Signal transduction by the 5-HT_{2A} receptor and its H452Y polymorphic variant

by

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The candidate confirms that the work submitted is her own and that appropriate credit has been given where reference has been made to the work of others.

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ABSTRACT

The 5-HT_{2A} receptor (5-HT_{2A}R) is implicated in neuropsychiatric disorders such as schizophrenia and is thought to mediate the actions of a number of hallucinogenic and antipsychotic drugs. Additionally, certain polymorphic variants of the receptor, such as an allele resulting in substitution of amino acid 452 histidine (H) with tyrosine (Y), have been linked to schizophrenia or altered therapeutic response to antipsychotics. The 5-HT_{2A}R utilises various intracellular signalling pathways, including the activation of phospholipase C (PLC) and phospholipase D (PLD) via recruitment of the small G-protein ADP-Ribosylation Factor (ARF). This thesis focusses on protein:protein interactions and signalling mechanisms of the 5-HT_{2A}R and H452Y-5-HT_{2A}R receptor variant.

Both ARF1 and PLD1 have previously been shown to bind to the carboxy-terminal tail (ct) of the 5-HT_{2A}R. In chapter three it is demonstrated that the 5-HT_{2A}R can activate PLD in an ARF-dependent manner, primarily through the PLD1 isoform. GST-fusion proteins of truncated and mutated variants of the receptor ct are used to show that ARF1 and PLD1 independently bind to distinct sites. Co-immunoprecipitation, GST-fusion protein studies and PLD activity assays demonstrate that the introduction of the H452Y mutation decreases the physical interactions between the receptor and PLD1, as well as decreasing 5-HT_{2A}R-mediated PLD activation. In chapter four, potential mechanisms of wild-type and H452Y-5-HT_{2A}R desensitisation are explored. It is shown that β-arrestin 2 (β-arr 2) confers a decrease in H452Y-5-HT_{2A}R-mediated PLC activity, despite having no significant effect upon wild-type 5-HT_{2A}R responses. The H452Y-5-HT_{2A}R variant is also shown, by GST-fusion protein studies, to bind β-arr 2 more strongly. The H452Y-5-HT_{2A}R additionally mediates increased levels of 5-HT-induced ERK phosphorylation compared to the wild type 5-HT_{2A}R, potentially through increased scaffolding of ERK activation complexes by receptor-bound β-arr 2. Chapter five focusses on possible interactions of the 5-HT_{2A}R with the Ca^{2+}-binding proteins annexin A2, S100B and the annexin A2 partner p11, together with the functional consequences of these interactions. Co-immunoprecipiation and GST-fusion protein

studies show that annexin A2 binds specifically to the 5-HT_{2A}R ct. Furthermore, annexin A2 (but not S100B or p11) is shown to result in an amplification of 5-HT_{2A}R-mediated PLC responses.

These findings provide a greater insight into some of the signal transduction mechanisms of the 5-HT_{2A}R and their perturbation in the H452Y polymorphic form of the receptor, and understanding of the molecular mechanisms underlying neuropsychiatric diseases in patient subgroups, potentially leading to improved therapeutic treatments.

ABBREVIATIONS

5-HT	5-hydroxytryptamine
5-HT_{2A}R	5-HT_{2A} receptor
5-HT_{2A}Rct	5-HT_{2A} receptor carboxy-terminus
5-HT_{2A}Ri3	5-HT_{2A} receptor 3rd intracellular loop
AA	arachidonic acid
AC	adenylate cyclase
Ala	alanine
AP-2	adaptor protein-2
ARF	ADP-ribosylation factor
Arg	arginine
Asp	aspartic acid
ATP	adenosine triphosphate
Bmax	binding maximum
Bq	becquerels
BRET	bioluminscence resonance energy transfer
BSA	bovine serum alumin
°C	degrees celsius
Ca^{2+}	calcium
cAMP	cyclic adenosine monophosphate
CNS	central nervous system
COS7	green monkey kidney fibroblast cell line
CREB	cAMP response element binding
C-terminus	carboxy-terminus
DAG	diacylglycerol
DMEM	Dulbecco's modified Eagle's medium
DNA	deoxyribonucleic acid
EBSS	Earle's balanced salt solution
EC50	median effective concentration
EDTA	ethylenediamine tetra-acetic acid
ER	endoplasmic reticulum
ERK	extracellular signal-regulated kinases
FRET	fluorescence resonance energy transfer
GABA	gamma-aminobutyric acid
GAP	GTPase-activating protein
GDP	guanine diphosphate
GEF	guanine-nucleotide exchange factor
GI	gastrointestinal tract
Glu	glutamate
Gly	glycine
GPCR	G protein-coupled receptor
G protein	guanine-nucleotide binding protein
GRK	G protein-coupled receptor kinase
GST	glutathione-S-transferase
GTP	guanine triphosphate
h	hours

HA	haemagluttinin
HBSS	Hank's buffered saline solution
HEK293	human embryonic kidney293
HL60	human promyelocytic leukemia cells
Hsp90	heat shock protein 90
IgG	immunoglobulin
Ile	isoleucine
InsP	inositol phosphate
IP	immunoprecipitation
JAK	janus kinase
K^+	potassium
K_D	dissociation constant
kDa	kiloDalton
l	litre
LB	Luria Bertani
LSD	lysergic acid diethylamide
M	molar
M_3	muscarinic$_3$
MAP kinase	mitogen-activated protein kinase
mg	milligram
Mg^{2+}	magnesium
mGluR	metabotropic glutamate receptor
min	minutes
ml	millilitre
mM	millimolar
mm	millimetre
MOPS	3-(N-morpholino) propane sulfonic acid
MUPP1	multi-PDZ domain protein I
µg	microgram
µl	microlitre
µm	micron
µM	micromolar
Na^+	sodium
NCS	normal calf serum
ng	nanogram
nm	nanometre
nM	nanomolar
NMR	nuclear magnetic resonance
N-terminus	amino terminus
p11	annexin 2 light chain
PA	phosphatidic acid
PBS	phosphate-buffered saline solution
PH	pleckstrin homology
PI	phosphoinositide
PIP_2	phosphatidylinositol 4,5-bisphosphate
PKA	protein kinase A
PKC	protein kinase C
PLA	phospholipase A

PLC	phospholipase C
PLD	phospholipase D
PrC	protein C
Pro	proline
PSD95	post synaptic density protein 95
PtdBut	phosphatidyl butanol
PtdCho	phosphatidyl choline
PVDF	polyvinyldifluoride
RGS	regulator of G protein
R-luc	Renilla luciferase
RNA	ribonucleic acid
S100B	S100 calcium binding protein B
S.D.	standard deviation
S.D.S.	sodium dodecyl sulphate
sec	second
S.E.M	standard error of the mean
SERT	serotonin transporter
SH3	SRC homology 3 domain
SNP	single nucleotide polymorphism
SR	sarcoplasmic reticulum
tm	transmembrane domain
TRPV	transient receptor potential varilloid
Tyr	tyrosine
VFTM	venus flytrap module
VPAC	vasoactive intestinal peptide receptor
YFP	yellow fluorescence protein

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CHAPTER 1.0

1.0 Introduction

1.1 GPCR Structure

The G protein-coupled receptor (GPCR) family constitutes one of the largest superfamilies of human proteins, and is the largest cell surface receptor family, with genes encoding functional GPCRs comprising more than 1% of the human genome (1,2). GPCRs are deployed within the plasma membrane and interact with extracellular ligands. These interactions induce conformational changes in the receptor, which are transmitted intracellularly. Thus GPCRs act to transfer signals across the cellular plasma membrane, activating G proteins and participating in protein:protein interactions. In this way GPCRs activate intracellular signal transduction pathways, ultimately resulting in a physiological response. Due to the variety of both potential receptor ligands and effector proteins, members of the GPCR superfamily demonstrate great functional diversity, acting to influence numerous physiological processes, such as cell growth and differentiation, metabolism, vision, smell and taste (3). Consequently, GPCRs are of immense pharmacological importance, with an estimated >50% of all clinical drugs in current use being targeted at these receptors (4,5).

Although GPCRs exhibit diverse primary structures, and low sequence homology, experimental studies have determined a common basic architecture. They are thought to be composed of: an extracellular amino-terminus (N-terminus); 7 relatively hydrophobic α-helical transmembrane domains, linked by three intra- and three extra-cellular loops of widely varying lengths and sequence composition; and an

intracellular carboxy –terminal tail (C-terminus) (6). The α-helical transmembrane domains are thought to group together within cell membranes to form an anti-clockwise orientated bundle, with the third transmembrane-spanning domain positioned towards the centre (7). Originally, structural GPCR models were based on the structure of *bacteriorhodopsin*, solved by NMR, electron diffraction and X ray-based crystallography (8-11). This structure has since been demonstrated to be unrepresentative of the transmembrane domains of mammalian rhodopsin structures (12). Subsequent sequence homology studies of *bacteriorhodopsin* with other GPCRs suggested that these receptors do display some elements of a common basic structure, with the presence of 7 α-helical transmembrane-spanning domains (13-15). These studies were followed with the publication of several higher resolution rhodopsin structures (16,17). In 2000, the inactivated (11-*cis*-retinal) 3-dimensional structure of bovine rhodopsin was determined by X-ray crystallography to 2.8-angstroms (7). This structure demonstrated a similar basic architecture to previous models, but with the addition of an eighth α-helical domain located distal to transmembrane-spanning α-helix 7, and orientated parallel to the membrane plane (7). Recently, in 2007, the first crystal structures of a non-rhodopsin receptor, the β_2-adrenergic receptor, were published (18-20). This was closely followed by the publication of the turkey β_1-adrenergic receptor (21), and the human adenosine A_{2A} receptor structure (22). Comparison of the solved structures has revealed the arrangement of the transmembrane helices to be loosely conserved between the three representative subclasses. However, dissimilarities between the models, such as the conformation of the extracellular regions, the position of ligand binding sites and the stability of a conserved interaction of the Asp-Arg-Tyr (DRY) motif within the third

intracellular loop with a Glu residue of the sixth transmembrane domain (7,18,19) (21-23), have highlighted the limitations of using rhodopsin as a general GPCR model and the need for a greater array of solved GPCR structures.

The GPCR superfamily has been classified into families using various techniques. The two most commonly used nomenclature systems, the A-F clan system and glutamate, rhodopsin, adhesion, frizzled/taste, secretin (GRAFS) system both categorise the GPCR superfamily based on the comparison of the 7 transmembrane domains of the receptors. The A-F clan system was developed using an informatics technique to analyse GPCRs of both vertebrates and invertebrates (24,25). Several conserved regions of alignment were used to generate a rhodopsin family fingerprint, which was used to scan the database, yielding GPCR families A-F (24). The GPCR superfamily has more recently been classified using the GRAFS system (26,27). Phylogenetic studies were used to categorise the superfamily into five human GPCR families, each containing members related by their presumed evolutionary origins, giving yield to glutamate, rhodopsin, adhesion protein, frizzled/taste 2 and secretin families (GRAFS) (27). The members of these families will be discussed in further detail below, using both GRAFS and A-F classification nomenclature.

1.2 Rhodopsin-like family (Class-A)

The rhodopsin-like family of receptors, otherwise known as Class-A receptors, comprise the largest family of GPCRs, with approximately 90% of known GPCRs being members of this group (28,29). Although many Class-A receptors are odorant or chemokine GPCRs, members of this group are diverse in terms of ligand, primary

structure and the physiological functions they may mediate (29). Rhodopsin-like receptors are divided into four subgroups. The α group contains the amine receptor group, which comprises 5-HT, dopamine, muscarinic, adrenergic and cannabinoid receptors among others. In addition the opsin, melatonin, prostaglandin and MECA (consisting of melanocortin, endothelial differentiation sphingolipid, cannabinoid and adenosine receptors) receptor groups are all contained within the α group. The β group is primarily composed of peptide-binding receptors and contains neuropeptide Y receptors, oxytocin receptors and endothelin receptors. The γ group comprises peptide- and lipid-binding receptors and includes the three opioid receptors, the AT_1 angiotensin receptor and the type 2 and 5 somatostatin receptors. Finally, the δ group comprises the olfactory receptors (the largest group of rhodopsin receptors), glycoprotein-binding receptors, the protease-activated receptors (PAR) and the leukotriene receptors (26,27,30-32).

As the name suggests, receptors belonging to the rhodopsin-like group display significant sequence homology to the rhodopsin receptor (27). Rhodopsin-like GPCRs generally display relatively low sequence homology within the family, although they do typically express a few specific very highly conserved domains. The majority of class-A receptors contain a highly conserved structurally stabilising disulphide bond between Cyss of the second extracellular loop (e2) and the third transmembrane spanning helix (tm3) (33-36). A highly conserved motif, Asp-Arg-Tyr (DRY), is located at the cytoplasmic face of the receptor, between the third transmembrane domain (tm3) and second intracellular loop (i2) (37). This motif is thought to be involved in stabilising structural interactions and has been proposed to

regulate agonist-induced conformational changes of the receptor, and subsequently the binding of the GPCR to the heterotrimeric G protein (38-43). A conserved Asp-Pro-xx-Tyr (NPxxY) motif is located at the junction between the base of transmembrane-spanning domain 7 (tm7) and the beginning of the C-terminal tail. The two amino acid residues found between Pro and Tyr tend to be hydrophobic (37). It has been proposed that the presence of the NPxxY domain interrupts the α-helical structure of the seventh transmembrane domain by forming a flexible hinge region, thereby creating a structural perturbation that may have various potential functional roles (44). This domain has been reported to be required for the efficient internalisation of some receptors, such as the δ-opioid receptor (45), the neurokinin-1 receptor (46) and the vasopressin-II receptor (47). However, alternative studies have demonstrated that the angiotensin II type I receptor (48) and the gastrin-releasing peptide receptor (49) undergo internalisation independently of the NPxxY motif. The presence of the NPxxY motif has additionally been demonstrated to influence receptor signalling. Studies have shown that the motif may play a key role in receptor interactions with the small G protein ARF and possibly Rho, and subsequent ARF-mediated signalling (50). Further signalling investigations have also revealed the involvement of the NPxxY motif in the activation of adenylyl cyclase, phospholipase C, MAP kinase and phosholipase D (50-53). Additionally, a conserved Cys of the carboxy-terminus is thought to undergo palmitoylation, thereby anchoring the carboxy-terminal tail domain at the cell membrane, creating a putative fourth intracellular loop. X-ray crystallography of the rhodopsin structure identified a short amphipathic α-helix existing parallel to the plane of the membrane, located between tm7 and the palmitoylated Cys (7). This region, the putative fourth intracellular loop,

is thought to exist in many rhodopsin family GPCR structures and is suggested to be one of the GPCR regions involved in G protein coupling (7,54).

The rhodopsin-like GPCR family includes two receptors for which the ligand-bound structures have now been solved to high resolution, thus providing insights into the mechanisms of GPCR ligand-binding and activation (7,18-20). The bovine rhodopsin receptor is known to be covalently bound to its ligand retinal, which is buried within a four-stranded β-sheet structure (55,56). Retinal undergoes isomerisation in response to light, inducing a conformational change in rhodopsin that brings about an association with the G protein transducin (57). When bound to the 11-cis retinal isomer (dark form of retinal), the receptor is known to be locked in an inactive form, held by an ionic interaction between Arg 135 of the DRY motif with the adjacent Glu 134 and Glu 247 of tm6 (40,55,56). Ligand-activation induces a break in this interaction and a subsequent outwards movement of tm6 in relation to tm3 (55,58). In contrast, the β-adrenergic receptor binds diffusible ligands. The e2 forms an α-helix stabilised by disulphide bonds, which reveals the ligand-binding site, making it accessible from the extracellular environment (18-20,55). Similarly to the rhodopsin receptor, the β-adrenergic receptor has an ionic interaction between the DRY motif of tm3 and tm6, although this interaction is not as strong in the β-adrenergic receptor, possibly decreasing the stability of the receptor (18-20,55).

1.3 Secretin receptor family (Class-B)

The class-B or secretin receptor family comprises 15 peptide-binding receptors, which express a high degree of sequence similarity (59). Members of this GPCR

family are activated by peptide ligands of approximately 30-40 amino acids, such as hormones, neuropeptides and autocrine factors (60). The receptor family includes the calcitonin and calcitonin-like receptors, the corticotrophin–releasing hormone receptors, the glucagon receptor, the gastric inhibitory polypeptide receptor, the glucagon-like peptide receptors, the growth-hormone-releasing hormone receptor, the pituitary adenylyl cyclase-activating polypeptide receptor, the parathyroid hormone receptors, the secretin receptor and the vasoactive intestinal peptide receptors (59). Secretin family GPCRs have a large extracellular N-terminus, within which a large proportion of the receptor family sequence diversity exists. However, almost all members of the secretin family contain conserved Cys residues within the N-terminus, which form disulphide bridges, acting to stabilise the receptor (61). Some of these conserved Cys residues are also thought to be closely involved in ligand binding (62,63). In addition, the e1 and e2 contain conserved Cys residues, which are also implicated in ligand binding (61,62). Although an exact mechanism has not been determined for the activation of class-B GPCRs, there are two popular theories. Dong et al proposed that agonist binding may induce a conformational change in the receptor, inducing the association of the N-terminus with tm6, acting to stabilise the receptor in its active conformation (64). Alternatively, the two-domain model proposes that class-B receptors bind ligand at the N-terminus, promoting interactions between the ligand and the transmembrane domain. This induces a conformational change in the receptor, increasing G protein interactions and thereby inducing the potential activation of downstream signalling pathways (65). Secretin family receptor-mediated signal transduction is reportedly initiated via interactions of the intracellular loops and carboxy-terminus of the receptor with Gs, Gq and Gi to

control their downstream pathways (66,67). However, secretin family receptors generally couple efficiently to Gs proteins as a common characteristic, thereby activating adenylyl cyclase (66).

1.4 Adhesion receptor family (Class-B)

The human adhesion receptor family is also a class-B clan member and contains 33 human and 31 mouse GPCRs, which have been further subdivided to form 7 sub-families. Although members of the adhesion receptor family also belong to the class-B GPCR clan, they possess markedly different N-termini to secretin family GPCRs (32). The N-termini are long and can be composed of as many as a few thousand amino acid residues (68). They are diverse in terms of size and amino acid composition (26), but are commonly rich in Ser/Thr residues, resulting in high levels of glycoslyation (28,69). Adhesion family GPCR N-termini frequently contain several functional domains such as immunoglobulin, cadherin and laminin G domains, some of which are thought to be primarily responsible for the adhesive properties of these receptors (70). Nearly all members of the adhesion receptor family possess a conserved GPCR proteolytic site (GPS), within the extracellular regions of the receptor, adjacent to the transmembrane domain (71,72). It is reportedly involved in the proteolytic cleavage of receptor N-termini, to yield two non-covalently linked subunits (71,73). This cleavage event is thought to be required for receptor folding and trafficking (74).

