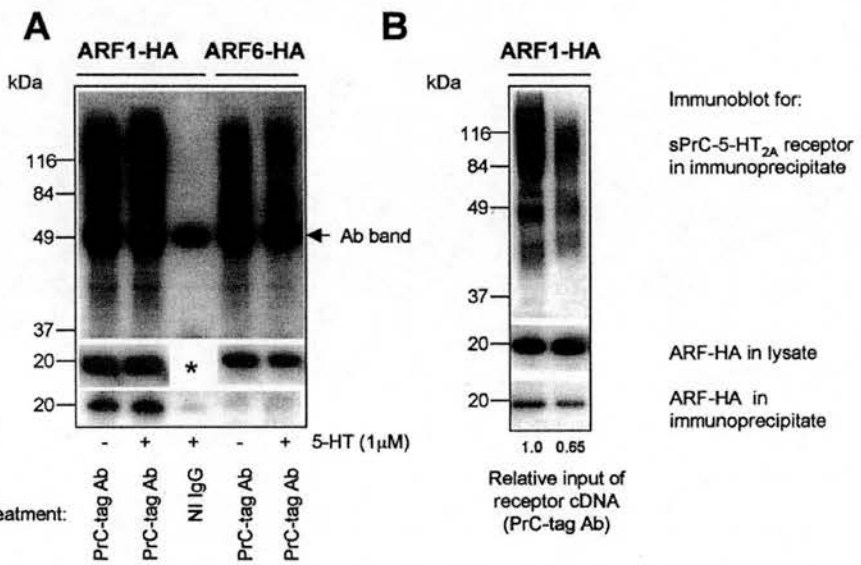

Fig. 2. ARF1-HA and ARF6-HA coimmunoprecipitation with the sPrC-5-HT_{2A} receptor. COS-7 cells were cotransfected with sPrC-5-HT_{2A}R and ARF1-HA or ARF6-HA. A and B, ARF1-HA and ARF6-HA capture in pulldown assays of the sPrC-5-HT_{2A}R carried out with PrC-tag antibody compared with a control procedure using NI IgG. In each case, at the top (anti-PrC-tag immunoblot on immunoprecipitates) are shown pulldown assays of receptor (diffuse bands caused by glycosylation), in which receptor was only detected when immunoprecipitated by PrC-tag antibody, not by NI IgG. The middle (anti-HA immunoblot on sample input) shows that ARF1-HA and ARF6-HA were expressed at similar levels in the compared samples. In A, the ARF input level for lane 3 (NI IgG control), designated by *, was the same as that for lane 2 because the same lysate was split between the two treatments with PrC-tag antibody or nonimmune IgG. The bottom (anti-HA immunoblot on immunoprecipitates) shows specific coimmunoprecipitation of ARF1-HA (or ARF6-HA) with the receptor. A low level of nonspecific pulldown of ARF can be seen in the NI IgG lane. In A, cells were challenged with either 5-HT (1 μM) for 5 min or control. Much more ARF1-HA was pulled down compared with ARF6-HA, even though there was only a small difference in the relative amount of these isoforms expressed in the total lysate. Addition of 5-HT seemed to cause increased coimmunoprecipitation of both isoforms, but the extent of this increase was greater for ARF1-HA. B, the effect of different levels of sPrC-5-HT_{2A}R expression on ARF1-HA coimmunoprecipitation. Cells were transfected with different amounts of expression plasmid for the sPrC-5-HT_{2A}R, with substitution by pcDNA3 vector, thus keeping ARF1-HA expression constant. Cells used for the blot in lane 1 had the standard level of receptor cDNA, whereas cells in lane 2 received 65% of this level. Densitometric analysis indicated that the amount of receptor immunoprecipitated in lane 2 was 71% of that in lane 1 and the amount of ARF1-HA coimmunoprecipitated was 62% of that in lane 1, although the ARF1-HA input level was similar in both samples. In equivalent aliquots of membrane preparations from these cells transfected with the standard or reduced (65%) amount of sPrC-5-HT_{2A}R plasmid, specific [^{3}H]ketanserin binding represented 627 ± 53 and 344 ± 41 dpm per assay, respectively (means ± S.E.M., n = 4).

noreactivity in the construct. Increasing the concentration of ARF1-HA present with each construct caused corresponding increases in the amount of ARF bound. ARF1-HA bound to the GST-5-HT$_{2A}$ct construct to a much greater extent than to the GST-5-HT$_{2A}$i3 construct. ARF6-HA also bound to both the GST-5-HT$_{2A}$ct and the GST-5-HT$_{2A}$i3 constructs, but to a much lesser extent than the ARF1-HA bound to either construct, requiring higher levels of added ARF6-HA to obtain detectable binding. To obtain an estimate of the affinity of ARF1-V5-His$_6$ (added in cytosolic extracts) for the GST-5-HT$_{2A}$ ct construct, we quantified the amount of ARF binding by comparison of densitometric values for V5-immunoreactivity with a coprocessed standard curve of known amounts of ARF1-V5-His$_6$ that had been purified by Co^{2+} affinity column (as above). The ARF-V5-His$_6$ ligand was provided for the interaction assay as an enriched cytosolic extract rather than affinity-purified material because binding was more robust and consistent using this approach. It is not clear whether this is a result of deleterious effects of the purification on ARF1-V5-His$_6$ conformation and function or whether unknown cytosolic factors are additionally required for optimal ARF binding. Working within the linear range of the standard curve for V5-immunoreactive band density against purified ARF1-V5-His$_6$ concentration, we added a range of cytosolic ARF1-V5-His$_6$ concentrations (1–25 ng) to interaction assays and derived values for the amounts of ligand bound from the corresponding V5-immunoreactive band densities. Nonlinear curve fitting of the saturation curve gave a value for affinity (50% saturation) of 1.7 ± 0.4 nM, with greater than 90% occupancy of available sites by 4 to 5 nM.