The majority of adhesion family receptor members are orphan GPCRs, thus both the ligand and precise function are elusive (32). Consequently, it has been generally

difficult to determine whether adhesion family GPCR members are linked to G proteins (73).

1.5 Glutamate receptor family (Class-C)

The class-C or glutamate family of GPCRs is composed of 22 receptors which are further subcategorised into 8 metabotropic glutamate, 1 calcium-sensing, 2 $GABA_B$, 3 taste, the GPRC6A and 7 orphan class C receptors (75). The N-terminus of glutamate family receptors is long, frequently containing more than 500 residues (76,77). It is involved in ligand recognition through a mechanism involving a conserved Venus Flytrap Module (VFTM) (76,78). With the exception of the GABA receptors, class-C glutamate family receptors express an additional conserved Cys-rich domain that links the VFTM to the transmembrane-spanning domain (75). The ligand recognition site forms a cavity within the N-terminus, flanked by two folded lobe regions, stabilised by disulphide bonds to either side (79). The lobes surrounding the ligand binding site are mobile and, in the absence of ligand, are proposed to oscillate between closed or open conformations. The presence of ligand is thought to stabilise the closed state of the ligand-binding region, thereby trapping a ligand such as glutamate at the binding site (80).

The transmembrane helices are linked by short intra- and extracellular loops, of which e2 is the longest. e2 and e3 are reported to be involved in G protein binding and selectivity (75). As is consistent with the structure of rhodopsin, tm3 of class-C receptors is reported to be particularly hydrophobic, indicating that it is localised at the centre of the 'transmembrane barrel' (7,75). Again in accordance with rhodopsin

structural studies, tm3 of glutamate receptors is linked via a disulphide bond to e2 (7,75). An xPKxY motif, similar to the highly conserved NPxxY motif of rhodopsin-like receptors is also conserved within tm7, which may be necessary for efficient coupling with G proteins (7,40,75). Additionally, members of the glutamate family receptors are also thought to express a putative eighth, amphipathic, helix, which is thought to be involved in G protein coupling (7,54,81).

Many GPCRs can bind allosteric ligands, which regulate the activity of a receptor by interacting at binding sites distinct from the orthosteric ligand-binding site. The binding of an allosteric ligand acts to stabilize a particular receptor conformation, thereby altering the receptor binding affinity for the orthosteric ligand, and thus influencing receptor activity. Class-C receptors are putatively regulated by many positive and negative allosteric modulators (82,83). It has been reported that allosteric compounds may bind at the transmembrane helices of class-C receptors, as opposed to the N-terminus, in a crevice located within tm3, tm5, tm6 and tm7 (83). Class-C orthosteric ligand binding sites are known to consist of very highly conserved amino acid sequences, allowing little agonist-selectivity between the subtypes (84). It is thought that allosteric binding sites are less conserved, providing a potentially more specific therapeutic target (84). Both negative and positive class-C allosteric modulators have been developed for potential therapeutic purposes, including CDPPB, an antipsychotic that acts as an allosteric enhancer of the metabotropic glutamate 2 (mGlu2) receptor, and LY-354740, a positive allosteric regulator of both mGlu2 and mGlu3 receptors, currently undergoing clinical trials for the treatment of anxiety-related disorders (84).

Class-C receptors were the first GPCRs demonstrated to form functional in vivo dimers (85,86). Many members of the class-C receptor family are thought to form homodimers and/or heterodimers. The metabotropic glutamate receptors and calcium-sensing receptor have been identified in homodimeric form, linked by disulphide bonds and by direct interactions between the ligand binding domains (75,79,85). Subsequently, a metabotropic glutamate-calcium sensing receptor (mGlu-CaSR) heterodimer was identified (87). The $GABA_B$ receptor also forms a heterodimer, composed of the splice variants $GABA_{B1}$ and $GABA_{B2}$, linked via coiled-coil domains within the C-terminus (88) and also via interactions between the VFTM domains (89). It is suggested that $GABA_B$ function is dependent upon the formation of $GABA_{B1}$ and $GABA_{B2}$ heterodimers (88).

1.6 Frizzled / Taste 2 receptor family (class B)

Although the frizzled and taste 2 receptor families comprise receptors that appear to have a diverse evolutionary history, the mammalian frizzled and taste 2 receptors are classified as one grouped under the GRAFS nomenclature system (27). However, under the A-F clan system the frizzled family, along with the adhesion family, were categorised as a part of the class-B secretin family (26). The frizzled family of GPCRs are receptors for Wnt glycoprotein ligands (32,90). Wnts are essential mediators of embryonic development, as well as being involved in many disease states (91,92). The family comprises only 11 human receptors, 10 frizzled receptors and 1 smoothened receptor (27,32). As is consistent with other members of the

GPCR superfamily, frizzled receptors are composed of an extracellular N-terminus, 7 transmembrane domains and an intracellular carboxy-terminus. The ligand-binding site is located within the N-terminus, and contains a Cys-rich domain (93). The C-terminus of frizzled proteins appears not to contain the Cys residue required to form the putative fourth intracellular loop described in many GPCRs (94). However, many frizzled proteins have been determined to contain the C-terminal motif serine/threonine-Xxx-valine, recognised as a PDZ-binding domain, thereby providing a potential site for the scaffolding of signalling complexes (94,95). As yet, the frizzled family of receptors have not been demonstrated to interact directly with G proteins, although data from signalling studies would suggest that frizzled receptors can signal via the Gi/o and Gq proteins (96).

The taste 2 receptors are a family of bitter taste receptors, comprising 40-80 human and rodent GPCRs (97). Under the GRAFS nomenclature system, the taste 2 receptors are categorised with the frizzled receptors. However, they have previously been classified under the clan system as a family distantly related to the rhodopsin receptor family, or as a novel separate family (98). They are thought to interact with a large, diverse group of bitter tasting ligands (98).

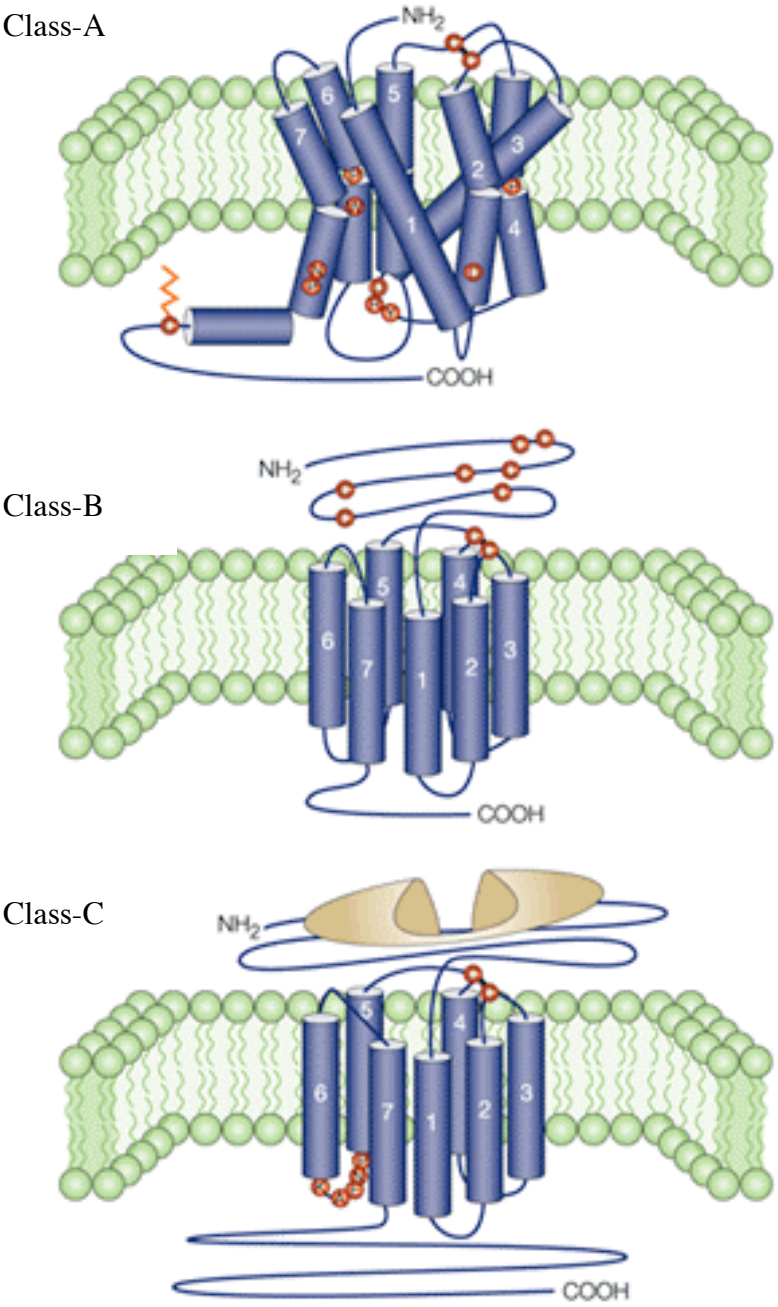


Figure 1.1 Diagram illustrating the basic architecture of class-A, class-B and class-C GPCRs. Structure of Class-A GPCRs (above), Class-B (middle) and Class-C (bottom), taken from George et al, 2002 (530).

1.7 G Proteins and signalling pathways

The transduction of a signal resulting from GPCR ligand-activation is enabled by heterotrimeric G proteins. These highly conserved proteins are multi-subunit structures, composed of a G protein α subunit (Gα) and a G protein βγ subunit (Gβγ) (99). They are often described as 'molecular switches', as they switch from an inactive GDP bound state to an active GTP bound state in response to GPCR activation, with their transition to the inactive state facilitated by the intrinsic GTP-ase activity of the Gα subunit (3). When in an active state, the G protein can interact with a plethora of downstream effector proteins, which are often enzymes, thereby promoting the transduction of the signal into the cell. There are 21 identified human Gα subunits, encoded by 16 genes. These are classified into four groups based on sequence homology. There are only 6 Gβ subunits, encoded by 5 genes and 12 Gγ subunits (100). However, this provides the potential for a putative >1000 G protein heterotrimer combinations (3), although it is unlikely that this results in >1000 functionally diverse heterotrimer combinations. Various receptors have been demonstrated to selectively use different heterotrimeric G protein combinations in the activation of particular signalling pathways (101,102).

The Gα subunit, (~ 41kDa), contains a highly conserved guanine nucleotide-binding pocket, composed of both a helical and a GTPase domain (103). The GTPase domain is conserved throughout the entire G protein family. It acts to hydrolyse GTP, resulting in structural changes due to changes to the conformation of three flexible switches (I, II and III) (103,104). The helical domain is a structure of six α-helices

that acts as a lid, thereby trapping bound nucleotides at the nucleotide-binding pocket (103,104). The Gβ subunit (~ 35 kDa) has an α-helical N-terminus which links to the N-terminal of the Gγ subunit via a coiled coil, the remaining major part of the Gβ subunit is composed of a seven bladed, β-sheet propeller structure (103). The Gγ subunits are small, (~7-8 kDa), and are primarily α-helical in structure (3). The C-terminal comprises a CAAx domain that undergoes post-translational isoprenylation at the Cys residue, and as a result, is important for membrane localisation (3,105). The G protein subunits interact with one another at various sites. Gβ and Gγ subunits form a tight heterodimer between the N- and C-termini of Gγ and the N-terminus and 5th and 6th blades of the Gβ subunit (106). The heterotrimer is formed via interactions between the N-terminal and switch II domains of the Gα subunit with Gβ (106).

The type of G protein that a GPCR couples to determines the signalling pathway and thus physiological responses that receptor activation will promote. Both the Gα and Gβγ subunits are capable of interacting with secondary effector proteins to regulate downstream signalling. As previously mentioned, the mammalian Gα subunits are categorised into 4 subgroups, dependent upon the effector protein they link to. The heterotrimeric G proteins are also classified into four groups, defined by their Gα subunit, and thus by their associated signalling pathway (107). Gαs proteins directly bind to adenylate cyclases, 12 transmembrane domain proteins that catalyse the cyclical conversion of cytoplasmic adenosine 5' triphosphate (ATP) to cyclic adenosine 3', 5' monophosphate (cAMP) (107). The second messenger cAMP in turn interacts with and regulates secondary effector proteins, such as protein kinase A, involved in various signalling mechanisms, thereby eliciting diverse physiological

responses, such as the activation of the transcription factor CREB, phosphorylase kinase, phosphodiesterases (negative regulators of cAMP production) and the opening of the L-type Ca^{2+} channel. cAMP also directly activates the cAMP- and AMP-regulated guanine nucleotide exchange factor, exchange protein activated by cyclic AMP (EPac1) which activates Rap1, ultimately resulting in the phosphorylation and activation of Mitogen-Activated Protein Kinase (ERK MAP Kinase) 1 and 2 (108). Gαs-mediated GTP hydrolysis and thus de-activation is specifically and permanently blocked by cholera toxin, which has ADP-ribosylation enzymatic activity and acts to ribsoylate the Gαs subunit, resulting in constitutive cAMP production.

The Gi/o family is composed of the $G\alpha i_{1\text{-}3}$, the $G\alpha o_{a\text{-}b}$, Gαz and the photoreceptor Gαt G proteins. Gαi/o subunits act to directly inhibit some adenylyl cyclase subtypes, thereby decreasing intracellular levels of cAMP. However, both Gα and Gβγ proteins can act as direct regulators of downstream signalling molecules in this pathway. Activated Gβγ subunits can directly interact with and thereby regulate K^+ channels, neuronal N-type Ca^{2+} channels and some subtypes of phosphatidylinositol 3-kinase (PI 3-K), phospholipase C-β (PLC-β) and adenylyl cyclases. The Gi/o pathway can be specifically inhibited by pertussis toxin, which catalyses the ADP-ribosylation of Gαi, Gαo and Gαt, thereby preventing GDP/GTP exchange and functionally effective G protein:receptor interaction. Thus this toxin has been an important experimental tool in many studies investigating Gi/o-mediated pathways.

The Gq/11 family of G proteins comprises Gαq, Gα11 and Gα14-16 G proteins. Both Gα and Gβγ subunits of this pathway can activate PLC-β1-4, thereby promoting the hydrolysis of membrane-bound phosphatidylinositol 4, 5-bisphosphate (PIP_2), to yield the second messengers inositol 1, 4, 5- trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 increases intracellular Ca^{2+} concentrations by triggering the release of Ca^{2+} stores from the endoplasmic reticulum. Ca^{2+} is required for the activation of many signalling molecules, including classical protein kinase C (PKC) isoforms. Additionally, DAG acts to recruit various PKC isoforms to the membrane, which are subsequently activated. PKC influences many physiological responses via its activity as a positive and a negative regulator of a diverse array of downstream signalling molecules such as phospholipase D isoforms (109), MAP kinases, Raf1 and Ras (110), the transcriptional activators c-fos, c-jun and NFκB (111) and the cyclin complex E/cdk2 (112).

Gα12 and Gα13 of the G12/13 family were the most recently identified subfamily of G proteins. These ubiquitously expressed proteins were first reported to bind and activate Rho-specific guanine nucleotide exchange factors (113,114). Subsequent Rho activation in turn influences cellular growth and cytoskeletal rearrangements (115). Further to these studies, Gα12 and/or Gα13 have been reported to interact with a wide array of effector proteins, including Regulator of G protein Signalling (RGS) proteins, cadherins, Hsp90, PLD, c-Src and PKC (107,115). G12/13 are involved in cell migration, proliferation and transformation, thus are of much interest in cell biology studies related to oncology (115).

As previously mentioned, G proteins act to communicate a signal from the membrane-bound GPCR to membrane-bound or intracellular second messengers. This is achieved via interactions between the G protein and the cytoplasmic face of the GPCR. There are various theories proposed to explain the formation of these interactions. It has been suggested that intracellularly localised G proteins may bind only to activated GPCRs (116,117). FRET studies have indicated that G proteins interact with α_{2A}-adrenergic receptors in a random fashion via intracellular collisions (118). However, alternative FRET studies have suggested that G proteins and GPCRs may be 'pre-coupled' prior to agonist-activation of the receptor (116,119). Once ligand-bound, it has been proposed that the GPCR undergoes a conformational change, putatively involving movement of the second, third or sixth transmembrane helices, within the bundle of helices, to expose crucial residues on its cytoplasmic face, to which the Gα-protein C-terminus binds (120,121). Residues in the loop linking α-helix 4 and β-sheet 6 of the Gα subunit (α4-β6) and the Gα N-terminal helix are also thought to be involved in interactions with the i3 or c-terminus of the receptor (122,123). The Gβγ subunit also independently binds the receptor, in addition to stabilising the Gα-GPCR interface (124). Receptor activation induces a conformational change in the Gα subunit, thought to involve rearrangements of switch regions and/or the N-terminal helix around the nucleotide-binding pocket, that results in the exchange of bound GDP for GTP (125,126). This instigates the release of the G protein from the receptor, and the dissociation of the heterotrimeric G protein to yield the monomeric Gα and heterodimeric Gβγ subunits. Once dissociated from one another Gα and Gβγ proteins are free to diffuse through the cell, coupling to effector molecules and thereby regulating intracellular signalling

pathways. Upon hydrolysis of GTP, Gα re-associates with Gβγ, to reform the heterotrimeric G protein.

1.8 Regulation of GPCR activation

Once a GPCR has been activated, the subsequent signal is generally regulated in order to avoid constitutive signal transduction. This is achieved in a variety of ways; receptors may be desensitized, down-regulated, or regulated by intracellular proteins. Desensitisation can be homologous, whereby agonist-activated receptors display reduced signalling. Alternatively, heterologous desensitisation involves a decrease in receptor output, in response to the activation of another GPCR. Following ligand activation of GPCRs, it has been established that the receptor adopts a conformation that is generally susceptible to rapid phosphorylation by G protein-coupled receptor kinases (GRKs), at specific Ser and Thr residues within the third intracellular loop and C-terminal tail of the receptor (127). This phosphorylation event promotes the binding of arrestin isoforms, which recognise both specific phosphorylated GPCR residues and the active conformation of the receptor within the intracellular domains (127). Once bound, arrestin acts to induce homologous receptor desensitisation, by creating steric hindrance of GPCR:G protein interactions, which interrupts G protein guanine nucleotide exchange, thereby decreasing G protein-stimulated signal transduction (128). In addition, the binding of arrestin to GPCRs can also promote clathrin-dependant receptor internalisation (127). Arrestins have been found to bind both to clathrin (in vitro) and the AP-2 adaptor complex (129). The AP-2 complex also binds to clathrin, dynamin and Eps-15, which are all molecules involved in clathrin-dependent endocytosis (129). As a result, a link is formed between the

GPCR and the clathrin endocytic machinery. Subsequently, the receptor can be internalised via clathrin-coated pit formation, resulting in endocytosis (129).

The mode of arrestin activation has been found to be both receptor and cell-type specific (130,131). The differential strength of arrestin interactions with different GPCRs (which is thought to be determined by particular groups of serine and threonine residues positioned in the carboxy-terminus of the GPCR) can have significant effects upon arrestin-initiated receptor down-regulation, resensitisation and various signal transduction pathways (132,133). Thus several GPCRs of the rhodopsin family have been classified as Class A or B, defined by their binding affinity for β-arrestin isoforms. Class A receptors only form a transient interaction with arrestin (127). Following internalisation, the receptor–arrestin complex dissociates, liberating the GPCR, which is rapidly dephosphorylated and recycled to the plasma membrane (127). In contrast, Class B GPCRs have been found to form a stable interaction with arrestin, which does not readily dissociate following internalisation (127). This strong interaction is thought to hinder the recycling of the receptor to the plasma membrane and possibly promote receptor degradation (127). In addition, arrestins are thought to be capable of acting as scaffolds for various signalling proteins, including members of the ERK MAP kinase pathway (134-136), and consequently, they can act as mediators of ERK 1 / 2 activation (137).

The RGS (Regulators of G protein Signalling) protein family comprises a group of proteins with intrinsic GTPase-activating protein (GAP) activity, related by common evolutionary origin. More than 30 mammalian RGS proteins have been identified to

date, all of which contain a conserved 120-130 amino acid RGS domain that is thought to account for GAP activity at Gα-GTP (138-140). Thus by directly binding Gα proteins, RGS proteins act to enhance the exchange of GTP for GDP by the activated Gα-GTP protein, thereby promoting Gα deactivation and sequestering signal transduction. The majority of RGS proteins are reported to confer GAP activity at Gαi/o and Gαq/11 proteins (140,141). However, an increase in Gαs-GTP hydrolysis and a decrease in Gαs-mediated signalling were reported in the presence of RGS-PX1 (141,142). Although RGS proteins typically act as inhibitors of GPCR signalling, some larger members of the RGS protein family are thought to act as scaffolds, thereby participating in multiprotein signalling complexes (140,143). Some RGS proteins are thought to selectively bind both particular Gα and GPCR family members (140). In small RGS proteins, receptor selectivity is reportedly conferred via particular amino acids of the N-terminal region (141,144), whilst large RGS proteins are more likely to bind GPCRs via protein interaction domains (141,145).

1.9 5-HT receptors

5-hydroxytryptamine (5-HT) is a ubiquitous monoaminergic neurotransmitter found in animals, plants, fungi and unicellular organisms. In humans, it is found throughout the central nervous system (CNS) and in the gastrointestinal tract (GI) and is synthesized from the amino acid tryptophan, which is derived from the diet (146). Tryptophan is decarboxylated by the enzyme tryptophan decarboxylase, yielding tryptamine, which is futher hydroxylated by the enzyme tryptamine 5-hydroxylase, to generate serotonin (147). Following its release from serotonergic neurons, 5-HT

activity is terminated primarily via uptake from the synapse into the neuron, mediated by the serotonin transporter (SERT), and subsequent catabolism by monoamine oxidase (148). 5-HT activity is mediated by the 5-HT receptor family of Class A GPCRs. The 5-HT receptor family comprises 7 subfamilies of rhodopsin-like receptors, which have since been further divided into 15 subtypes (149). With the exception of the ionotropic 5-HT_3 receptor, members of the 5-HT receptor family are coupled to G proteins.

The 5-HT_1 subfamily is composed of the 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, 5-HT_{1E} and 5-HT_{1F} receptor sub-types (149). These receptors all bind the pertussis sensitive Gi/o proteins, thereby inhibiting adenylyl cyclase (AC) activation, cAMP formation and subsequently act as negative regulators of protein kinase A (PKA) (146). PKA is an important regulator of the transcription factor CREB-1 and Rap-1 (which is involved in ERK1/2 activation), thereby this receptor family can mediate the downstream regulation of genes such as c-fos and MAP kinase activation pathways. 5-HT_1 receptor signalling can be mediated through a wide range of additional downstream signalling proteins, including ERK, Akt, Janus Kinase, and p38 kinase (146). The 5-HT_2 subfamily of GPCRs is further divided into 3 sub-types, the 5-HT_{2A}, 5-HT_{2B} and 5-HT_{2C} receptor (149). Each of these receptors preferentially couples to Gq/11 proteins, which activate PLC (150). Other pathways/second messengers that the 5-HT_2 GPCR subfamily can couple to include PLD, PLA_2, AC, the Janus Kinase (JAK) pathway and the ERK pathway (50,151-155). The 5-HT_3 receptor is a ligand-gated, cation selective ion channel, located both within the central and peripheral nervous systems (156,157). The receptor is composed of five subunits, each surrounding a

central ion-conducting pore (156,157). Each subunit comprises an extracellular, transmembrane and intracellular domain. The agonist-binding site is located within the extracellular domain (156). Upon ligand-activation, the 5-HT_3 cation channel opens, allowing an influx of Ca^{2+} and Na^{+} ions and efflux of K^{+} ions, resulting in a rapid depolarisation (156). The 5-HT_5 subfamily of receptors are the least understood and studied of the 5-HT receptor family. The family is divided into two subtypes, 5-HT_{5A} and 5-HT_{5B} (149). Only the 5-HT_{5A} receptor is expressed in the human, but both are expressed in the mouse and the rat (158,159). The 5-HT_{5A} receptor has been demonstrated to couple to G proteins and appears to preferentially interact with the Gi/o protein (160). Finally, members of the 5-HT_4, 5-HT_6 and 5-HT_7 subfamilies all preferentially couple to the Gs protein, thereby activating adenylate cyclase and protein kinase A (161)}. However, they are categorised into separate receptor classes due to very low sequence homology (<35%) (149). The 5-HT_4 receptor exists as 10 different C-terminal splice variants, although all variants demonstrate very similar coupling properties, and are all known to additionally couple to the G_{13} protein to activate the small G protein, Rho (146,162). The 5-HT_7 receptor exists in 4 splice variants which are known to demonstrate differing patterns of internalisation, although all splice variants couple similarly and additionally couple to the G_{12} protein to also activate Rho (146).

Via the activation of these receptors, 5-HT is involved in many central roles within the CNS, such as thermoregulation, emotion, sleep, appetite, motor function, cognition and the vomiting reflex. In addition, 5-HT is suspected to be involved in the regulation of circadian rhythms by influencing the hypothalamic control of the

secretion of certain hormones from the pituitary gland (163). 5-HT is also reported to invoke physiological effects upon the GI tract (164-166). There is evidence to suggest that the 5-HT_3 receptors are involved in the mediation of intestinal secretion, GI smooth muscle control and vomiting and 5-HT_4 in the modulation of intestinal secretion, the stimulation of GI motility and GI smooth muscle relaxation/contraction (166). 5-HT has also been demonstrated to invoke numerous effects upon the cardiovascular system via its actions as a peripheral vasoconstrictor (167), and through indirect pathways mediated via the central nervous system (168). Thus, it is not surprising that 5-HT is thought to be involved in various patho-physiological states. The serotonergic system has been implicated in hypertension, GI conditions such as irritable bowel syndrome and nausea and psychiatric disorders including schizophrenia, anxiety disorders, obsessive-compulsive disorder, Alzheimer's disease, depression, anorexia and addiction (166-169, 172). As a result the serotonergic system is of much pharmaceutical interest, and the 5-HT receptors are the targets of many pharmacological studies and currently available drugs.

The 5-HT_{2A} receptor (5-HT_{2A}R), a subtype of the 5-HT_2 subfamily, has evoked much interest due to its suspected involvement in hallucinogenic effects of drugs such as lysergic acid diethylamide (LSD), psilocybin and mescaline (169-171) and also in antipsychotic drug activity (172-174). Additionally, recent research has indicated that expression patterns, and thus the distribution of the 5-HT_{2A}R within the central nervous system, may differ in untreated patients afflicted with neuropathological conditions, e.g. schizophrenia, when compared to a normal control group (172). This

suggests the involvement of the receptor in the pathology of neuropsychiatric phenotypes, such as schizophrenia.

Consequently, the 5-HT family, and in particular the 5-HT_{2A}R, have been the subject of many structural and functional research studies. The N-terminus of 5-HT_2 family receptors tends to be poorly conserved, and in common with the majority of family A GPCRs, has not been demonstrated to have a clear functional role (175). Mutagenesis studies on various 5-HT family member tm2 regions have indicated that residues within this domain may be involved in ligand binding (176). Sealfon et al demonstrated that the 5-HT_{2A}R second transmembrane domain (tm2) is also involved in conformational arrangements relevant to G protein coupling (175,177). It has also been proposed that tm2 may be important for the conformational changes seen during receptor activation (168,177). The residues of both the 1st intra- and extra-cellular loops are poorly conserved throughout the 5-HT family, although they are always short, consisting of 6-7 residues (175). The 3rd trans-membrane spanning domain (tm3) contains several conserved residues, including the DRY motif and a Cys residue that serves to form a stabilising disulphide bond with a highly conserved Cys residue of the long 2nd extra-cellular loop. The Arg residue of the D/ERY motif in the 5-HT_{2A}R is thought to be involved in receptor activation by forming an interaction with a Glu in tm6, that acts to stabilise the inactive receptor conformation (178,179). Further mutagenesis studies using various 5-HT receptor sub-types have determined that this domain also includes residues essential for ligand-binding. Some studies have suggested that a highly conserved Asp residue of the 5-HT_2 receptor tm3, D155 in the rat model, (positioned after the DRY motif of tm3), is involved in

interactions with the terminal amine moiety of various agonists, including 5-HT (180). In addition to facilitating optimal ligand binding, this residue is also thought to be involved in the targeting of the receptor to the membrane (181). Residues of tm5 are also thought to be involved in ligand binding and it has been proposed that binding of some ligands could induce a change in the orientation of tm5 (175). However, these interactions are suggested to be species specific; the rat and human 5-HT_{2A}R express an Ala and Ser residue at residue 242 of tm5 respectively, which is suggested to result in a higher affinity of N-1-substituted tryptamines at the rat receptor as opposed to the human receptor, and vice versa with respect to the binding of N-1-unsubstituted tryptamines (182). The 3rd intra-cellular loop (i3), although it is always relatively long, varies widely both in terms of its length and amino acid composition (175). With the exception of the rat and mouse 5-HT_{5A}R, the third extracellular loop contains two highly conserved Cys residues, with the potential to form a disulphide bond. Signalling assays using chimeric receptor constructs and GST-fusion protein studies have determined i3 to be involved in G protein coupling (175,183,184). Experiments detecting signalling downstream of the 5-HT_{2A}R, but substituting the 5-HT_{2A} i3 with a 5-HT_{1B} i3, resulted in the inhibition of adenylyl cyclase (AC) as opposed to the typical activation of PLC (184). This receptor domain has also been implicated in the binding of β-arrestins, calmodulin and small GTPases (185,186). tm6 and tm7 represent the trans-membrane spanning domains with the highest sequence homology among the 5-HT receptor family (175). Mutagenesis studies focused on 5-HT family receptor tm6 and tm7 domains suggest that residues of these domains are also involved in ligand binding (175,187,188). Further research focused on the sixth transmembrane-spanning domain of the 5-HT_{2A}R elucidated the

possible involvement of tm6 residues in receptor conformation changes required for G protein-coupling (179). Many 5-HT receptors, including the 5-HT_{2A}R express a highly conserved Cys in the proximal region of the C-terminal tail, close to the junction with tm7 (7). As previously discussed, this is a putative palmitoylation site, potentially giving rise to a 4th intracellular loop and acting to anchor an eighth cytoplasmic helix at the membrane (7). 5-HT receptor C-terminal domains have been implicated with a variety of functional roles. They are also thought to be involved in ligand binding and receptor activation, in addition to playing a putative role in targeting the receptor to various sites. PDZ-domain protein target motifs located at the distal region of 5-HT_2 receptor C-terminal tails promote interactions with various signalling proteins, thereby influencing receptor trafficking and signalling (189,190). This motif has been demonstrated to be required for 5-HT_{2A}R binding of the PDZ-domain protein post-synaptic density 95 (PSD95) (189,190). PSD95 is a scaffolding protein, participating in the formation of multiprotein signalling complexes (189-191). Co-expression of PSD95 and the 5-HT_{2A}R has been shown to promote 5-HT-induced inositol phosphate production (191). The binding of PSD95 to the 5-HT_{2A}R is also reported to result in an inhibition of agonist-mediated receptor internalisation (191). Additionally, the PDZ-protein recognition motif has been shown to be important for the dendritic targeting of the 5-HT_{2A}R in cultured cortical pyramidal neurons (7,189,192).

1.10 Main signalling pathways of the 5-HT_{2A}R

The 5-HT_{2A}R is proposed to signal primarily through 3 distinct yet interconnected pathways, via the activation of phospholipase C, phospholipase D and phospholipase

A_2. The conventional 5-HT_{2A}R-induced signal transduction pathway occurs via GPCR interaction with heterotrimeric G proteins of the Gq/11 group. The carboxy-terminal part of the third intracellular loop (i3) of the receptor is thought to be responsible for forming direct interactions with the α subunit of the heterotrimeric Gq protein (Gαq) (168). It has also been proposed that GPCR:Gαq association may be facilitated by residues of the second and seventh transmembrane segments, which promote helix:helix interactions (177). The binding of the G protein to the GPCR promotes the replacement of GDP by GTP on the G protein α subunit, leading to dissociation of the α subunit from the βγ subunits. Gαq is then able to diffuse away and bind to phospholipase C (PLC), which it activates. The activation of PLC results in an increase of diacyl glycerol (DAG) and inositol phosphates and consequently the activation of protein kinase C (PKC) and a rise in intracellular Ca^{2+} levels.

1.10.1 Phospholipase C

In the 1950s a novel enzymatic activity was described that resulted in the incorporation of [^{32}P] into phospholipids, following pancreatic acinar tissue stimulation by carbachol (193). Following subsequent studies, this enzyme was characterised as phospholipase C (PLC), a membrane bound enzyme, capable of catalysing the formation of inositol (1, 4, 5)-trisphosphate and 1, 2-diacylglycerol from the membrane phospholipid, phosphatidyl inositol (4, 5)-bisphosphate (PIP_2) (194-198). There are thirteen currently identified mammalian PLC isoforms, which are categorised into six groups and further divided into subtypes, to yield; four PLCβ subtypes (1-4); two PLCγ subtypes (1-2); three PLCδ subtypes (1, 3 & 4); PLCε; PLCζ; and two PLCη subtypes (1-2) (199). The basic structure of a PLC protein

comprises: a pleckstrin homology (PH) containing N-terminal domain, of approximately 120 residues that interacts with the heads of the membrane phospholipids (200-202); four EF-hand motifs that provide a flexible linkage region to the enzyme's catalytic core (203-205); the catalytic core, composed of an X and a Y domain, which contains the residues involved in the hydrolysis of phosphoinositols (203); and a C-terminal C2 domain (203,204). Each sub-family also express its own characteristic regulatory domain, which acts to define the signalling pathway in which the protein will participate (199). The PLCγ isoforms, composed of two Src homology 2 (SH2) domains and an Src homology 3 (SH3) domain, flanked to either side by a split PH domain (203) are reported to form interactions with PLD2 via the SH3 domain, thus acting as an intermediate between PLC and PLD signalling pathways (206).

The C-termini of PLCβ enzymes contain regulatory domains that form interactions with both activated GTP-bound Gαq subunits (207,208), and in addition PDZ domain proteins (209), thereby potentially promoting the scaffolding of signalling complexes. Analysis of the crystal structure of PLCβ has shown the C-terminus to exist as a long coiled coil structure, thereby exposing key residues involved in interactions with GTP-loaded Gαq subunits (207,208). Active Gαq is thought to be selective in its binding to PLCβ isoforms, preferentially binding PLCβ1, followed by PLCβ4, then PLCβ3 and finally PLCβ2 (210,211). The PLCβ isoforms also contain an intrinsic GTPase-activating protein (GAP) functionality within their C-terminus, which acts to catalyse the hydrolysis of GTP on the active form of Gαq to form the inactive GDP-bound Gαq subunit (212). Gβγ subunits, liberated via the activation of

Gi/o proteins, are also capable of activating PLCβ isoforms, but with approximately 50-100 fold less potency than Gαq (213). The Gβγ subunit forms interactions with the PH domain of PLCβ N-terminal tails (214), and also with the catalytic domain of PLCβ2 (215). PLCβ3 and PLCβ2 appear to be the only isozymes for which Gβγ subunits demonstrate a high affinity (216,217). It has been proposed that Gαq and Gβγ subunits can simultaneously interact with PLCβ isoforms, and demonstrated that Gβγ is incapable of activating PLCβ in the absence of Gαq (199,218).

Following PLC activation, the enzyme acts to hydrolyse the membrane phospholipid phosphatidyl inositol (4, 5)-bisphosphate (PIP_2), by catalysing its cleavage just before the phosphate group, thereby yielding 1, 2 diacylglycerol (1, 2-DAG) and (1, 4, 5)-inositol triphosphate (1, 4, 5-IP_3) (219,220). These are both important intracellular second messengers. 1, 4, 5-IP_3 increases intracellular Ca^{2+} levels by diffusing into the cytosol and binding to $InsP_3$ receptors, localised on the membranes of the endoplasmic reticulum (ER) and the sarcoplasmic reticulum (SR) (221,222). These receptors are ligand-gated Ca^{2+} channels, thus activation by 1, 4, 5-IP_3 results in the release of Ca^{2+} into the surrounding cytoplasm (221). The subsequent rise in intracellular Ca^{2+} concentration serves to activate the ryanodine receptor-operated channel on the SR, which leads to a further increase in the Ca^{2+}, thereby acting to increase cellular depolarization (223,224). 1, 2-DAG stays at the membrane and induces the translocation of protein kinase C (PKC) isoforms to the membrane, which it then stimulates (221,225,226). This results in the activation of downstream PKC-mediated signalling cascades.