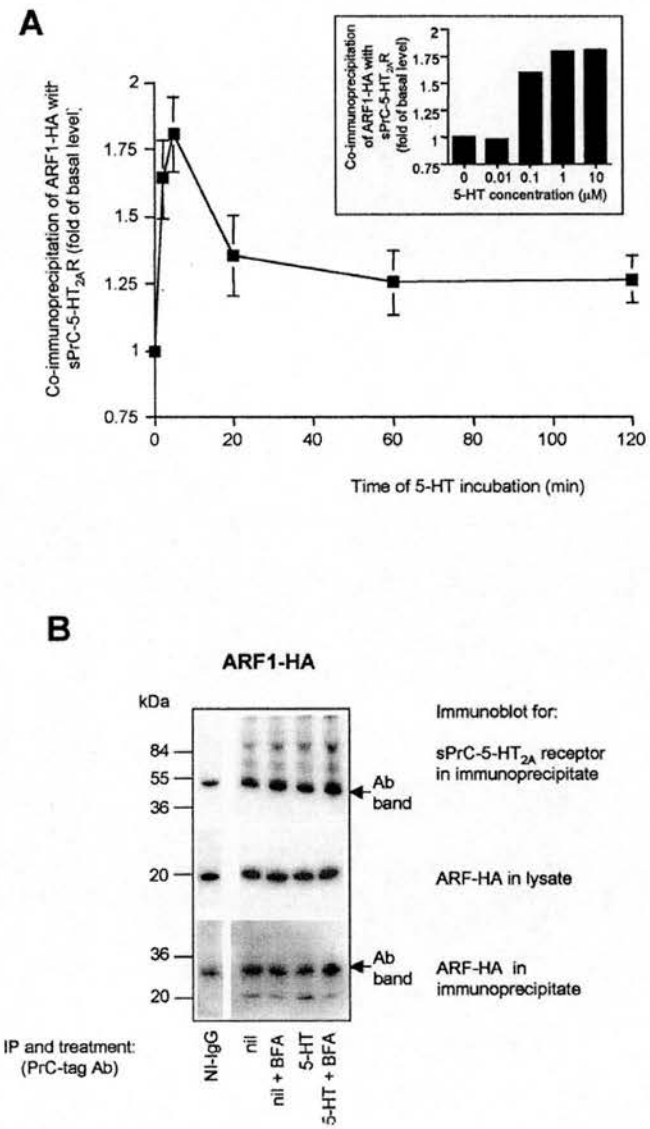

Fig. 3. Characterization of ARF1-HA association with the sPrC-5-HT$_{2A}$ receptor. A, incubation of cells with 5-HT caused a time- and concentration-dependent increase in the amount of ARF1-HA coimmunoprecipitating with the sPrC-5-HT$_{2A}$R. The main figure shows that incubation with 5-HT (1 μM) caused a rapid rise in the association of ARF1-HA to 1.81 ± 0.14 of basal at 5 min, reducing thereafter. Values are means ± S.E.M., n = 4 to 6 at each point. Inset, a single experiment showing a concentration-response curve to 5-HT (10 min) in which a maximal response was reached by 1 μM. This experiment was repeated twice more with similar results. B, effects of BFA on the levels of ARF1-HA associating with the receptor under basal and 5-HT-stimulated conditions. The first lane shows a control immunoprecipitation procedure with nonimmune mouse IgG (NI IgG) instead of the PrC-tag antibody, which was used in all other lanes: nil, no stimulation; nil + BFA, BFA (100 μM, 20 min); 5-HT, 5-HT (10 μM, 10 min); and 5-HT+ BFA, BFA 10 min, followed by 5-HT plus BFA for a further 10 min. Pulldown of receptor (reprobed with the PrC-tag antibody) is shown in the top, input of ARF1-HA in the lysate is shown in the middle, and coimmunoprecipitation of ARF1-HA in PrC-tag antibody-directed pulldown assays is shown in the bottom. Because in these experiments captured proteins were solubilized directly in Laemmli buffer, nonspecific bands can be seen, reflecting the presence of the antibody or control immunoglobulin used for pulldown assays.