1.10.2 Phospholipase A_2

5-HT_{2A} receptor activation can also result in an increase in intracellular arachidonic acid (AA) levels. This increase in AA concentrations has previously been demonstrated to be phospholipase A_2 (PLA_2) dependent (227). Although there is some evidence showing that PLA_2 activation can occur as a downstream effect of agonist-induced phospholipase C activity, it is now established that PLA_2 activation and thus AA release largely occur as part of an independently activated signalling cascade (227). The phospholipase A_2 enzymes have been categorised into three groups (228), of which only one, cytosolic PLA_2 is reported to undergo receptor-mediated activation (229). The potential mechanisms of 5-HT_{2A}R activation of PLA_2 were investigated by Kurrasch-Orbaugh et al (227). Pre-incubations of NIH3T3-5-HT_{2A}R cells with pertussis toxin resulted in partial inhibition of 5-HT-induced AA release, thereby implicating the involvement of Gαi/o proteins in this pathway (230). Inhibition of Gβγ (by the Gβγ-binding domain of the β-adrenergic receptor kinase), resulted in decreased 5-HT_{2A}R-mediated AA release, in addition to an inhibition of 5-HT_{2A}R-mediated AA release seen following inhibition of MEK, with the specific inhibitor PD 098,059, thereby implicating involvement of both MEK-activated ERK 1/2, and Gβγ in the PLA_2 pathway (230). In addition, selective inhibition of the Gα12/13 subunit, Rho and p38 also resulted in a decrease in 5-HT_{2A}R-mediated AA release (230). Thus, two pathways have been implicated in the mediation of 5-HT_{2A} receptor-activated PLA_2 pathway; one involving Gα12/13 activation of Rho and p38 activation; and another involving the activation of ERK 1, 2, as a result of the activation of Gαi/o and Gβγ subunits and subsequent activation of Ras and Raf. PLA_2 is reportedly activated by the binding of Ca^{2+}, which induces its translocation to

the membrane and subsequent phosphorylation of a Ser at residue 505, reportedly by ERK 1/2 or p38 MAPK (231,232). Following activation, cytosolic phospholipase A_2 induces AA release by hydrolysing AA-containing phospholipids at the sn-2 position (233,234).

1.10.3 Phospholipase D

There are two mammalian phosphatidylcholine-specific PLD isoforms, PLD1 and PLD2, encoded for by two PLD genes, both with two splice variants (235-237). Subcellular PLD localisation is reportedly isoform-dependent; PLD2 has been demonstrated to localise predominantly within lipid raft fractions at the plasma membrane (236,238,239), and is possibly associated with the Golgi apparatus (240). Alternatively, PLD1 is found throughout the cell, but typically localises to Golgi structures, the endoplasmic reticulum and the late endosomes (236,241). There have also been reports of PLD1 localisation at the plasma membrane, although the extent to which this occurs may be cell-type-dependent (242,243) and it is further established that PLD1 can undergo translocation to the plasma membrane subsequent to GPCR activation (244). The enzymes also appear to demonstrate isoform-specific cellular functions; PLD1 is reportedly involved in agonist-induced secretion, actin reorganisation, cell adhesion and migration (199,245-247), whilst various studies have implicated PLD2 activity in the endocytosis and recycling of membrane receptors (241,248).

Active PLD acts to hydrolyse phospholipids, primarily phosphatidyl choline (PtdCho), at the terminal phosphodiester bond, thereby producing phosphatidic acid

(PA) and a free polar head group (choline). Whilst PLD-generated choline is used as a precursor for phosphatidyl choline regeneration, and can be used by cholinergic neurons for the synthesis of acetylcholine (249), phosphatidic acid (PA) is thought to be primarily responsible for PLD-mediated signalling, and is implicated in a diverse array of cellular functions, including the activation of the Ras/Raf/MEK/ERK cascade. There is evidence that PA acts to promote the translocation of the Ser/Thr kinase cRaf-1 to the cell membrane, where it can be activated by the membrane bound Ras (250-253), resulting in MEK phosphorylation and subsequent ERK activation. It has also been suggested that PA production may result in an increase in the production of endocytic vesicles, to which Ras-Raf complexes may attach, in addition to scaffolding proteins linked to MEK and ERK 1 / 2 (253). PA is additionally demonstrated to activate type I PI-4P 5-kinase, which is reportedly involved in actin polymerisation and subsequently, membrane ruffling (254-257). Furthermore, PA is shown to interact with (and have an essential role in the activation of) target of rapamycin, a mediator of translational regulation (258,259). PA has also been demonstrated to bind the small G protein ARF, kinesin and N-ethylmaleimide-sensitive factor (260).

PA can additionally be further metabolised to diacylglycerol (DAG) via phosphatidic acid phosphohydrolase (PAP)-catalysed dephosphorylation (261,262). Agonist activation of the M_3 receptor has been demonstrated to result in a second, delayed period of DAG elevation following the original PLC-mediated DAG release, probably due to the relatively slow conversion of DAG from PA by PAP (263). However, DAG is also phosphorylated by diacylglycerol kinases, to reform PA

(254). Contrary to previous thought (264), PA-generated DAG is also thought to be involved in the membrane translocation and subsequent activation of PKC isoforms (265,266).

Furthermore, PA can also be converted to lysophosphatidic acid (LPA) via the actions of phospholipase A_1 or A_2 (267,268). This metabolite can also be reconverted to the original PA, via lysophosphatidic acid acyltransferases catalysis (254). Although the complete range of molecular mechanisms through which LPA exerts its activity are not entirely understood, it is known to cause functional activation of various membrane-bound Edg family G protein-coupled receptors (269). LPA is demonstrated to be involved in a myriad of physiological roles: it has been shown to display growth-factor-like activity and promote cellular proliferation (270); it is required for the activation of protein kinase D (271); and has additionally been established to activate PKC, inhibit the formation of cAMP and promote Ca^{2+} release via Edg family G protein activity (272).

It is well established that the 5-HT_{2A}R is also capable of activating alternative transduction pathways, including a pathway that involves the recruitment of the small G protein ADP-ribosylation factor (ARF), and the subsequent activation of phospholipase D (PLD) (50). Isolated cytosolic extracts found to be capable of activating PLD were identified as ARF proteins by Cockcroft et al, 1994 (273). It has now also been demonstrated that both ARF and RhoA function as activators of PLD in signal transduction pathways from many GPCRs, including the 5-HT_{2A}R (50). ARF1 has since been acknowledged as the isoform involved in PLD activation

downstream of the 5-HT_{2A}R (274). The binding of ARF1 to the 5-HT_{2A}R has been found to increase both when ARF1 is GTP-loaded and when the receptor is stimulated with 5-HT. GST-fusion protein constructs of 5-HT_{2A}R domains have been used to illustrate that ARF1 appears to bind with the highest affinity to the C-terminal domain, where residues 376–384 have been implicated in ARF1 coupling. I further demonstrate within this thesis that PLD binds to the distal regions of the C-terminus, although the exact mechanism of PLD activation by ARF within the inferred receptor:ARF:PLD complex is unknown. This pathway is discussed in further detail in section 3.1.

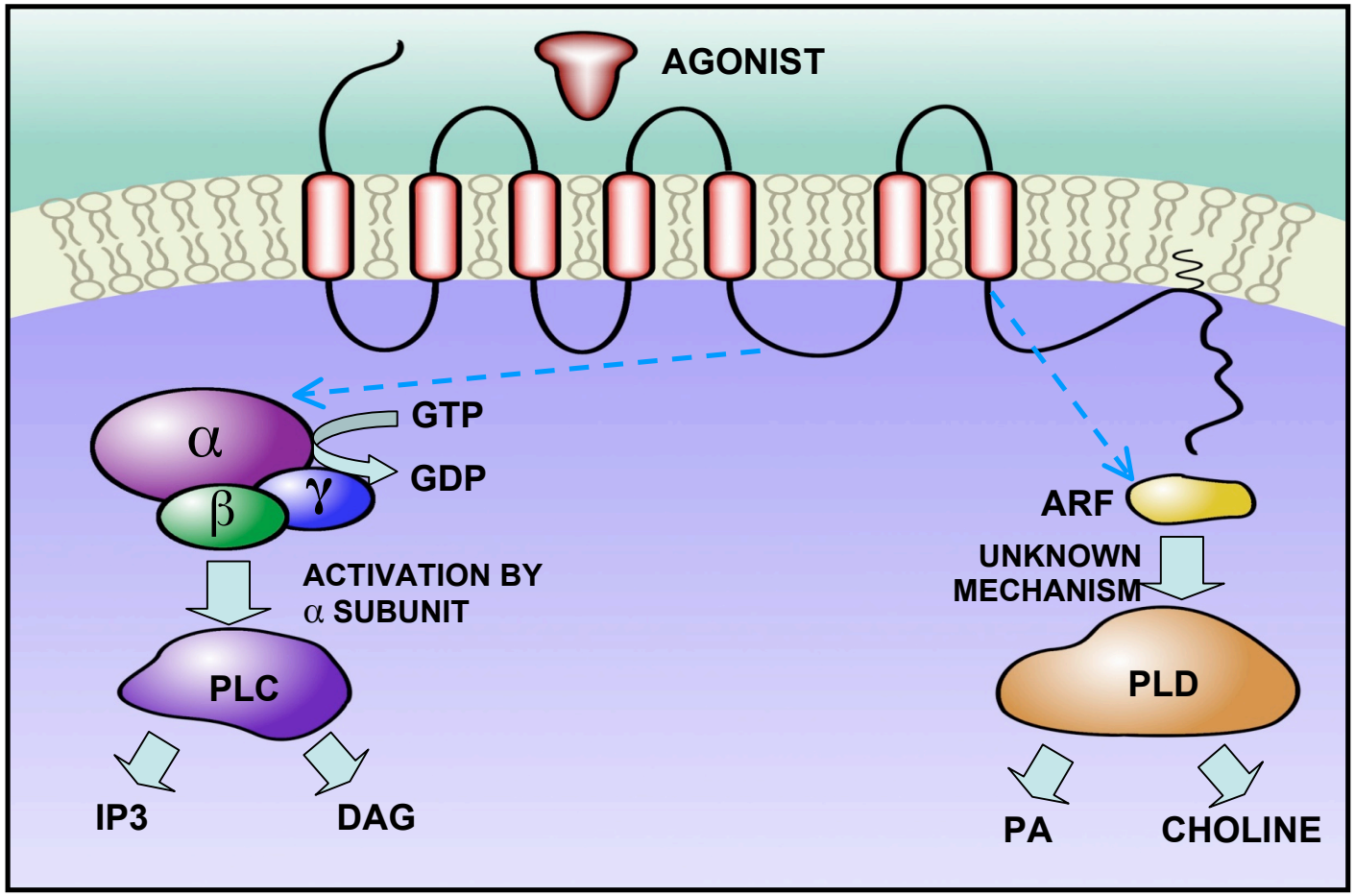


Figure 1.2 5-HT_{2A}R-mediated PLC activation and ARF-dependent PLD activation. Schematic diagram illustrating Gq/11 activation of PLC to generate inositol triphosphates and diacylglycerol and ARF-dependent PLD activation, resulting in phosphatidic acid and choline production.

1.10.4 Ligand-directed signalling

It is now widely established that the signalling pathway which an activated receptor mediates can be influenced by the structure of the bound ligand (275). Different ligands are thought to act to stabilise different conformational states of the receptor, thereby resulting in differential activation of the various secondary effector proteins. This phenomenon, termed agonist-directed trafficking of receptor stimulus, or functional selectivity, has been demonstrated using various receptor types, including β_2-adrenergic (276), dopamine (277), μ-opioid (278-280) and the 5-HT_{2A} receptors (281-283). Experiments investigating 5-HT_{2A}R activation by a variety of agonists have provided evidence to suggest that IP accumulation and AA release occur at differing degrees dependent upon the ligand stimulating the receptor, indicating that there is a differential activation of PLC and PLA_2 pathways (281-283). Additionally, the PLC and PLA_2 pathways have actually been determined to have different 'receptor reserves' (227). These studies demonstrated that PLA_2 activation actually requires a much smaller proportion of activated 5-HT_{2A}R than PLC activation needs, providing further evidence for the independent nature of PLA_2 signalling (227).

1.11 Serotonergic involvement in schizophrenia

Schizophrenia is widely regarded to be a multi-factorial disease, with neurodevelopmental and dopaminergic disturbances being some of the most popular hypothosise. However, there is also strong support for the involvement of the serotonergic system. Following the discovery that serotonin receptors act as mediators of lysergic acid diethylamide (LSD) activity (284,285), there has been

much research regarding the possible involvement of the serotonergic system with the disease state schizophrenia (170). The physiological effects induced by serotonergic hallucinogens such as LSD, are thought to present in a similar manner to symptoms those of a psychotic patient (286). Thus, it has been proposed that alterations in the functioning of the serotonergic system could result in the development of a psychotic disease state such as schizophrenia. Habituation and pre-impulse inhibition are cognitive processes that allow the mind to focus on relevant stimuli by filtering out superfluous information (286). It has been proposed that schizophrenia may occur as a result of disruptions to these responses, which could potentially result in the development of psychotic symptoms (286,287). Serotonergic hallucinogens have also been shown to induce alterations of both habituation and preimpulse inhibition startle responses in mouse models (288). Thus further inferring that abnormalities of serotonergic systems could underlie some schizophrenia-induced psychotic symptoms.

In particular, the 5-HT_{2A}R is of particular interest with regard to schizophrenia, due to the known agonist actions of hallucinogens such as LSD, mescaline, psylocibin as well as the antagonist actions of atypical psychotics at these receptors (284,285) (168,289). The localisation of the 5-HT_{2A}R within the pre-frontal cortex has been found to be altered within schizophrenic patients and patients thought to be at risk of developing schizophrenia (172), and post-mortem studies have revealed there to be reduced 5-HT_{2A}R binding sites in the prefrontal cortex of schizophrenia sufferers (290). Additionally the therapeutic effect of some antipsychotic agents has been

shown to increase following co-administration with additional antagonists of the 5-HT_{2A}R (286).

1.12 Clozapine, an atypical antipsychotic with affinity for the 5-HT_{2A}R

Typical antipsychotics are well established to exert their effects by influencing the dopaminergic system (291). Conversely, atypical antipsychotics have relatively high affinity for 5-HT receptors, in particular the 5-HT_{2A}R (292,293). These drugs are reported to exhibit a higher ratio of affinity and antagonism at serotonin receptors than at dopamine receptors (294-296). The atypical antipsychotics are of much interest, as they are effective agents in the treatment of schizophrenia, and in particular the treatment of negative symptoms, but do not present the same risk of extrapyramidal side effects seen with the typical antipsychotics (291). Clozapine, developed in the 1960s, is the prototypical atypical antipsychotic (297). It was found to be effective in the psychotic, negative and cognitive treatment of schizophrenia and in particular was found to be unique in its effective treatment of previously treatment-resistant schizophrenic patients, in addition to evoking minimal extrapyramidal side effects (298). However, its use was restricted in the 1970s following the finding that the use of the drug could result in development of potentially fatal agranulocytosis (297). Clozapine acts as a competitive antagonist upon 5-HT_{2A}R-initiated phospho-inositide (PI) turnover, mediated by PLC activation (293). It is reported to induce cortical 5-HT_2 receptor downregulation in rats (292), and is also suggested to induce the in vitro internalisation of the 5-HT_{2A}R, in addition to promoting an in vivo subcellular redistribution of the receptor; actions which are

not normally observed with receptor antagonists (299). Recent research has additionally implied that clozapine may contribute towards activation of the MEK/ERK MAP kinase pathway (300).

1.13 Single Nucleotide Polymorphic (SNP) forms of the 5-HT_{2A}R

To date, 17 single nucleotide polymorphisms (SNPs) have been identified within the 5-HT_{2A}R gene, 9 within the promoter region and 7 within the coding region. Of the SNPs known to exist in the 5-HT_{2A}R gene promoter, A1438G is the most widely studied, and different studies have looked at its association with seasonal affective disorder, Alzheimers disease, alcoholism and suicide, with a mixture of results in all cases. This SNP is in complete linkage disequilbrium with a non-coding T102C SNP, thus meta-analysis associations of one apply equally to the other. Of the 7 SNPs identified within the coding region of the 5-HT_{2A}R gene, only 5 are non-synonymous; T25N of the N-terminus, I197V of i2, and S421F, A447V and H452Y of the ct. Approximately 9% of individuals are reported to express the H452Y minor allele within the distal region of the 5-HT_{2A}Rct. Meta-analysis studies have concluded that there is a significant association between heterozygous expression of the Tyr 452 minor allele and a poor clozapine response in schizophrenic patients (301,302). An additional meta-analysis study did not report a significant association, but did identify the Tyr 452 allele to be expressed with a greater frequency among the clozapine resistant (303). Heterozygous and homozygous expression of the Tyr 452 allele has since been (304)linked to various neuropsychological disorders, such as attention deficit hyperactivity disorder (ADHD) (305), bipolar disorder (306), and also poor memory (307). In addition, studies using the platelets of eight homozygous

His 452 individuals and eight heterozygous H452Y individuals determined there to be differences in the signalling of the Tyr 452 allele. Despite there being no significant differences in the levels of PKC isoforms, Gαq/11 subunits or [^{125}I]LSD binding between the two platelet groups, there was a significant difference in 5-HT-stimulated Ca^{2+} mobilisation by the heterozygous H452Y platelets: the peak amplitude was smaller and the overall shape of the Ca^{2+} mobilisation peak was blunted, when compared to homozygous His 452 platelets (308). A further study has suggested that there is a substantial decrease in both PLC and PLD activation by NIH3T3 cells overexpressing the 452 Tyr variant of the 5-HT_{2A}R, when compared to control, wild-type 5-HT_{2A} receptor expressing cells (309). In addition, the wild-type 5-HT_{2A} receptor variant, was reported to undergo rapid desensitisation as a result of prolonged periods of incubation with 5-HT, prior to PLC assay (309). However, the H452Y variant of the receptor displayed a significantly reduced pattern of desensitisation when stimulated in an analogous manner (309). It was thus suggested that the H452Y-5-HT_{2A}R variant may exist in a partially desensitized state prior to agonist incubation (309).

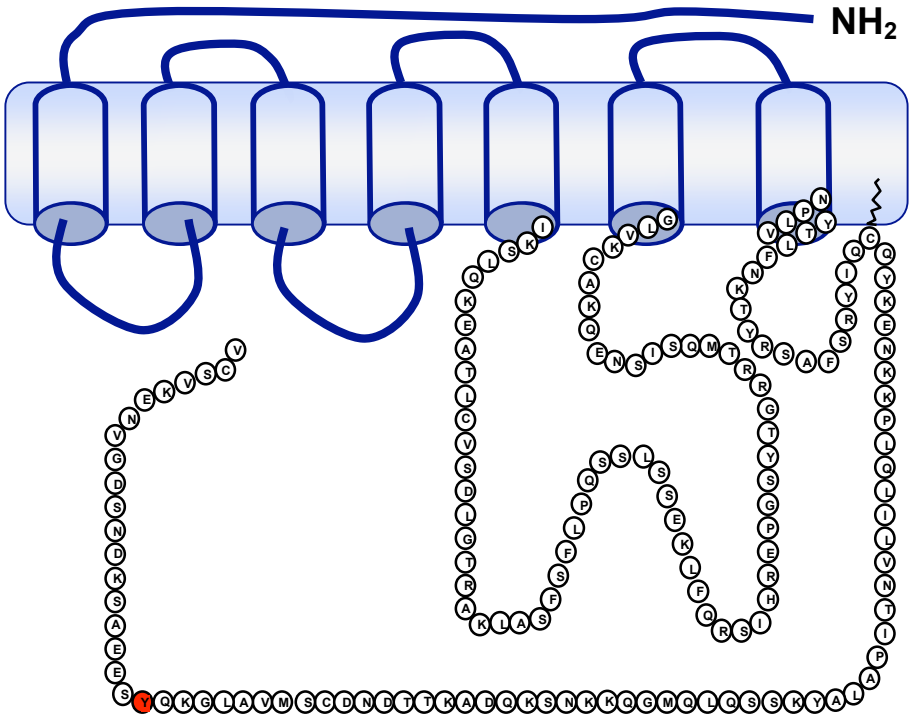


Figure 1.3 H452Y polymorphic variant of the 5-HT$_{2A}$R. Diagram to illustrate the locus of the H452Y polymorphism (in red) within the distal region of the 5-HT$_{2A}$Rct.

1.14 Aims of the study

It follows that due to the proposed involvement of the 5-HT_{2A}R in pathophysiological disorders such as schizophrenia, its signalling mechanisms are of particular pharmacological interest. The aim of this thesis was to gain a further understanding of 5-HT_{2A}R-mediated signal transduction, and to compare this to the signalling of a physiologically relevant polymorphic variant of the receptor (H452Y-5-HT_{2A}R). Following suggestions that the H452Y polymorphism may influence Gq/11-mediated signalling downstream of the 5-HT_{2A}R (308,309), it was proposed to further investigate this effect, comparing the desensitisation of Gq/11-mediated responses downstream of each receptor, and the potential effect of β-arrestin isoforms and Ca^{2+}-binding proteins on this pathway. However, the 5-HT_{2A}R has also been shown to mediate signalling through less conventional and well-studied pathways, such as the ARF-dependent activation of PLD. Robertson et al, 2003 previously identified the active isoform involved in 5-HT_{2A}R-mediated ARF-dependent PLD signalling to be ARF1, and the locus of its binding to be at the beginning of the 5-HT_{2A}Rct (274). Subsequent to these studies it was proposed to investigate which PLD isoforms are involved in this pathway, and in order to further understand the mechanism of ARF-dependent PLD activation, to explore the dynamics and locus of PLD binding at the 5-HT_{2A}Rct, in addition to examining any possible effects of the H452Y polymorphism on this signalling pathway.

CHAPTER 2.0

2.0 Materials & Methods

2.1 Preparation of mammalian cDNA

2.1.1 Site-directed mutagenesis

cDNAs of mutated receptor constructs were generated by colleagues in house using in vitro site-directed mutagenesis. A brief outline of the protocol follows: the parent plasmid was denatured to yield two single stranded parent plasmids and incubated with a mutagenic and a selection primer (which contains a mutation in the recognition sequence for a restriction enzyme site). The two primers were simultaneously annealed to the single stranded DNA, and following elongation, ligated with T4 DNA ligase. Selected mutated and control plasmids were transformed into *mutS Escherichia Coli (E.Coli)* strains (defective in mismatch repair) and DNA from the pooled bacterial population was isolated. Mutated plasmids were isolated based on resistance to a second restriction enzyme, introduced by the selection primer. The DNA was again transformed into bacterial cells, and as unmutated plasmids were digested during the second restriction digest, only mutated plasmids were transformed. cDNA was then recovered as described in section 2.1.3. p.45.

2.1.2 Transformation of competent cells

50 µl of either BL21 (>10^6 cfu/µg suspension) or JM109 (10^8 cfu/µg suspension) competent strains of *E.Coli* were incubated with 1 µg of ampicillin-resistant plasmid cDNA for 20 min on ice. The cells were then heat shocked at 42°C for 45 s, prior to a further 10 min incubation on ice. 900 µl of sterile Luria Bertani (LB) broth (10 g/l

bacto-tryptone, 5 g/l yeast extract, 10 g/l NaCl; pH 7.0) was then added to the cells and incubated at 37°C for approximately (~) 1 h. 100 μl of the cell suspension was then spread onto LB agar plates (LB broth, 1.5% bacto-agar w/v, 12.5 μg/ml ampicillin), which were incubated overnight at 37°C.

2.1.3 Plasmid purification

Plasmid purification was performed using the Qiagen HiSpeed Plasmid Maxi Kit. A single bacterial colony was selected and used to inoculate a starter culture of 3 ml LB-broth, supplemented with ampicillin (12.5 μg/ml) to ensure the selection of plasmid containing cells. The starter culture was then left to incubate with rotation for (~ 8 h, 37°C). Following incubation, 1 ml was added to 100 ml of LB-broth (with 12.5 μg/ml ampicillin) and incubated (overnight, 37°C) in the shaking incubator. Bacterial cells were then harvested via centrifugation (15 min, 7000 g, 4°C). The supernatant was then removed and the remaining pellet resuspended in 10 ml Buffer P1 (Qiagen resuspension buffer; 50 mM Tris-Cl pH 8.0, 10 mM EDTA), containing 100 μg/ml RNase A solution. Cells were lysed with the addition of 10 ml Buffer P2 (Qiagen lysis buffer; 200 mM NaOH, 1% SDS w/v). The solution was mixed thoroughly by inversion and incubated for 5 min after which 10 ml chilled Buffer P3 (Qiagen neutralisation buffer; 3.0 M potassium acetate; pH 5.5) was added to prevent further cell lysis and the solution mixed thoroughly. The lysate was poured into a QIAfilter cartridge and incubated at room temperature for 10 min, before being filtered into a HiSpeed maxi tip, previously equilibrated with 10 ml Buffer QBT (Qiagen 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 passed through, the

HiSpeed maxi tip was washed through with 60 ml Buffer QC (Qiagen wash buffer; 1.0 M NaCl, 50 mM MOPS pH 7.0, 15% isopropanol v/v), and the DNA eluted into a 50 ml tube using 15 ml Buffer QF (Qiagen elution buffer; 1.25 M NaCl, 50 mM Tris HCl pH 8.5, 15% isopropanol v/v). The DNA was precipitated by incubating the eluate with 10.5 ml isopropanol (5 min, room temperature (RT)). The resulting DNA solution was transferred to a 30 ml syringe and filtered through a QIAprecipitator to isolate the DNA, which was subsequently washed using 2 ml 70% ethanol. The QIAprecipitator was left to dry before 1 ml Buffer TE (10 mM Tris Cl pH 8.5, 1 mM EDTA) was filtered through into a 1.5 ml Eppendorf, eluting the DNA. The DNA eluate was passed back through the QIAprecipitator to enable maximal DNA retrieval.

2.1.4 Agarose gel analysis of DNA

Agarose gel (1% agarose non-low melting point, concentrate standard Tris-borate-EDTA buffer) was heated until melting point and ethidium bromide added to a final concentration of 0.5 μg/ml. The molten gel was allowed to cool before being poured to a depth of 3-5 mm into a sealed electrophoresis tank containing a comb positioned 0.5-1.0 mm above the plate, in order to create sample wells. Once the gel was set it was covered with electrophoresis buffer (concentrate standard tris-borate-EDTA buffer). 10 μl DNA samples were then added to 1 μl loading buffer (0.4% bromophenol blue in glycerol) and loaded into the gel, which was run at 100 V (25-30 mA, ~ 2-3 h), before being examined under a UV light. Approximate relative concentrations of DNA were estimated from band brightness.

2.1.5 Spectrophotometric quantification of DNA concentration

DNA concentrations were quantified when required by spectrophotometric analysis. 5 μl of the appropriate DNA was added to a plastic cuvette containing 0.5 ml ddH_2O and the solution mixed. The absorbance at 260 nm (A_{260}) was determined using a spectrophotometer (Shimadzu UV-1201). DNA concentrations were calculated by multiplying the A_{260} reading by 100 to account for the dilution factor and then by 50, as an A_{260} of 1 corresponds to a DNA concentration of approximately 50 μg/ml. DNA samples were typically found to have a concentration between 0.5–1.0 μg/μl.

2.2 Cell culture

2.2.1 Cell maintenance

COS7 cells (ECACC) were routinely cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Invitrogen, Paisley, UK), supplemented with 10% normal calf serum (Gibco), 100 units/ml penicillin (Gibco) and 100 μg/ml streptomycin (Gibco) and maintained at 37°C in a 5% CO_2, 95% O_2 humidified environment.

C6 glioma cells (ECACC) were maintained in Ham's F12 Medium (Sigma, Poole, Dorset, UK), supplemented with 10% foetal calf serum (Gibco) and 50 units/ml penicillin (Gibco) and 50 μg/ml streptomycin (Gibco) and kept at 37°C (5% CO_2, 95% O_2).

Cells were passaged twice weekly when they had reached ~ 90% confluence. DMEM was removed by aspiration and cells were washed for 20 s in 20 ml pre-warmed HBSS (Gibco). Cells were then exposed to 10 ml trypsin-EDTA (0.05% trypsin,

0.02% EDTA, 0.085% NaCl; Gibco) for 1 min, after which the trypsin-EDTA was removed and cells incubated at 37°C for 5 min. Following cell detatchment, cells were re-suspended in 8 ml DMEM and counted using a haemocytometer (Marienfeld). Cells were then seeded into flasks (75 or 175 cm^2) or plates (12- or 24-well) (Greiner Bio-One, Gloucestershire, UK) at 21 x 10^5, 45 x 10^5, 1 x 10^5 or 0.5 x 10^5, respectively.

2.2.2 Cryopreservation of cultured cells

Exponential phase cells were detached using trypsin-EDTA, as above, and re-suspended in appropriate growth medium. Cells were centrifuged (200 g, 5 min), before the supernatant was removed and the pellet resuspended in DMEM supplemented with 10% DMSO. 1 ml aliquots were then frozen in an insulated box (-70°C, overnight), prior to transfer to liquid nitrogen (–140°C).

2.2.3 Thawing of cryopreserved cells

Aliquots of cells were removed from liquid nitrogen (–140°C) and quickly defrosted in a water bath (37°C, 1 min). The cell suspension was transferred to 10 ml pre-warmed DMEM supplemented with 10% normal calf serum (Gibco), 100 units/ml penicillin (Gibco) and 100 μg/ml streptomycin (Gibco) in a 75 cm^2 flask. Cells were incubated for 24 h, before the addition of fresh medium.

2.2.4 Transient transfections

Cells were grown to ~ 60% confluency, then transfected with the appropriate cDNA, generally in the $pcDNA_{3.1}$ expression plasmid, using either Gene Juice Transfection

Reagent (Novagen, Merck Biosciences, Nottingham, UK), or Fugene-6 Transfection Reagent (Roche, Charles Avenue, Burgess Hill, West Sussex, UK). Transfection reagents were used according to the manufacturers instructions, a 3:1 or 5:1 transfection reagent:cDNA ratio was used for COS7 or C6 glioma cells respectively. Prior to cell transfection with 5-HT_{2A} receptor cDNA, medium was removed from cells and replaced with DMEM containing 2% Ultroser G (USG; Pall Biosciences, Portsmouth, Hampshire, UK) in order to remove any traces of 5-HT present in normal serum. Transfection Reagent-cDNA complexes were incubated for ~ 15 min before being added to the cells in appropriate quantities; 5 or 10 μl of total cDNA was added to 75 or 175 cm^2 flasks respectively and 0.33 or 0.7 μl of total cDNA was added to a single well of a 24 or 12 well plate respectively. Cells were then grown at 37°C in a 5% CO_2, 95% O_2 humidified environment, before experimental use. Inositol phosphate and western blot studies were used to determine the optimal conditions for the transfection of COS7 cells using either Gene Juice Transfection Reagent, or Fugene-6 Transfection Reagent. Transfection of 1.0 x 10^5 cells/well (of a 12-well plate) for 48 hours was found to result in maximal expression of both Flag-tagged muscarinic 3 receptor and HA-tagged 5-HT_{2A} receptor. These conditions were used for all experiments shown within this thesis.

2.3 Preparation of cell extracts over-expressing proteins

COS7 cells were seeded in 175 cm^2 flasks and transfected with appropriate DNA as described in section 2.2.4 p48. Cells were quiesced approximately 4 h prior to harvest, after which they were washed once with Hanks' Balanced Salt Solution (HBSS). Cells were then lysed in 2 ml extraction buffer (PBS pH 7.5, 20% glycerol

w/v, 1.0% CHAPS w/v, 1.0% sodium deoxycholate w/v, 1 mM sodium orthovanadate, 1% v/v protease inhibitor cocktail set III; Calbiochem) on ice for 40 min. Alternatively, cells over-expressing proteins which localised to cellular compartments were isolated with 2 ml buffer (PBS pH 7.5, 1 mM dithiothreitol, 1 mM sodium orthovanadate, 1% v/v protease inhibitor cocktail set III; Calbiochem), followed by homogenisation (Ystral polytron) (setting 3, ~ 15 s. Cell lysates were then centrifuged (20 min, 11,600 g, 4°C), before 250 µl aliquots of supernatant were taken and stored at -40°C until use.

2.4 Ligand binding experiments

COS7 cells were seeded into 175 cm^2 flasks and transfected with appropriate cDNA as outlined in section 2.2.1 p.47 and 2.2.4 p.48, respectively. Transfected cells were quiesced for 4 h prior to lysis. Cells were detached from the flask surface and harvested in ice cold ketanserin binding buffer (50 mM Tris, 5 mM $MgCl_2$, 1 mM EGTA; pH 7.2) supplemented with 1:100 protease inhibitor cocktail set III (Calbiochem) and disrupted with an Ystral homogeniser (setting 3, ~ 15 s). Cell lysates were transferred to a 2 ml skirted plastic vial and centrifuged (10 min, 2000 g, 4°C), to pellet nuclear debris. The supernatants were transferred to clean 2 ml vials and further centrifuged (30 min, 12,000 g, 4°C) to pellet cell membranes. The supernatant was removed and the pellet resuspended in 2 ml cold ketanserin buffer by trituration. The membranes were washed by centrifuging (15 min, 12,000 g, 4°C), before resuspension in 2 ml ketanserin buffer. The membrane suspension was mixed thoroughly by trituration until homogenous, and 100 µl aliquoted in triplicate into 1.5 ml screw-topped Eppendorfs. Receptor expression was assessed by incubating

membranes with 0.8 nM [^{3}H]ketanserin (72.2 Ci/mmol) and a range of concentrations of unlabelled ketanserin (0.1-300 nM), or 10 μM mianserin to determine non-specific binding (total volume 0.5 ml, 60 min, 37°C). Binding was quenched by the addition of 1 ml ice-cold ketanserin binding buffer and membranes pelleted by centrifugation (20 min, 12,000 g, 4°C). Supernatant was removed by aspiration. The bottom of each Eppendorf tube was cut off and placed into 6 ml polyethylene vials, to which 4 ml of scintillation fluid (perkin-elmer) was added. Bound [^{3}H]ketanserin was detected using a Beckman LS 5801 scintillation counter, on a 4 min per sample, [^{3}H] disintegrations per min (DPM) programme. Homologous displacement of [^{3}H]ketanserin binding by a series of concentrations of unlabelled ketanserin was assessed and used to evaluate receptor expression levels.

2.5 Protein Assay

The Pierce BCA Protein Assay Kit was used to determine protein concentrations of COS7 cell membrane preparations identical to those in ligand binding studies, as described in section 2.4. p.50. BSA standards were prepared by serially diluting a 2.0 mg/ml BSA stock solution in ketanserin buffer to give a range of 0-2000 μg/ml final BSA concentrations. 25 μl membrane preparation was added in triplicates to a 96-well plate. 200 μl BCA working reagent, (50:1, Reagent A:Reagent B) was added to each well and mixed by shaking for 30 s. The plate was incubated at 37°C for 30 min, before being allowed to cool back to RT. Protein concentrations were determined by measuring absorbance at 562 nm, using a Thermo Varioskan Flash plate reader and unknowns were quantified by comparison with the concurrently run BSA standard curve.

2.6 Cell Surface Biotinylation

Procedures were based on the use of a membrane-impermeant covalent biotinylation agent (NH-SO_4-biotin) and the capture of solubilised biotinylated proteins on (reversibly binding monomeric) agarose beads, following the manufacturers recommended protocols (Sigma and Pierce, respectively). COS7 cells were seeded into 175 cm^2 flasks and transfected with appropriate cDNA as outlined in sections 2.2.1. p.47 and 2.2.4. p.48, respectively. Medium was aspirated from the flasks and cells were washed with 10 ml PBS per flask at RT. PBS was removed by aspiration and flasks were put on ice to cool. Cells were incubated with 1 mM NH-SO_4-biotin in PBS (2 h, 4°C), before removal by aspiration and incubation with glycine (75 mM) in PBS (10 min, 4°C) to quench the biotinylated agent and reduce non-specific binding. Glycine was removed by aspiration and cells washed with 5 ml ice cold PBS. Cell membranes were solubilised in 2 ml/flask solubilisation buffer (10% glycerol, 1% CHAPS, 0.5% deoxycholic acid, 1:100 protease inhibitor cocktail set III, PBS) and centrifuged (20 min, 12,000 g, 4°C). The combined supernatant from 2 flasks was retrieved and 1.8 ml was transferred to 2 x 2 ml skirted vials. Aliquot 'A' was left on ice, whilst the other was incubated with monomeric avidin immobilised on agarose beads (1 h, 4°C) with rolling, to bind biotin. Monomeric avidin agarose beads were prepared in advance; beads supplied in suspension were centrifuged (30 s, 12000 g, 4°C) and supernatant removed, before being washed x 2 in PBS. The beads were pelleted and incubated with 2 mM biotin in PBS (30 min, 4°C) with rolling, in order to displace any biotinylated proteins isolated from the membrane suspension. The bead solution was centrifuged (30 s, 12,000 g, 4°C) and supernatant removed and labelled 'B'. Receptor expression was assessed by incubating 100 µl

membranes 'A' and 'B' with 0.8 nM [^{3}H]ketanserin (72.2 Ci/mmol) at 37°C, for 60 min. Non-specific binding was determined by the addition of 10 μM mianserin. The binding reaction was terminated by the precipitation of proteins through the addition of 100 μl 0.05% bovine γ-globulin in water and 900 μl 30% polyethylene glycol-8000 in PBS at 4°C. After vigorous vortexing tubes were kept on ice for 15 min before centrifugation (20 min, 12,000 g, 4°C). The supernatant was completely removed by 2 rounds of aspiration. The bottom of the Eppendorf tubes were cut off and placed in 6 ml polyethylene vials, to which 4 ml of scintillation fluid was added. Bound [^{3}H]ketanserin was detected using a Beckman LS 5801 scintillation counter, (4 min, [^{3}H] DPM programme).