The differential signaling properties of the wild-type 5-HT$_{2A}$R and the N376D mutant 5-HT$_{2A}$R (Mitchell et al., 1998, 2003) suggest that the NPxxY motif, at the junction of the ct and the 7th transmembrane domain, may participate in the binding of ARF1 to the receptor. To test this theory, we carried out signaling experiments, coimmunoprecipitation, and GST-fusion protein studies with both the wild-type and N376D mutant form of the receptor. Figure 7A shows that 1 μM 5-HT-induced PLD activation by the wild-type sPrC-5-HT$_{2A}$R was significantly reduced by BFA (100 μM) or by coexpression of T31N-ARF1-HA but not T27N-ARF6-HA. Corresponding responses of the N376D-sPrC-5-HT$_{2A}$R examined in the same experiments showed no significant inhibi-

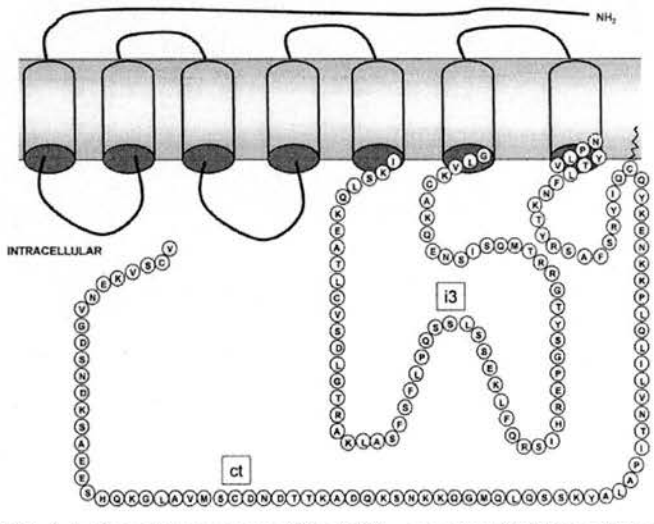

Fig. 4. Amino acid sequences of the 5-HT$_{2A}$ receptor third intracellular loop (i3) and carboxy-terminal (ct) tail inserts incorporated into GST-fusion proteins.

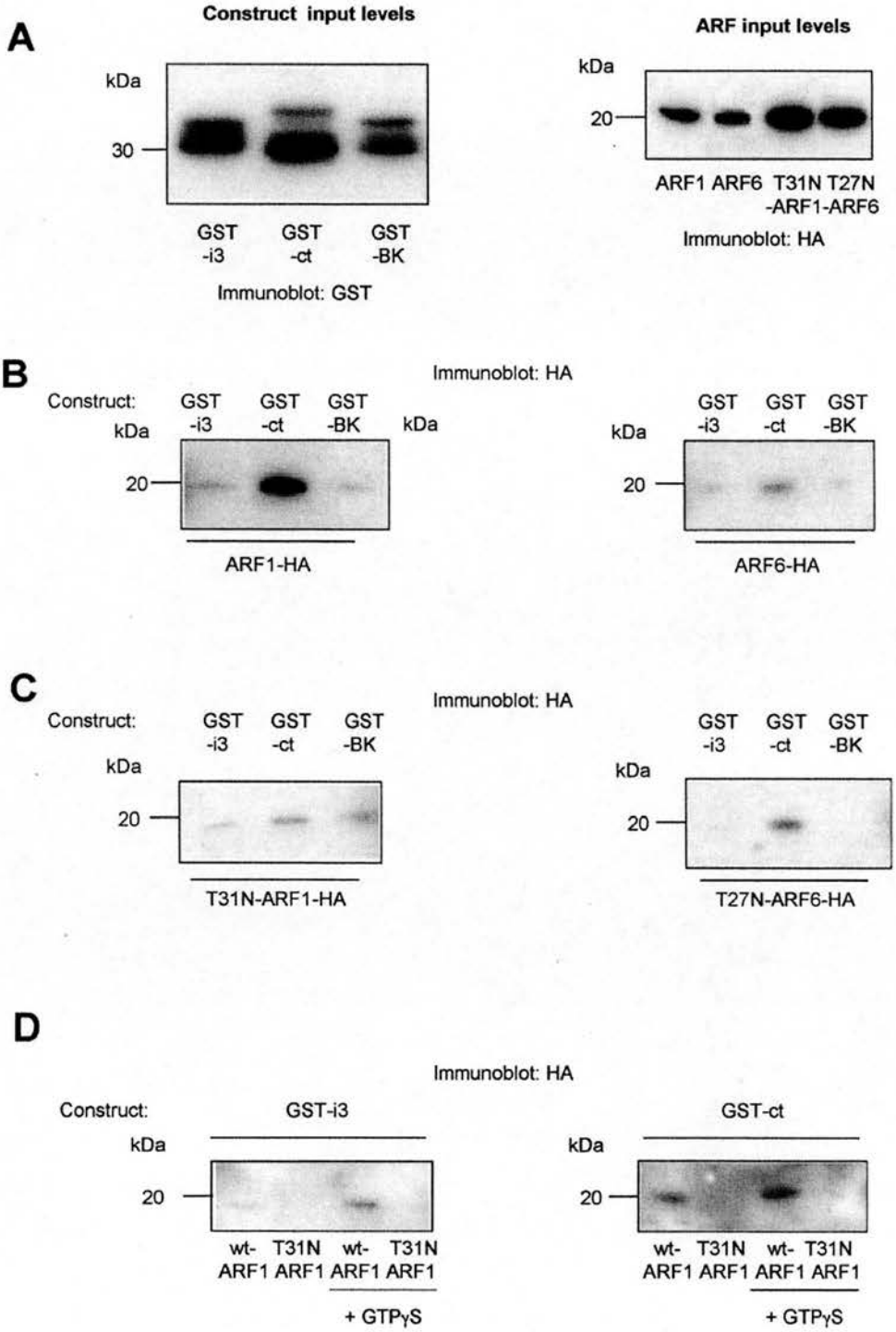

Fig. 5. Interactions of ARF isoforms with GST fusion proteins of domains from the 5-HT_{2A} receptor. GST-5-HT_{2A}i3, GST-5-HT_{2A}ct, and (control) GST-BK channel (STREX exon) constructs were incubated with cellular extracts enriched in particular HA-tagged ARF isoforms. A, input levels of fusion protein constructs and ARF isoforms were balanced in terms of GST immunoreactivity and HA-immunoreactivity, respectively. The fusion protein construct input levels are shown for GST-5-HT_{2A}i3 (GST-i3), GST-5-HT_{2A}ct (GST-ct), and GST-BK running at apparent molecular masses of approximately 36, 40, and 34 kDa, respectively. Unconjugated GST ran at approximately 29 kDa. The ARF input levels are shown for ARF1-HA, ARF6-HA, T31N-ARF1-HA, and T27N-ARF6-HA. B and C, association of the indicated ARF form with GST-5-HT_{2A}i3, GST-5-HT_{2A}ct, and GST-BK constructs, respectively. ARF1-HA bound selectively to the GST-5-HT_{2A}ct construct, with little binding to the GST-5-HT_{2A}i3, or GST-BK constructs. ARF6-HA showed a similar profile but bound to a much lesser extent than ARF1-HA. The T31N mutation in ARF1-HA severely reduced the ability of the protein to bind to the GST-5-HT_{2A}ct construct, but the equivalent mutation in ARF6-HA had no discernible effect. In D, GST-5-HT_{2A}i3 and GST-5-HT_{2A}ct constructs were incubated with cellular extracts enriched in the indicated HA-tagged ARF isoforms in the presence or absence of GTPγS (100 μM). GTPγS increased the binding of wild-type ARF1-HA to both GST-5-HT_{2A}i3 and GST-5-HT_{2A}ct constructs, but did not alter the lack of binding seen with T31N-ARF1-HA.