2.7 GST-fusion protein studies

2.7.1 Transformation of competent cells

BL-21 RIL *E. coli* were transformed with plasmids encoding GST-fusion protein constructs as described in section 2.1.2. p.44. The transformed bacterial cells were plated onto 2 x YT plates (16 g/l bacto-tryptone, 10 g/l yeast extract, 5 g/l NaCl, 1.5% bacto-agar w/v; pH 7.0), supplemented with 12.5 μg/ml ampicillin to ensure the selection of transformed cells.

2.7.2 GST-fusion protein expression

5 ml of 2 x YT broth (16 g/l tryptone, 10 g/l yeast extract, 5 g/l NaCl), supplemented with 12.5 μg/ml ampicillin, was inoculated with a single transformed BL-21 colony and incubated overnight with rotation at 37°C. 3 ml of the culture was added to 300 ml pre-warmed 2 x YT broth, supplemented with 2% w/v sterile glucose and 12.5

μg/ml ampicillin and incubated (2 h, 37°C), with shaking (200 rpm), until the cell culture had reached OD_{600} 0.6-0.8. Isopropyl-β-D-thiogalactoside was then added to a final concentration of 0.5 mM, in order to induce protein expression. The cells were then incubated for a further 3 h (37°C, 200 rpm).

2.7.3 Glutathione-sepharose capture of GST-fusion proteins

The cells were pelleted by centrifugation (10 min, 7500 g, 4°C), the supernatant removed and the pellet re-suspended in 6 ml 'Bugbuster' lysis reagent (Novagen) and left to lyse for 10 min at RT. The bacterial cell lysate was then centrifuged (25 min, 11,600 g, 4°C) and 1 ml of the resulting supernatant added to 200 μl Glutathione-4B Sepharose bead suspension (pre-washed in PBS; pH 7.5). The beads lysate mixture was incubated with the lysate (30 min, 4°C) with rolling, to allow the GST fusion proteins to bind to the glutathione-conjugated beads. The beads were then pelleted and washed 4 x with a 1 ml volume of PBS, supplemented with 1% v/v protease inhibitors, cocktail set III. 200 μl of PBS and 200 μl of pre-prepared cell extracts over-expressing proteins of interest (section 2.3 p.48) were added to the beads and incubated overnight at 4°C. The beads were then pelleted washed 2 x in GST binding buffer (PBS pH 7.5, 20% glycerol w/v, 1.0% CHAPS w/v, 1.0% sodium deoxycholate w/v, 5 mM EDTA , 1% v/v protease inhibitors cocktail set III) and then 3 x in dilute GST binding buffer (1:20 dilution in PBS pH 7.5). Following the careful removal of the supernatant buffer, beads were resuspended in Laemmli buffer (50 mM Tris, 2% SDS w/v, 5% mercaptoethanol v/v, supplemented with 8 mM urea) and boiled for 5 min.

2.8 Co-immunoprecipitation

COS7 cells were seeded into 75 cm^2 or 175 cm^2 flasks and transfected with appropriate cDNAs, as outlined in sections 2.2.1. p.47 and 2.2.4. p.48 respectively. Transfected cells were quiesced 4 h prior to lysis. Cells were washed with HBSS, before being solubilised in 1 ml/75 cm_2 or 2 ml/175 cm_2 of IP buffer, (20% glycerol, 0.5% CHAPS, 0.5% deoxycholic acid, 1 mM sodium orthovanadate, 1:100 protease inhibitor cocktail set III, NaCl 150 mM, HEPES 20 mM; pH 7.4) for approximately 40 min, with agitation. Flask contents were then scraped thoroughly, and the resulting cell lysates transferred to 1.5 ml Eppendorf tubes. Lysates were centrifuged (10 min, 11,500 g, 4ºC), after which supernatants were transferred to 1.5 ml Eppendorf tubes containing 20 µl Protein G-Sepharose 4B fast flow beads (diluted 1:1 with IP buffer). The lysate/bead mixture was incubated (~ 45 min, 4ºC) with rolling, in order to pre-clear the lysate by capturing any endogenous proteins non-specifically bound to protein-G beads. The beads were then pelleted by centrifugation (30 s, 12,000 g, 4ºC) and 470 µl of the pre-cleared supernatants were added to tubes containing 40 µl Protein G-Sepharose suspension and the appropriate primary antibody, or an equivalent concentration of species-specific, non-immune IgG as a negative control. Calcium chloride (1 mM) was also added to the tubes when using calcium-dependent antibodies. Lysates were left to incubate (overnight, 4ºC) with rolling. 56 µl of the remaining lysate was added to 10 µl 2 x Laemmli buffer (50mM tris, 2% w/v sodium dodecyl sulphate, 5% v/v mercaptoethanol, supplemented with 8 M urea) and stored at -40ºC, to be used as an indication of total levels of protein expression. The beads were then pelleted by centrifugation (30 s, 12,000 g, 4ºC) and the supernatant removed. The protein G beads were then washed

3 x with 1 ml IP buffer. Following the final wash, 30 µl of 2 x Laemmli buffer/1 ml of original supernatant was added to the samples, which were then heated at 70 or 100ºC (depending on protein content) for 5 min.

2.9 Antibody cross-linking to protein G-sepharose

In experiments to investigate co-immunoprecipitation of arrestins with HA-tagged wild-type and H452Y-5-HT_{2A}R, the mouse monoclonal HA antibody (clone 12CA5, Roche) was covalently cross-linked to protein G-sepharose CL-4B beads prior to use to avoid IgG bands on Western blots obscuring the protein of interest. 180 µl beads were conjugated with 90 µg 12CA5 or non-immune mouse IgG as indicated and the solubilised extract was immunoprecipitated at a rate of 40 µl antibody-derivatised beads per 2 ml extract from 1 x 175 cm^2 flask. Crosslinking was carried out according to the protocol supplied by Dynal/Invitrogen for crosslinking to protein G Dynabeads but with the beads being collected at each stage by brief centrifugation in a bench-top Eppendorf centrifuge rather than by magnet. Briefly, IgG binding was carried out for 40 min at room temperature in citrate-phosphate buffer pH 5.0 with rolling. After thorough washing in the same buffer and then 0.2 M triethanolamine pH 8.2, cross-linking was achieved with 20 mM freshly made dimethyl pimelimidate (DMP Pierce/Perbio) in triethanolamine buffer for 30 min at room temperature with rolling. Following quenching of remaining DMP with 50 mM Tris pH 7.5 for 15 min the derivatised beads were washed and then finally resuspended in PBS with 0.1% Tween-20 prior to use.

2.10 SDS-PAGE and immunoblot

Co-immunoprecipitation samples were added to 1 μl loading gel solution (0.04% w/v bromophenol blue in glycerol)/5 μl sample and the volume increased to 20 μl with 2 x Laemmli buffer. The sample solutions were then mixed and centrifuged (30 s, 12,000 g, 4ºC). A 4-12% Bis-Tris polyacrylamide gradient gel, designed to optimally separate proteins in the range 40-400 kDa, was rinsed thoroughly with ddH_2O. The gel was placed into an X-cell *Surelock* Mini-cell and the tightly sealed inner and outer buffer chambers filled with running buffer, MOPS (50 mM MOPS, 50 mM Tris base, 1 mM EDTA, 0.1% w/v SDS; pH 7.7) or MES (50 mM MES, 50 mM Tris base, 1 mM EDTA, 0.1% w/v SDS; pH 7.3). 20 μl of the samples were loaded into the wells and 5 μl of pre-stained molecular weight marker protein solution was also loaded into the gel. The gel was then run for approximately 1 h at 200 V, thereby inducing separation of the sample proteins according to molecular weight.

2.10.1 Western Blot

The proteins on the gel were transferred to 0.2 μm pore polyvinylidene difluoride (PVDF) membranes by electroblotting. Prior to blotting membranes were cut and soaked in methanol for ~ 30 min, before being rinsed in ddH_2O. Filter paper and blotting pads were pre-soaked in transfer buffer (bicine 500 mM, bis-tris 500 mM, EDTA 20.5 mM, chlorobutanol 1 mM; pH 7.2, supplemented with 10% methanol). Once the gel was run it was removed from the plastic cassette and placed on top of a pre-soaked piece of filter paper. The membrane was placed on top of the gel, over which a second piece of filter paper was placed and any air bubbles removed. The gel/membrane/filter paper bundle was placed in the blotting module between six pre-

soaked blotting pads, with the membrane closest to the anode core. The inner chamber of the blotting module was filled with transfer buffer and the outer chamber was filled with ddH_2O. The blot was run at 30 V for 90-120 min.

2.10.2 Immunoblotting

The blot was then left to block in 5% Marvel PBS solution (overnight , 4ºC) and then removed from the block and washed in 0.1% Tween-20 PBS solution x 3. The blot was then exposed to 2 ml 1:250 rat monoclonal anti-HA tag antibody, conjugated to horseradish peroxidase (HRP) (3F10, Roche), diluted in 5% Marvel, 0.5% Tween PBS, for ~ 1 h, at RT. The blot was then washed 5 x in 0.1% Tween-20 PBS solution, after which the HA antibody bands were visualised by incubation of the blot with luminol (Cell Signalling Technology, New England Biolabs) for 1 min and exposure to ECL film (Amersham Biosciences, Buckinghamshire, UK).

2.11 Cellular PLC activity assay

2.11.1 Cell Labelling

Prior to assay, 12-well plates of COS7 cells were transfected with appropriate cDNAs. When using 5-HT receptor cDNA, cells were maintained in DMEM with 2% USG (to avoid 5-HT content of normal serum). Otherwise the DMEM was supplemented with 10% normal calf serum. 1 day prior to assay medium was removed from the plates by aspiration and replaced with 0.5 ml Earle's balanced salt solution (EBSS), supplemented with 10 mM HEPES (pH 7.5), glucose (0.18%) and labelled with 1.5 μCi / well [^{3}H]inositol (specific activity 18.5 Ci/mmole). Plates were then left to incubate at 37°C (5% CO_2, 95% O_2) for ~ 18 h.

2.11.2 PLC Assay of cells transfected with 5-HT_{2A}

Immediately prior to assay, the cell medium was removed from the plates by aspiration and replaced with EBSS, supplemented with 10 mM HEPES (pH 7.5), glucose (0.18%) and bovine serum albumin (BSA) (0.2%). Lithium chloride was then added to each well to a final concentration of 10 mM and the plates left to incubate for 15 min prior to agonist incubation. For time-course assays, cells were stimulated with 5-HT (10 μM) for up to 3 h. Alternatively, for concentration-response assays, cells were stimulated with a range of agonist concentrations (carbachol: up to 200 μM; 5-HT: up to 3 μM) for 3 h. Reactions were terminated by removal of the medium and the addition of 0.5 ml ice-cold formic acid (10 mM).

2.11.3 *Separation of [^{3}H]inositol phosphates using anion exchange columns*

[^{3}H]inositol phosphates were isolated by adding the PLC assay samples to 5 ml columns containing 1 ml Dowex anion exchange resin (1 x 8; formate form; 200-400 mesh). An increasing gradient of ammonium formate/formic acid were added to the resin to elute the [^{3}H]inositol phosphates. Columns were initially washed through with 15 ml ddH_2O, followed by 5 ml ammonium formate (50 mM). The [^{3}H]inositol phosphate samples were eluted into 20 ml Zinsser scintillation vials using 10 ml of ammonium formate (1.0 M)/formic acid (0.1 M). The columns were finally washed with 5 ml ammonium formate (2 M)/formic acid (0.1 M), followed by 15 ml ddH_2O water, ready for re-use. 500 μl aliquots of the eluted samples were then transferred in duplicate to 6 ml polyethylene vials, to which 4 ml of scintillation fluid was added. Levels of [^{3}H] were detected using a Beckman LS 5801 scintillation counter, (4 min, [^{3}H] DPM programme).

2.12 Cellular PLD Activity Assay

2.12.1 *Cell labelling*

72 h prior to assay 12-well plates of COS7 cells were transfected with appropriate cDNAs, as outlined in section 2.2.4 p.48, with USG used as a serum substitute when using 5-HT receptors, as outlined in section 2.11.1. p.58. Approximately 18 h prior to assay, cells were quiesced in DMEM (0.5 ml/well) containing [^{3}H]palmitate (3µl/well; specific activity 47.5 Ci/mmol).

2.12.2 PLD Assay

Cells were incubated at 37°C (5% CO_2, 95% O_2) for ~ 18 h. Following incubation, the media was replaced with 0.5 ml/well MEM, containing HEPES (25 mM; pH 7.5), and fatty-acid free BSA (1.0% w/v) and the plates incubated (20 min, 37°C). Immediately prior to assay, butan-1-ol was added from a 600 mM MEM solution to a final concentration of 30 mM. Cells were then stimulated with agonist and incubated for 30 min. Reactions were terminated by the removal of assay medium and the addition of 500 µl ice-cold methanol/well.

2.12.3 Separation of [^{3}H] phosphatidyl butanol using solvent extraction and thin layer chromatography

Membranes were scraped from the base of the wells and transferred into 2 ml glass vials, to which 500 µl chloroform and 400 µl ddH_2O was added, yielding a final methanol/chloroform/water ratio of 5:5:4. Vials were sealed, mixed by vortex and left overnight at 4°C. Samples were then centrifuged (20 min, 5000 g, RT), to facilitate the separation of the samples into an upper aqueous layer and a lower

organic layer. The aqueous layer was removed, after which 250 µl of the lower organic layer was transferred into a clean glass vial and allowed to evaporate overnight in a fume hood. Samples were resuspended in 50 µl of a 19:1 chloroform:methanol solution and spotted onto lanes of LK5D thin layer chromatography plates, using a stream of nitrogen to dry the spots between sample loading. The plates were allowed to run for 1-1.5 h, in the organic phase of a running solvent mixture (ethyl acetate, 2,2,4-trimethylpentane, acetic acid, ddH_2O, in the ratios 110:50:20:100). The area of the plate corresponding to the migration of [^{3}H]phosphatidyl butanol ([^{3}H]PtdBut), approximately 5 cm from the plate origin (previously identified using iodine staining of a phosphatidyl butanol standard (McCulloch, 1998)), was scraped from the plates in 6 x 0.5 cm bands and transferred to 6 ml polyethylene vials, into which 3.2 ml scintillation fluid was added. Levels of [^{3}H] were detected using a Beckman LS 5801 scintillation counter, (4 min, [^{3}H] DPM programme).

2.13 Data Analysis

All quantitative data was calculated as mean ± standard error of the mean (SEM), acquired from a number (n) of individual experiments. Statistical significance of differences where appropriate was evaluated by Wilcoxon test, Mann-Whitney U-test or one-paired T-test, with $p < 0.05$ selected as criterion for significance. The Graphpad Prism programme (Graphpad Software, CA, USA) was used to analyse and plot non-linear error-weighted curve fittings and to determine the concentration of a drug which could produce 50% of the maximum response (EC_{50} value), using the logistic Hill equation.

Quantitative densitometry values were derived using the ScanAnalysis program (Biosoft, Cambridge, UK).

2.14 Materials

2.14.1 *Chemicals and Reagents*

All chemicals and reagents were from Sigma Chemical Company (Poole, Dorset, UK), unless otherwise stated.

2.14.2 Plasmids

N-terminally haemaglutinnin tagged human phospholipase D1b (HA-PLD1) and mouse phospholipase D2 (HA-PLD2) in pCGN vector were a kind gift from Mike Frohman, State University of New York. C-terminally FLAG-tagged β-arrestin 1 (FLAG-βarr1) and β-arrestin 2 (FLAG-βarr 2) in $PcDNA_3$ were kindly gifted by Miles Houslay, University of Glasgow. β-arrestin 1 (βarr 1) and β-arrestin 2 (βarr 2) in pCMV5 were kind gifts from the Lefkowitz lab, Duke Medical Centre. Human ARF1-HA ad ARF6-HA in plasmid pXS were kind gifts from Julie Donaldson, NIH. Plasmids encoding GST-fusion proteins of human $5\text{-}HT_{2A}R$ C-terminal tail, truncations of the C-terminal tail, K385 variant of the C-terminal tail and third intracellular loop in pGEX-2T, in addition to the N-terminally FLAG tagged human M_3 muscarinic receptor (sFM_3), N-terminally Protein C- and HA-tagged human $5\text{-}HT_{2A}$ receptor and its N376D mutant form in $pcDNA_{3.1}$ were created in collaboration with Eve Lutz. All plasmids were generated in-house, unless otherwise stated.

CHAPTER 3.0

3.0 PLD activation by the wild-type 5-HT$_{2A}$ receptor and its H452Y mutant form

3.1 Introduction

Phospholipase D (PLD) activity was initially demonstrated over 60 years ago in cabbage (310), although it was not until 1975 that it was first identified in mammals (311). PLD is now known to exist in fungi, plants, protozoa, bacteria and animals, thereby indicating its probably fundamental involvement in essential cellular functions (199). PLD activity is implicated in a wide range of physiological responses including endocytosis, exocytosis, secretion, vesicle trafficking, calcium mobilisation, cytoskeletal rearrangements and mitogenesis (199). Active PLD acts to hydrolyse phospholipids, primarily phosphatidyl choline (PtdCho), at the terminal phosphodiester bond, thereby producing phosphatidic acid (PA) and a free polar head group (choline).

3.1.1 Phospholipase D Structure

Phospholipase D enzymes contain several conserved motifs, including catalytic HxxxxKxD (HKD) sequences. Both PLD1 and PLD2 contain 2 HKD motifs, existing between residues 455-490 and 892-926 of the N- and C-terminal regions of the human PLD1 isoform, respectively (237). Mutagenesis studies during which Lys 898 of PLD1 was replaced with an Arg yielded an inactive K898R PLD1 variant, highlighting the critical role of the HKD motif in catalytic activity (312). The equivalent mutation in PLD2, K758R, was also found to result in a similar loss of catalytic activity (312). PLD activation is thought to require an association of the N- and C-termini and thus the two HKD domains, thereby forming an active catalytic

centre (313). It has been further demonstrated that HKD association is an essential prerequisite for Ser/Thr phosphorylation of the N-terminus of PLD1 (314).