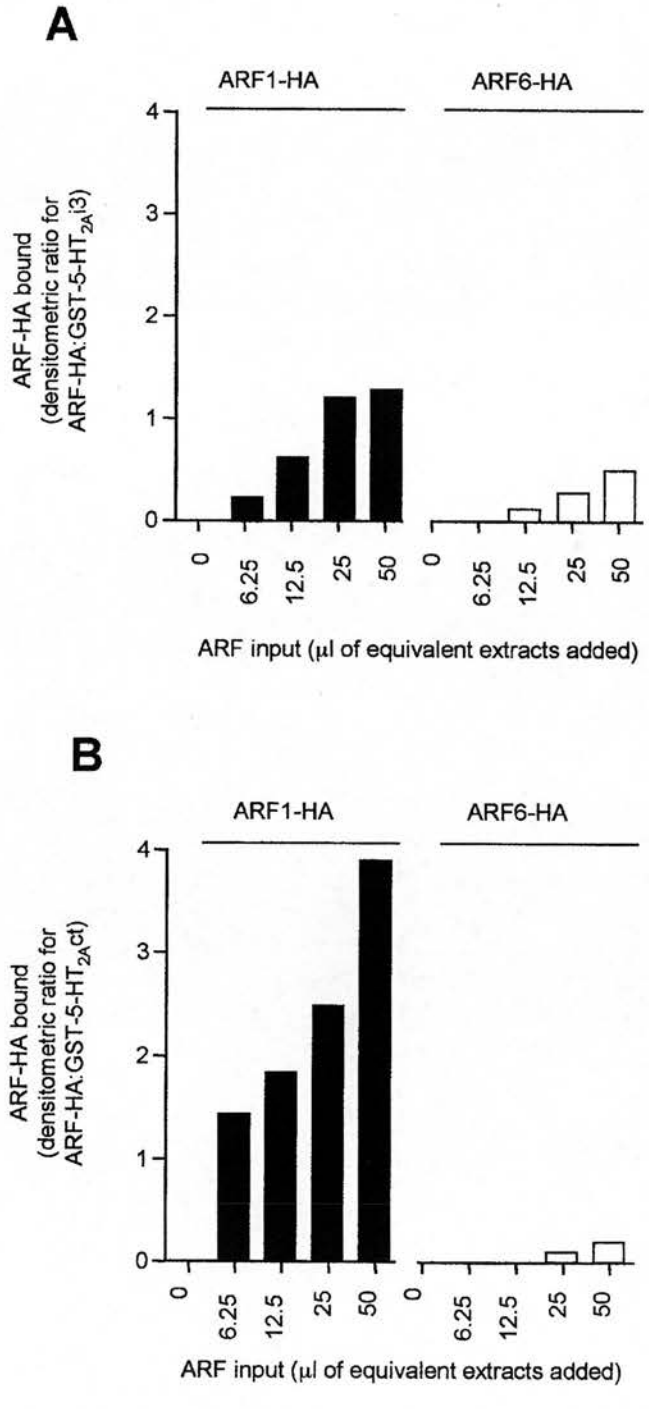

Fig. 6. Concentration-dependence of ARF1-HA and ARF6-HA binding to the GST fusion proteins of domains from the 5-HT$_{2A}$ receptor. GST-5-HT$_{2A}$i3 and GST-5-HT$_{2A}$ct constructs were exposed to increasing amounts of cellular extracts containing ARF1-HA and ARF6-HA. The content of HA-immunoreactive ARF per microliter of the ARF1-HA and ARF6-HA extracts was shown to be equivalent. A, binding of ARF1-HA and ARF6-HA to the GST-5-HT$_{2A}$i3 construct. ARF1-HA bound to the construct in a concentration-dependent manner, as did ARF6, but to a much lesser extent. B, binding of ARF1-HA and ARF6-HA to the GST-5-HT$_{2A}$ct construct. ARF1-HA bound in a concentration-dependent manner, whereas the binding of ARF6-HA was minimal and detectable only at the highest levels of ARF6-HA input.

tion. Figure 7B shows the results of coimmunoprecipitation experiments in COS-7 cells cotransfected with ARF1-HA and either the wild-type sPrC-5-HT$_{2A}$R or its N376D mutant form. After stimulation with 5-HT (1 μM, 5 min) or control, solubilized extracts were immunoprecipitated with HA-tagged antibody, and 5-HT$_{2A}$Rs associated with the immunoprecipitate were assayed as specific [^{3}H]ketanserin binding. Low levels of nonspecific [^{3}H]ketanserin binding were present in each case, and these showed no discernible differences between samples. In cells transfected with the wild-type sPrC-5-HT$_{2A}$R but not those expressing the N376D mutant, significant levels of specific [^{3}H]ketanserin binding became associated with the HA tag immunoprecipitate after 5-HT stimulation. When NI IgG was substituted for the HA tag antibody during immunoprecipitation, no specific [^{3}H]ketanserin binding was captured. Using the [^{3}H]ketanserin binding protocol there was no evidence for significant basal association between ARF1-HA and sPrC-5-HT$_{2A}$R (6 $\pm$ 8% of specific binding), unlike the data shown in Figs. 2 and 3. There were a number of methodological differences (such as shorter time for immunoprecipitation and the addition of glycerol) between this procedure and that used previously, which may result in the differences in basal association. 5-HT stimulation clearly caused an increased interaction between the sPrC-5-HT$_{2A}$R and ARF1-HA. Comparison of the amount of specific [^{3}H]ketanserin binding associated with ARF1-HA immunoprecipitates and the input levels of binding in solubilized extracts gave an estimate that 30 $\pm$ 5% of sPrC-5-HT$_{2a}$Rs were associated with ARF1-HA after 5-HT stimulation. This value is in reasonably close agreement with values estimated from the immunoprecipitation/immunoblot experiments (above).

Figure 7C shows an experiment to investigate whether the N376PLVY motif in the 5-HT$_{2A}$R ct domain may form part of the binding site for ARF1-HA. GST-fusion protein constructs of the wild type (Asn376-Val471) 5-HT$_{2A}$ct, the mutant (N376D-Val471) 5-HT$_{2A}$ct and the truncated (Lys385-Val471) 5-HT$_{2A}$ct were prepared. Equal amounts of these constructs (and GST alone) were determined by Coomassie blue staining and by GST immunoreactivity at the predicted molecular mass before interaction assays with ARF1-HA. The ratios of the densitometric values for bound ARF1-HA immunoreactivity to fusion protein input were then calculated on an arbitrary scale relative to that for the wild-type construct. Both individual images and the mean densitometry ratios for bound ARF1-HA:construct input showed a clear reduction in binding (to around 40% of wild type) by the N376D mutation and a further loss (to around 20%) by deletion of the Asn376-Asn384 sequence (Fig. 7C).

Discussion

The present findings demonstrate that a negative mutant construct of ARF1, but not ARF6, inhibits the activation of PLD, but not PLC, by the 5-HT$_{2A}$R. This indicates a selective functional role for the ARF1 isoform in the PLD signaling pathway of the 5-HT$_{2A}$R. PLD but not PLC responses of the 5-HT$_{2A}$R were correspondingly reduced in a concentration-dependent manner by BFA, an inhibitor of the ARF-GEFs, BIG1/2, which are reported to show selectivity for ARF1 rather than ARF6 (Morinaga et al., 1999). Further evidence consistent with a functional role for ARF1 in BFA-sensitive

PLD responses came from experiments assessing the BFA sensitivity of 5-HT_{2A}R PLD responses in cells coexpressing negative-mutant ARFs, T31N-ARF1-HA or T27N-ARF6-HA. The inhibitory effect of the negative-mutant ARF1 construct pre-empted any further inhibition by BFA, suggesting that they both acted within the same pathway, whereas negative mutant ARF6 was without effect. Other GPCRs may show different selectivity for ARF isoforms. In COS-7 cells express-

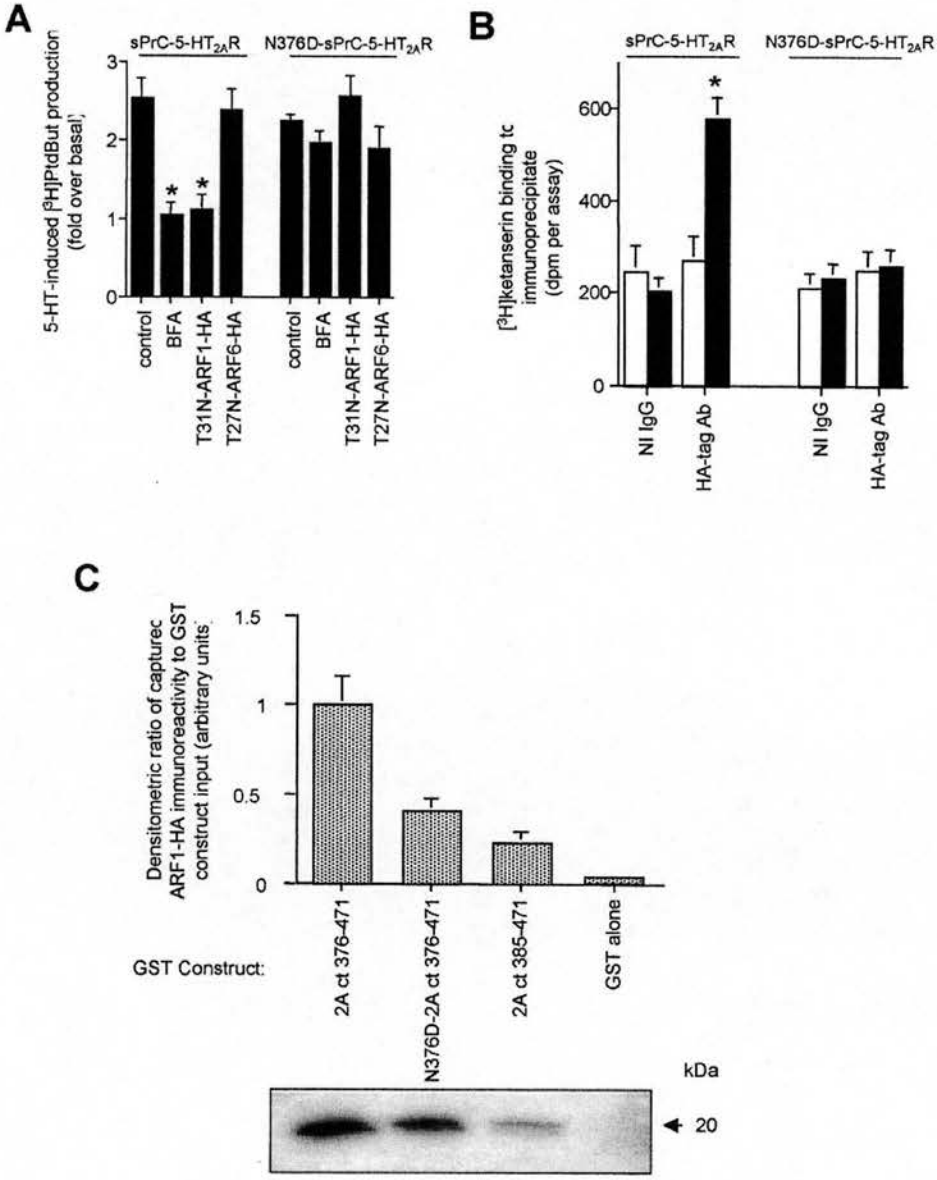

Fig. 7. Interactions of ARF isoforms with wild-type and N376D mutant sPrC-5-HT_{2A} receptors. A, 1 μM 5-HT-induced PLD signaling responses of wild-type and N376D mutant sPrC-5-HT_{2A}R. Comparison of their susceptibility to inhibition by BFA (100 μM) or negative mutant forms of ARF1 (T31N-ARF1) or ARF6 (T27N-ARF6). In control and BFA-treated cells, an amount of empty vector equivalent to that used for the mutant ARFs was cotransfected with the receptor constructs. Values are means ± S.E.M., $n = 6$, $^*p < 0.05$ by Wilcoxon test compared with control (5-HT alone). BFA and T31N-ARF1-HA significantly reduced responses of the wild type, but the T27N-ARF6-HA construct had no detectable effect. B, amount of [^{3}H]ketanserin binding to HA-tag directed immunoprecipitates in cells cotransfected with ARF1-HA and either wild-type or N376D mutant sPrC-5-HT_{2A}R. Nonspecific binding of [^{3}H]ketanserin in the presence of 10 μM mianserin fell in the range of 163 to 241 dpm/assay in all cases and showed no discernible difference between samples. Control procedures were carried out with an equivalent amount of nonimmune mouse IgG (NI IgG). Cells were pretreated with 5-HT (1 μM, 5 min, ■) or control (□). Values are means ± S.E.M., $n = 5$, *, $p < 0.05$ by Mann-Whitney U test compared with corresponding HA-immunoprecipitate without 5-HT prestimulation and to control immunoprecipitation with NI IgG. Significant levels of specific [^{3}H]ketanserin binding (above nonspecific binding) were recovered only in HA-tag directed immunoprecipitates from cells in which the wild-type sPrC-5-HT_{2A}R had been prestimulated with 5-HT. The input levels of specific [^{3}H]ketanserin binding in solubilized extracts immediately before immunoprecipitation were similar for the wild-type and N376D mutant receptors (1166 ± 154 and 1450 ± 125 dpm per sample, respectively). C, matched levels of GST-fusion proteins incorporating the (Asn376–Val471) wild-type 5-HT_{2A} receptor ct domain, the corresponding N376D mutant, or a truncated Lys385–Val471 sequence, as well as GST alone, were attached to glutathione-Sepharose beads and incubated with equivalent levels of ARF1-HA. Immunoreactivity for bound ARF1-HA was quantified by densitometry and the ratio to the level of input for each GST-fusion protein construct was calculated. These ratios were then normalized to that found for the wild-type ct construct. Values are means ± S.E.M., $n = 5$. A typical example of ECL film images for HA-immunoreactivity bound to these constructs is shown at bottom.

ing the M_3 muscarinic receptor and in A10 smooth muscle cells, PLD responses to carbachol and to angiotensin II or ET-1, respectively were attenuated by T31N-ARF1 and by T27N-ARF6 (Shome et al., 2000; Mitchell et al., 2003), whereas some other GPCRs such as P_{2u} and $PAC_{1\text{-}hop1}$ receptors may show selectivity for ARF6 over ARF1 (Ronaldson et al., 2002; Mitchell et al., 2003).