Mammalian PLD1 and PLD2 isoforms also contain N-terminal Phox homology (PX) domains at residues 81-212 and 65-196, respectively (237,315). PX domains are conserved regions found in many proteins, which bind phosphoinositides and are involved in the targeting of proteins to membranes (316). Both PLD1 and PLD2 function is thought to be dependent upon binding of the phosphoinositide PI-4,5P_2 (235-237,315). Moreover, the PX domains are also thought to be an essential prerequisite for effective PLD activity (317-319). These domains have been reported to form interactions wih PKC isoforms (320), in addition to being involved in the modulation of pleckstrin homology (PH) domain function (see below) (321).

PLD isoforms additionally contain PH domains, thought to be involved in PLD translocation to the plasma membrane (322). The PH domains are found within residues 219-328 and 203-311 of PLD1 and PLD2, respectively (237). Both PLD 1 and 2 isoforms undergo palmitoylation of two adjacent Cys residues (240-241 and 223-224 respectively) within the PH domain, which acts to facilitate the association of the enzyme with membrane phosphoinositides (315,318,323).

3.1.2 Regulation of phospholipase D isoforms

Mammalian phospholipase D isoforms undergo functional modulation by several intracellular regulatory factors, including the small G proteins ADP-ribosylation factor (ARF) and Rho, phosphoinositides, PKC, and PKC-dependent

phosphorylation. PKC-mediated PLD activation was recognised on account of the discovery that PKC- activating factors, such as phorbol esters, induce PLD activation (324,325). Further experiments determined that purified PKC extracts could induce PLD activation in a concentration-dependent manner in Chinese hamster lung fibroblast membranes (326). In 1996, recombinant and purified PKCα were shown to interact with and activate PLD1 synergistically with ARF (327). Moreover, PKC inhibitors such as staurosporine, bisindolylmaleimide and Ro31-8220 have been used to demonstrate that the agonist dependent-GPCR activation of PLD is dependent upon the activation of PKC in a variety of cell types (328-331). Although PKC isoforms have been demonstrated to activate the two PLD isoforms with similar potencies (109,332), PLD activation is thought to be PKC isoform specific; PKCα and PKCβ are reported to be the primary PKC isoform activators of PLD (332,333). Furthermore, although PKCδ has been previously demonstrated to be an equally effective activator of PLD in HL60 cells (334), it has additionally been implicated to act as a negative regulator of PLD1 in melanoma cell lines (335,336). PKC-mediated PLD regulation is thought to involve direct interactions and phosphorylation (254). However, phosphorylation-independent PLD activation by PKC has also been demonstrated (327), and has been reported to involve interactions within the N-terminal PX domain of PLD1 (318). This was confirmed by the mutagenesis of human PLD1 at a GVPLE sequence at position 87 of the protein, (a non-phosphorylatable motif), to yield PIM87 PLD1, which resulted in a loss of responsiveness to PKC (320).

The small G protein, Rho, was first determined to be an activator of PLD in neutrophils, by Bowman et al in 1993 (337). Subsequently, RhoA, Rac1 and Cdc42 have been identified as the primary Rho GTPase activators of PLD (235,337,338). Rho proteins have been demonstrated to form direct GTP-dependent interactions with purified PLD1 (235,334,339), and with the use of yeast two-hybrid assays, RhoA was further determined to bind residues 712-1074 of the human PLD1 C-terminal tail domain (340). The subsequent point mutagenesis of PLD1 at residue 870 from the original Ile to an Arg, although inducing no effect upon the expression or activation profile of PLD1, conferred insensitivity to regulation by Rho GTPases (341). Furthermore Rho family GTPases are thought to be involved in the GPCR-mediated activation of PLD: M_3 muscarinic acetylcholine receptor-mediated PLD activation in HEK cells was attenuated with the addition of the specific Rho inhibitors toxin B from *Clostridium difficile* and C3 exoenzyme from *Clostridium botulinum,* thus suggesting the involvement of Rho in M_3 receptor-mediated PLD activation (342). Rho-mediated activation of PLD has since been demonstrated to occur independently of Gq protein activation (50).

ARF proteins, primarily ARF1 and ARF6, are able to directly activate PLD1, and to a lesser extent PLD2, in a GTP-dependent manner (235,237,343,344). ARFs are 20kDa GTPases that form part of the Ras superfamily. There are 6 mammalian ARF proteins, categorised into 3 classes to yield: class I, comprising ARF1, ARF2, and ARF3; class II, containing ARF4 and ARF5; and class III, consisting of ARF6 (345). With the exception of ARF6 (which is largely associated with the plasma membrane and endosomes), the majority of ARF family members are typically localised in the

cytosol. However, ARF1 has been demonstrated to translocate to the membrane following activation of the M_3 muscarinic receptor in COS7 cells (244), and in neutrophils (346,347).

3.1.3 Regulation of phospholipase D isoforms by members of the ARF family

ARF1-6 share several conserved structural features: all ARFs contain an N-terminal amphipathic helix, which is typically myristoylated at a Gly residue, following the removal of Met (345). It has been demonstrated that ARF1 myristoylation induces guanine nucleotide exchange factor (GEF)-dependent GDP-GTP exchange at physiological Mg^{2+} concentrations, in the presence of phospholipid vesicles (348). Although activated ARF ($ARF_{GTP\gamma S}$) can bind membrane phospholipids regardless of its myristoylation profile, ARF_{GDP} is only capable of binding membrane phospholipids when myristolyated (349). Following myristolyated ARF_{GDP} membrane association, the N-terminus associates with an interswitch domain. However, the N-terminal myristoyl group destabilises this interaction, allowing GTP access to the ARF-GTP binding site. Thus ARF_{GTP} is formed and a more stable interaction occurs with the plasma membrane (348-350). ARF family proteins express an identical consensus sequence for GTP binding and hydrolysis. Conformational changes of this region following GTP binding are thought to result in the exposure of the myristoylated N-terminus, switch 1 (amino acids 41-51 of ARF1) and switch 2 (amino acids 68-81 of ARF1) regions, which are implicated in plasma membrane (348,351) and effector binding (345,352) respectively.

ARF activation is regulated via ARF-guanine nucleotide exchange factors (ARF-GEFs), which facilitate GDP-GTP exchange. These proteins all contain a Sec7 domain that catalyses nucleotide exchange (351,353). ARF switch 1 and switch 2 regions are thought to be responsible for associating with the Sec7 domain, which inserts residues into the GTP binding site, thereby disrupting the phosphate and Mg^{2+} binding sites and promoting nucleotide dissociation (351,353). ARF deactivation is facilitated by ARF-GTPase activating proteins (ARF-GAPs), which required a zinc finger motif and a conserved arginine residue within the GAP domain for maximal ARF-GTPase activity (354,355).

ARF-mediated PLD activation was first demonstrated in permeabilised (cytosolic deficient) HL-60 granulocyte cells (356-358), although it was not until 1993 that the 16 kDa PLD-activating cytosolic factor was characterised as ARF (273,359). Via the reconstitution of PLD activity in cytosol depleted HL60 cells by recombinant ARF1 and the continued ARF dependence of PLD activity through several purification steps, these studies indicated that ARF proteins activate PLD in a direct manner (273,359). The N-terminal region of ARF1 has been isolated as an essential ARF domain for PLD activity, and is thus thought to be potentially involved in interactions with PLD (360). Furthermore the ARF binding site for PLD is reported to be distinct from regions required for activation of cholera toxin (361) and coatamer binding (360). The putative site within PLD for interaction with ARF remains unknown, although it is determined not to exist within the initial 325 amino acids of the N-terminus that are required for activation by PKC (318).

The first evidence that ARF proteins were involved in GPCR-mediated PLD activity was provided in 1995 by Rumenapp et al; stable M_3 muscarinic receptor-expressing HEK cells were incubated with the specific ARF-GEF-inhibitor brefeldin A (BFA), which was found to induce a decrease in carbachol-stimulated PLD activity, despite eliciting no effect upon receptor cell surface expression or G protein coupling (362). Furthermore, brefeldin A was determined not to act as a direct inhibitor of PLD (362). ARF has since been demonstrated to activate PLD in a variety of cell and receptor systems including: N-formylmethionyl-leucylphenylalanine (fMLP) receptor-dependent PLD activity in human neutrophils (346); VPAC receptor-mediated PLD activation in CHO cells (363); somatostatin receptor-dependent PLD activation in clonal β-cells (364); and endothelin-1, angiotensin II, and platelet-derived growth factor receptor-mediated PLD responses in vascular smooth muscle A10 cells (365). Mitchell et al first demonstrated that ARF and Rho can associate with GPCRs to activate PLD via a mechanism independent of heterotrimeric G protein Gq/11 activation in 1998 (50). Furthermore, the ARF/Rho-dependent receptor-mediated activation of PLD was found to be dependent upon the sequence existing at the junction between the 7^{th} transmembrane domain and the carboxy-terminal tail of the receptor, within which the NPxxY motif is contained (50). Receptors containing an alternate DPxxY motif, were found to activate PLD in an apparently ARF/Rho–independent manner (50). Moreover, mutation of the Asp to Asn conferred BFA/C3 exoenzyme sensitivity onto PLD responses of previously insensitive receptors, such as the gonadotrophin releasing hormone receptor (GnRH), and the reciprocal mutation conferred insensitivity onto BFA/C3-sensitive receptors such as the 5-HT_{2A}R (50).

Both ARF and PLD proteins have been demonstrated to form direct interactions with GPCRs in a variety of receptor and cellular contexts. ARF GTPases were first demonstrated to interact directly with GPCRs by co-immunoprecipitation of ARF1/3 with the M_3 receptor (50). The ARF binding site on the 5-HT_{2A}R has been determined to exist within the initial 9 amino acids of the receptor C-terminal tail (274). PLD2 has been selectively co-immunoprecipitated with both the μ-opioid receptor (366), and metabotropic glutamate receptors (367). There is also evidence to suggest that PLD1, and to a lesser extent PLD2, can be co-immunoprecipitated with the M_3 muscarinic receptor (unpublished observations). The binding of PLD1 has been localised to the distal regions of the M_3 muscarinic receptor C-terminal tail, with the use of a truncated receptor construct and GST-fusion proteins of M_3 muscarinic receptor domains (unpublished observations).

It has been suggested that ARF and Rho act synergistically to activate PLD (346,368). Further studies that utilised a PKC-unresponsive PLD1 mutant demonstrated that PKC also displays synergistic activation of PLD1 with RhoA (320). However, the extent to which Rho, ARF and PKC can activate PLD is thought to depend upon the specific receptor and cell type setting (235,369). For example, following M_3 muscarinic receptor activation in HEK-293 cells, both ARF1 and RhoA are demonstrated to undergo recruitment to the plasma membrane and independently activate PLD1 (199,362,370). Alternatively, in parallel experiments investigating PLD signalling in cardiomyocytes, PAR1 receptor-induced PLD stimulation was found to be PKC-independent and brefeldin A-sensitive, thus ARF-dependent, whilst

endothelin-1 receptor-mediated PLD activity was determined to be ARF-independent (371).

3.2 Results

3.2.1 Identification of PLD isoforms utilised by the 5-HT$_{2A}$R

Various GPCRs have been demonstrated to interact with and activate phospholipase D isoforms, some of which are suggested to demonstrate PLD-isoform specific preferences (372). In order to elucidate which PLD isoforms mediate the 5-HT$_{2A}$R response, COS7 cells were co-transfected with Protein C epitope-tagged 5-HT$_{2A}$R (sPrC-5-HT$_{2A}$R) and either control (empty pcDNA$_{3.1}$ vector), wild-type HA tagged PLD1 or PLD2 (Figure 3.1a, 3.1b), or dominant-negative mutant (Δ^-)PLD1 or PLD2 isoforms (Figure 3.1c, 3.1d). Δ^-PLD1 and Δ^- PLD2 are catalytically inactive due to the replacement of the usual Lys (K) with an Arg (R) residue, at amino acid 898 (PLD1 K898R) and 758 (PLD2 K758R), respectively, and thereby act to reduce the rate of substrate processing by the wild-type endogenous enzymes and hence block the effects of intracellular PLD signalling pathways (312,373). Cells were stimulated with 3 μM 5-HT for 0-10 minutes to assess PLD activity (Figure 3.1a, 3.1c), or 0-60 minutes to investigate PLC activity (Figure 3.1b, 3.1d). PLD responses were measured via the quantification of tritiated phosphatidyl butanol production ([^{3}H]PtdBut), and PLC via tritiated inositol phosphate production ([^{3}H]InsP). [^{3}H]PtdBut or [^{3}H]InsP production is illustrated as a fold of the respective unstimulated control. The control cells in Figure 3.1a (expressing 5-HT$_{2A}$R plus empty vector) evoke an endogenous PLD response to approximately three-fold of the basal. Additional expression of recombinant PLD2 does not appear to significantly

increase 5-HT_{2A}R-mediated PLD activity above control responses, however, additional expression of recombinant PLD1 isoform is shown to significantly increase 5-HT_{2A}R-mediated PLD activity over control responses at 2-10 min 5-HT incubation times (Figure 3.1a) ($p < 0.05$, Wilcoxon test) to approximately eight-fold over basal, thus suggesting that 5-HT_{2A}R-induced PLD activation is PLD1-dependent. Neither PLD1 nor PLD2 responses in the corresponding PLC assay (Figure 3.1b) were found to significantly vary from control responses. Figure 3.1c demonstrates PLD responses in the presence of Δ^- PLD1 and Δ^- PLD2 constructs. Δ^- PLD2 expression was not found to significantly influence 5-HT_{2A}R-mediated PLD activity, however expression of the dominant negative PLD1 isoform (Δ^- PLD1) was found to significantly inhibit [^{3}H]PtdBut production when compared to control responses ($p < 0.05$, Wilcoxon test), further implicating the involvement of the PLD1 isoform in 5-HT_{2A}R-mediated PLD activity. Both Δ^- PLD1 and Δ^- PLD2 expression were demonstrated to have no significant effect upon corresponding 5-HT-induced PLC responses (Figure 3.1d). Pilot experiments indicated that [^{3}H]PtdBut values in unstimulated COS7 cells transfected with the wild-type-5-HT_{2A}R, H452Y-5-HT_{2A}R (discussed later in chapter), or empty vector were not discernably increased through the duration of the assays. All plotted points represent means ± SEM, n = 8.

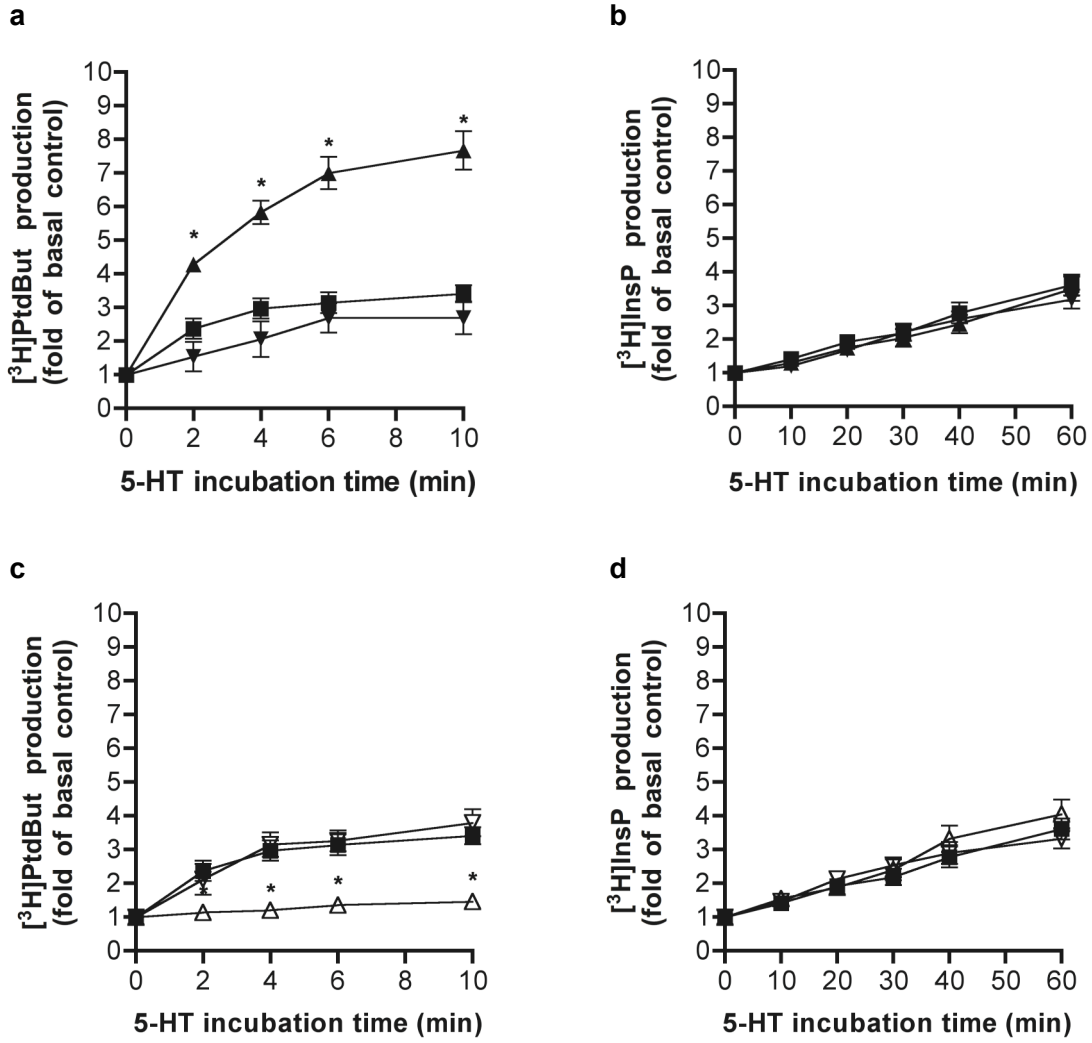


Figure 3.1 5-HT-induced PLD and PLC responses in COS7 cells co-transfected with 5-HT$_{2A}$R and PLD1, PLD2, Δ$^-$ PLD1 or Δ$^-$ PLD2. PLD and PLC responses of COS7 cells co-transfected with 5-HT$_{2A}$R and pcDNA$_{3.1}$ as control (■), PLD1 (▲) or 2 (▼) isoforms (Figures a and b), or dominant negative mutant (Δ$^-$) PLD1 (△) or 2 (▽) isoforms, K898R-PLD1 or K758R-PLD2 respectively (Figures c and d). Cells were stimulated with 5-HT (3 μM) at a range of incubation times, PLD activity is represented by [^{3}H]phosphatidyl butanol ([^{3}H]PtdBut) production (Figures a and c). PLC activity is represented by [^{3}H]inositol phosphate ([^{3}H]InsP) production (Figures b and d). The points plotted represent means ± SEM, n = 4. Values for 5-HT-induced [^{3}H]Ptd But production in the presence of wild-type PLD1 were significantly greater than control and those in the presence of Δ$^-$ PLD1 were significantly less than the control from 2-10 mins (* $p < 0.05$, Wilcoxon test).