Coimmunoprecipitation experiments demonstrated that the sPrC-5-HT_{2A}R, under basal conditions, could specifically bind ARF1-HA and to a lesser extent ARF6-HA. The binding of ARF1-HA was increased by 5-HT stimulation of the receptor in a concentration- and time-dependent manner. Only minor increases in the association of ARF6-HA with the sPrC-5-HT_{2A}R could be seen. The presence of BFA during the stimulation with 5-HT reversed the 5-HT–induced association of ARF1-HA, although we know from confocal immunofluorescence imaging that BFA does not prevent agonist-induced translocation of ARF1-HA to the plasma membrane (data not shown). This evidence that ARF1 is the isoform predominantly docking to the receptor in a BFA-sensitive manner is consistent with the data on PLD activation. Estimates of the proportion of 5-HT_{2A} receptors that was associated with ARF1-HA after 5-HT stimulation ranged between 30 and 37% depending on the approach taken, with corresponding estimates of basal association between 6 and 23%. Interaction of other proteins, such as $G_{q/11}$ and perhaps arrestins, with the 5-HT_{2A}R may well mean that relevant binding sites were not accessible to ARF1 in part of the receptor population.

The GST-fusion protein experiments suggested that the ct domain of the 5-HT_{2A}R provides a binding site for ARFs at which ARF1 shows a higher affinity than ARF6. Comparison of the immunoreactivity for ARF1-V5-His_6 bound to the GST-5-HT_{2A}ct construct with known amounts of purified ARF1-V5-His_6 allowed an estimate of the affinity of interaction. This was in the low nanomolar range (1.7 ± 0.4 nM), which is of lower affinity than that for arrestin interaction with the M_3R i3 domain (Wu et al., 1997) but higher than the corresponding interaction of $G\beta\gamma$ (Wu et al., 1998). The 5-HT_{2A} receptor i3 domain shows only low affinity for ARF in vitro but may still represent an auxiliary binding site in vivo. In contrast, the i3 domain effectively binds arrestins in similar experiments (Gelber et al., 1999). The interaction of ARF1-HA with the ct or i3 domain of the 5-HT_{2A}R seemed to be facilitated by GTPγS, suggesting that occupancy of its nucleotide recognition site by GTP rather than GDP promotes the interaction. Correspondingly, the GTP-binding-defective mutant ARF1 construct (T31N-ARF1-HA) showed an almost complete lack of specific binding to the ct or i3 domain GST fusion proteins that was unmodified by GTPγS. The lower level of ARF6-HA binding seemed to be little affected by T27N mutation of ARF6-HA, but this was not investigated further. The means by which agonist induces increased (BFA-sensitive and GTP status-sensitive) binding of ARF1 to the 5-HT_{2A}R is not clear. Involvement of BIG1/2 is implicated by the BFA sensitivity, but it is not known whether agonist activation of the receptor might facilitate GTP loading of ARF1 by direct protein-protein interaction, by regulation of BIG1/2, or by other means. However, GTP binding operates a conformational switch in ARFs that might contribute to additional protein-protein interactions (Goldberg, 1998).

Although the tm7 NPxxY motif has been implicated as a critical determinant of ARF coimmunoprecipitation and ARF-dependent signaling in rhodopsin family GPCRs, the precise site of ARF binding to the ct of the 5-HT_{2A}R remains to be elucidated. Mutation of this motif to DPxxY strongly inhibits BFA-sensitive, ARF-mediated activation of PLD (Fig. 7A; Mitchell et al., 1998) and sPrC-5-HT_{2A}R coimmunoprecipitation with ARF1-HA (Fig. 7B). Using GST-fusion proteins of the 5-HT_{2A}R ct domain, mutation of Asn376 to Asp causes a marked (60%) reduction in ARF1-HA association and removal of the Asn376–Asn384 segment reduces association further to around 20% of the wild-type values. This suggests that the majority of the key elements involved in 5-HT_{2A}R-ARF1 interaction, at least under these circumstances, may lie within the Asn376–Asn384 segment. Structural modeling based on rhodopsin and secondary structure predictions (PHD predict; http://cubic.bioc.columbia.edu/predictprotein/) suggest that the Pro377 residue is likely to form a pronounced kink in the tm7 helix and that Thr381–Lys385 may form a flexible hinge to an eighth helical segment that runs in the plane of the membrane until a palmitoylation anchor at Cys397 (Konvicka et al., 1998; Palczewski et al., 2000; Yeagle et al., 2000; Visiers et al., 2002). In the case of rhodopsin, activation of the receptor newly exposes to the intracellular surface an epitope that includes residues equivalent to Leu378–Tyr380 here (Abdulaev and Ridge, 1998), consistent with the idea that receptor activation may reveal residues involved in ARF association. The predicted fourth intracellular loop of rhodopsin, in particular residues equivalent to Asn384–Gln386 here, is involved in interaction with the α and γ subunits of transducin (Ernst et al., 2000; Marin et al., 2000). Interactions between amino acids in the NPxxY motif and the subsequent seven residues are thought to influence heterotrimeric G protein activation by both rhodopsin and the 5-HT_{2C}R (Prioleau et al., 2002; Fritze et al., 2003). Elements of this surface might also contribute to ARF docking. The interaction of 5-HT_{2A}R with $G\alpha_{q/11}$, however, is also thought to involve the carboxyl portion of the i3 loop (Roth et al., 1998).

Additional functional roles have been proposed for the N/DPxxY motif. The most consistent evidence is for a role linking the tm2 and tm7 helices (Sealfon et al., 1995). The NPxxY motif and the Y residue in particular have been proposed to constitute an internalization motif in some but by no means all GPCRs (Hunyady et al., 1995). Mutation of the Asn or Asp residue to Ala generally causes massive disruption of signaling pathways and of internalization, whereas reciprocal mutation of Asn or Asp seems to have relatively minor effects other than on ARF-dependent PLD activation (Sealfon et al., 1995; Le Gouill et al., 1997; Mitchell et al., 1998). In the case of the 5-HT_{2A} receptor, we confirmed that the NPxxY motif, rather than the DPxxY mutant motif, was necessary for functional BFA-sensitive and T31N-ARF1-HA-sensitive PLD responses from the receptor for 5-HT-induced coimmunoprecipitation of the receptor with ARF1-HA and for the major part of in vitro binding of ARF1-HA to the ct domain of the receptor.

There is increasing evidence that particular GPCRs can interact with diverse scaffolding and signaling proteins other than their conventional partners, the heterotrimeric G-proteins (Brady and Limbird, 2002; Premont and Hall, 2002). Receptor ct segments may dock bivalent adapter proteins containing PDZ or other domains, signaling proteins, and modulators of signaling functions (Dev et al., 2001; Oakley et

al., 2001; Brady and Limbird, 2002). In the 5-HT_2 receptor family, the distal ct residues are targeted by the PDZ-domain proteins PSD-95 and MUPP-1 (Backstrom et al., 2000; Becamel et al., 2001; Xia et al., 2003), interactions that may modify the signaling function of the receptors. A novel PDZ domain protein, tamalin, has been shown to bind to both mGluR1/5 receptors and the ARF-GEF ARNO (Kitano et al., 2002). It is conceivable that an analogous arrangement might occur in the case of the 5-HT_{2A} receptor, locating an ARF-GEF in the proximity of ARF.

ARF may not be the only small G protein that can interact with GPCRs. We showed that Rho A can be coimmunoprecipitated in a complex with NPxxY GPCRs (Mitchell et al., 1998), and there is evidence that both $G\alpha_{13}$ and $G\alpha_q$ may interact with Rho-GEFs to facilitate Rho function (Sagi et al., 2001). Other small G proteins of unknown identity have also been found to associate with the formyl-Met-Leu-Phe receptor (Polakis et al., 1989).

In summary, these experiments provide intracellular signaling, coimmunoprecipitation and in vitro domain interaction evidence for ARF association with the 5-HT_{2A}R, corresponding to its functional activation of PLD. Furthermore, ARF1 rather than ARF6 seems to participate in this mechanism through GTP-dependent interaction with a ct domain of the receptor.

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