3.2.2 Assessment of possible pathways mediating 5-HT$_{2A}$R-evoked PLD activation

A prominent role for ARF1 but not ARF6 in 5-HT$_{2A}$R-mediated PLD responses was established by the use of dominant negative constructs in previous work from the lab to assess the input from different potential activating factors (274,374). To assess the dependence of 5-HT$_{2A}$R-mediated PLD activity upon different potential activating factors, various inhibitory agents and dominant negative constructs were used. To investigate the influence of RhoA on 5-HT$_{2A}$R-mediated PLD activity, the effects of [T19N] RhoA, a dominant negative RhoA construct, were investigated on 5-HT- and thrombin-stimulated PLD activity (Table 3.1). COS7 cells were triple-transfected with Protein-C-epitope tagged 5-HT$_{2A}$R, PLD1 or pcDNA$_{3.1}$ empty vector as control, and [T19N] RhoA or pcDNA$_{3.1}$ control, and stimulated with either 3 μM 5-HT, or 0.5 U/ml thrombin. Transfection with additional PLD1 evokes significantly higher 5-HT-induced PLD responses than those seen with control. In the absence of the dominant negative [T19N] RhoA mutant 5-HT responses were amplified from 2.63 ± 0.35 to 5.86 ± 0.97 fold of basal, and in the presence of mutant RhoA they were amplified from 2.72 ± 0.28 to 5.33 ± 0.69 fold of basal ($p < 0.05$, Wilcoxon test). Thrombin, which is an activator of protease activated receptors (PAR)-1 and -4 (that can activate PLD in a Rho-dependent manner), also induced a significant increase in PLD activity above control in sPrC-5-HT$_{2A}$R-transfected cells that was increased by transfection of wild-type PLD1. In the absence of [T19N] RhoA thrombin-evoked responses were amplified by PLD1 from 3.13 ± 0.42 to 4.72 ± 0.51 fold of basal ($p < 0.05$, Wilcoxon test), but the addition of [T19N] RhoA conferred a significant reduction in PLD responses to 2.58 ± 0.37 fold of basal ($p < 0.05$, Wilcoxon test), with no significant increase over control, which was 2.38 ± 0.39 fold of basal. All

values are the mean of 6 separate results, displayed as mean ± SEM. These observations suggest lack of involvement of RhoA in 5-HT$_{2A}$R-mediated PLD responses in COS7 cells whereas responses of a control receptor, the thrombin PAR-1 R (which is known to engage closely in RhoA signalling) was clearly affected.

The influence on PKC and Rho upon 5-HT$_{2A}$R-regulated PLD activity was further investigated with the use of PIM87/IR-PLD1, a mutant PLD construct which exhibits a selective loss of responses to PKCα and Rho. COS7 cells were transfected with Protein C epitope-tagged 5-HT$_{2A}$R (sPrc-5-HT$_{2A}$R) and either pcDNA$_{3.1}$ as a control, wild-type PLD1, or PIM87/IR-PLD1. Cells were then stimulated with either 3 μM 5-HT, or 300 nM PDBu and assayed for PLD activity (Table 3.2). Both wild-type PLD1 and PIM87/IR-PLD1 cells demonstrated significantly increased 5-HT-induced PLD activity over control responses to 5-HT of 1.85 ± 0.10 fold increment over basal ($p < 0.05$, Wilcoxon test), with the PIM87/IR-PLD1 construct conferring only slightly lower amplification of 5-HT-induced PLD responses when compared to wild-type PLD1 with responses of 5.27 ± 0.67 and 6.08 ± 0.58 fold increment over basal respectively. When incubated with PDBu, a potent protein kinase C activator, again a significant increase over control was seen in wild-type PLD1 expressing cells, at 9.79 ± 0.88 compared to 4.64 ± 0.42 fold increment over unstimulated basal ($p < 0.05$, Wilcoxon test), but PtdBut-induced PLD responses were not increased over control by PIM87/IR-PLD1 and were significantly lower than responses in the presence of wild-type PLD1 ($p < 0.05$, Wilcoxon test). This suggests that neither Rho nor PKC are important in the amplification of 5-HT$_{2A}$R-elicited PLD responses

caused by additional wild-type PLD1 and therefore, by implication, that ARF may be a mediator.

Table 3.3 shows the 5-HT-induced PLD activity of COS7 cells transfected with sPrC-5-HT_{2A}R and either $pcDNA_{3.1}$, as control, or wild-type PLD1 in the presence of the PLC and tyrosine kinase inhibitors U 73122 and AG 213. Neither inhibitor was found to reduce endogenous responses to 5-HT, or the amplified responses in the presence of additional PLD1 expression. 5-HT-induced PLD activity in controls, with U 73122 or with AG 213, was 2.43 ± 0.26, 3.11 ± 0.48 and 3.01 ± 0.33 fold of basal whereas corresponding responses in the presence of additional PLD1 were 4.91 ± 0.53, 5.40 ± 0.69 and 5.12 ± 0.78 fold of basal. All PLD1 expressing cells were found to evoke significantly higher PLD responses than control cells ($p < 0.05$, Wilcoxon test).

Table 3.4 demonstrates the 5-HT-induced PLD activity of COS7 cells transfected with sPrC-5-HT_{2A}R and either $pcDNA_{3.1}$, as control, or wild-type PLD1 and incubated with selected protein kinase C inhibitors or pertussis toxin. Bisindoylmaleimide 1 (BIM1), acts to block the PKC binding site for ATP, thereby inducing the kinase domain to adopt an open conformation, and rendering it inactive (375). CGP 41251 is a derivative of staurosporine, and a structurally distinct protein kinase C inhibitor (376). Pertussis toxin acts to catalyze the ADP-ribosylation of G_i, G_o and G_t alpha subunits, thereby preventing their downstream signalling. Again, none of the inhibitory agents acted to significantly reduce either control 5-HT_{2A}R-elicited PLD responses or their amplification in the presence of additional wild-type

PLD1. Without additional PLD1, 5-HT-induced PLD responses for control, BIM1, CGP 41251 and pertussis toxin samples were 2.95 ± 0.33, 3.24 ± 0.39, 2.53 ± 0.61 and 3.06 ± 0.44 fold of unstimulated basal. In the presence of additional PLD1 the corresponding responses were 6.82 ± 0.83, 6.13 ± 0.91, 7.01 ± 0.53 and 6.19 ± 0.71 fold of basal. All PLD1 overexpressing cells were found to evoke significantly higher PLD responses than control cells ($p < 0.05$, Wilcoxon test). All values are the mean of 6 separate results, displayed as mean ± SEM. In these experiments we could find no evidence for involvement of PKC of Gi/Go in 5-HT$_{2A}$R-elicited PLD responses or their amplification in the presence of additional wild-type PLD1.

Figure 3.2 illustrates 5-HT$_{2A}$R- and gonadotrophin releasing hormone receptor (GnRHR)-mediated PLD responses in the presence/absence of PLD1 and [Q209L/D277N] Gαq, a dominant negative Gαq mutant construct, that acts to disrupt Gq coupling to PLC signalling pathways (377). [Q209L/D277N] Gαq does not appear to confer any decrease in COS7 cell 5-HT$_{2A}$R-mediated PLD responses, either in the presence or absence of wild-type PLD1, following stimulation with 3 μM 5-HT. Control responses to 5-HT in the absence or presence of [Q209L/D277N] Gαq and corresponding responses in the additional presence of extra PLD1 were 3.13 ± 0.23, 2.90 ± 0.22, 8.12 ± 1.46 and 6.76 ± 0.56 fold of basal. In contrast, in cells transfected with the mouse GnRHR, GnRHR-mediated PLD responses were significantly decreased by [Q209L/D277N] Gαq, both in the absence or presence of additional PLD1. Control responses to GnRH in the absence or presence of [Q209L/D277N] Gαq and corresponding responses in the additional presence of extra PLD1 were 4.38 ± 0.45, 1.98 ± 0.12, 9.83 ± 1.07 and 4.84 ± 0.53 fold of basal

respectively. Statistically significant increments in evoked PLD responses were seen due to additional PLD1 compared to vector alone in the case of both the 5-HT_{2A}R and the GnRHR ($p< 0.05$, Wilcoxon test). [Q209L/D277N] Gαq significantly attenuated facilitation of GnRHR but not 5-HT_{2A}R-mediated PLD responses caused by additional wild-type PLD1 ($p< 0.05$, Wilcoxon test). All values are the mean of 8 independent results, displayed as mean ± SEM. These observation suggest that while Gq may play a role in PLD signalling by the GnRHR, this is not the case for the 5-HT_{2A}R.

Previous work has established a strong inhibitory influence of dominant negative ARF1 ([T13N] ARF1) on 5-HT_{2A}R-mediated PLD activation (274,374). Table 3.5 illustrates the influence of the dominant negative [T31N] ARF1 construct upon 3 μM 5-HT stimulated PLC activity. COS7 cells were triple transfected with sPrC-5-HT_{2A}R, PLD1 or $pcDNA_{3.1}$ empty vector as control, and [T31N] ARF1 (that demonstrates a reduced affinity for GTP), or again, $pcDNA_{3.1}$ control. Cells were stimulated with 5-HT and assayed for PLC activity. PLC responses are represented as [^{3}H]inositol phosphate ([^{3}H]InsP) production as fold of basal, unstimulated control. It is evident that transfection with PLD1, or dominant negative [T31N] ARF1 does not evoke any discernable or significant change in PLC activity (Wilcoxon test). Control 5-HT_{2A}R-mediated PLC responses and those in the presence of [T31N] ARF1 were 2.69 ± 0.31 and 2.77 ± 0.35 fold of basal while corresponding values in the additional presence of extra wild-type PLD1 were 2.86 ± 0.40 and 2.53 ± 0.28 fold of basal respectively. All values are the mean of 8 independent results, displayed as mean ± SEM.

Treatment	[^{3}H]PtdBut production (fold of unstimulated control)	
	sPrC-5-HT$_{2A}$R + pcDNA$_{3.1}$	**sPrC-5-HT$_{2A}$R + PLD1**
5-HT (3 μM)	2.63 ± 0.35	5.86 ± 0.97*
5-HT (3 μM) + [T19N] RhoA	2.72 ± 0.28	5.33 ± 0.69*
thrombin (0.5 U/ml)	3.13 ± 0.42	4.72 ± 0.51*
thrombin (0.5 U/ml) + [T19N] RhoA	2.38 ± 0.39	2.58 ± 0.37†

Table 3.1 Effects of dominant negative RhoA construct on 5-HT$_{2A}$R-mediated and thrombin/PARR-mediated PLD responses in the absence or presence of additional PLD1. PLD responses of COS7 cells co-transfected with sPrC-5-HT$_{2A}$R and pcDNA$_{3.1}$ or PLD1, in the presence or absence of the dominant negative [T19N]RhoA construct, following stimulation with either 3 μM 5-HT or 0.5 U/ml thrombin. * indicates response significantly greater than response in the absence of PLD1 and † indicates response significantly less than response in the absence of [T19N]RhoA ($p < 0.05$, Wilcoxon test). Values are means ± SEM from 6 separate determinations, n = 3.

	Activator-induced [^{3}H]PtdBut production (fold increment over unstimulated control)		
	+ $pcDNA_{3.1}$	**+ PLD1 wt**	**+PIM87/IR-PLD1**
5-HT (3 μM)	1.85 ± 0.10	6.08 ± 0.58 *	5.27 ± 0.67 *
PDBu (300 nM)	4.64 ± 0.42	9.79 ± 0.88 *	3.93 ± 0.48 †

Table 3.2 Effects of co-transfection of wild-type PLD1 or a PLD1 construct defective in activation by PKC or Rho (PIM87/IR-PLD1) on PLD responses to 5-HT or phorbol 12, 13-dibutyrate (PDBu) in sPrC-5-HT_{2A}R-expressing COS7 cells. Values are means ± SEM, n - 3. Statistically significant increments in evoked PLD responses compared to vector alone and reduction compared to PLD1 wild-type are indicated, * and † respectively ($p < 0.05$, Wilcoxon test).

Treatment	[^{3}H]PtdBut production (fold of unstimulated control)	
	sPrC-5-HT$_{2A}$R + pcDNA$_{3.1}$	**sPrC-5-HT$_{2A}$R + PLD1**
5-HT (3 μM)	2.43 ± 0.26	4.91 ± 0.53*
5-HT (3 μM) + U 73122 (20 μM)	3.11 ± 0.48	5.40 ± 0.69*
5-HT (3 μM) + AG 213 (100 μM)	3.01 ± 0.33	5.12 ± 0.78*

Table 3.3 Effects of PLC and tyrosine kinase inhibitors on 5-HT$_{2A}$R-mediated PLD responses in the presence or absence of additional PLD1. PLD responses of COS7 cells co-transfected with sPrC-5-HT$_{2A}$R and pcDNA$_{3.1}$ or PLD1 and stimulated with either 3 μM 5-HT alone, or in combination with 20 μM U 73122 or 100 μM AG 213. All responses were found to be significantly greater in the presence of additional PLD1 (*$p < 0.05$, Wilcoxon test) but no significant inhibitory effects of U 73122 or AG 213 were detected. Values are means ± SEM, n = 3.

Treatment	[^{3}H]PtdBut production (fold of unstimulated control)	
	sPrC-5-HT_{2A}R + $pcDNA_{3.1}$	**sPrC-5-HT_{2A}R + PLD1**
5-HT (3 μM)	2.95 ± 0.33	6.82 ± 0.83*
5-HT (3 μM) + BIM1 (3 μM)	3.24 ± 0.39	6.13 ± 0.91*
5-HT (3 μM) + CGP 41251 (3 μM)	2.53 ± 0.61	7.01 ± 0.53*
5-HT (3 μM) + Ptx (200 ng/ml; 16 h)	3.06 ± 0.44	6.19 ± 0.71*

Table 3.4 Effects of protein kinase C inhibitors and pertussis toxin on sPrC-5-HT_{2A}R-mediated PLD responses in the absence or presence of additional PLD1. PLD responses of COS7 cells co-transfected with sPrC-5-HT_{2A}R and $pcDNA_{3.1}$ or PLD1 and stimulated with either 3 μM 5-HT alone, or in combination with, 3 μM BIM1, 3 μM CGP 41251 or 200 ng/ml pertussis toxin for 16 hours. All responses were found to be significantly greater in the presence of additional PLD1 (*$p < 0.05$, Wilcoxon test). Values are means ± SEM, n = 3.

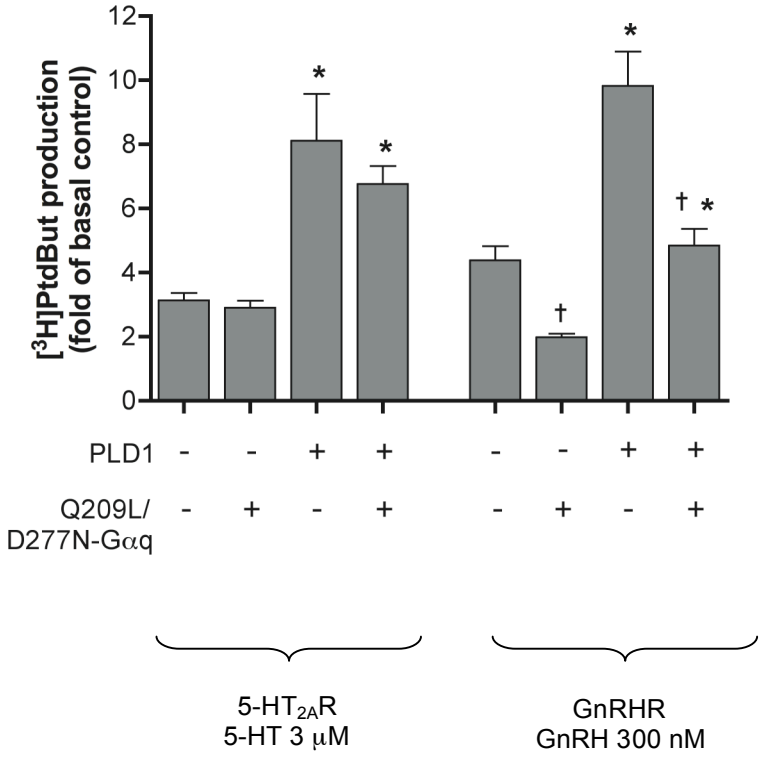


Figure 3.2 5-HT/GnRH-induced PLD responses in the presence/absence of PLD1 and [Q209L/D277N] Gαq. PLD responses of COS7 cells transfected with 5-HT$_{2A}$R or GnRHR, in the presence or absence of PLD1 and [Q209L/D277N] Gαq, following stimulation with 3 μM 5-HT or 300 nM GnRH. * indicates response significantly greater than response in the absence of PLD1 and † indicates response significantly reduced in the absence of [Q209L/D277N] Gαq ($p < 0.05$, Wilcoxon test). Values are means ± SEM, n = 4.

Treatment	[^{3}H]InsP production (fold of unstimulated control)	
	sPrC-5-HT_{2A} R + $pcDNA_{3.1}$	**sPrC-5-HT_{2A} R + PLD1**
5-HT (3 μM) + $pcDNA_{3.1}$	2.69 ± 0.31	2.86 ± 0.40
5-HT (3 μM) + [T31N]ARF1	2.77 ± 0.35	2.53 ± 0.28

Table 3.5 Effects of dominant negative ARF1 construct on 5-HT_{2A}R-mediated PLC responses in the absence or presence of additional PLD1. The table illustrates the 5-HT-stimulated PLC responses of COS7 cells triple-transfected with sPrC-5-HT_{2A}R, $pcDNA_{3.1}$ or PLD1 and $pcDNA_{3.1}$ or the dominant negative [T31N] ARF1 construct. Values are means ± SEM from 8 separate determinations, n = 4. There were no statistically significant differences between responses (Wilcoxon test).

3.2.3 PLD signalling pathway of the wild-type 5-HT$_{2A}$R - co-immunoprecipitation experiments

To further understand this signalling pathway, potential physical associations of the 5-HT$_{2A}$R and PLD isoforms were investigated; Figure 3.3 illustrates the results of co-immunoprecipitation experiments in which HA-tagged PLD1 (HA-PLD1) or PLD2 (HA-PLD2) constructs were investigated as potential interaction partners of the sPrC-5-HT$_{2A}$ receptor. COS7 cells were co-transfected with sPrC-5-HT$_{2A}$ receptor and either the HA-PLD1 isoform or the HA-PLD2 isoform. Mouse monoclonal HPC4 anti-PrC antibody (Roche) was used to isolate sPrC-5-HT$_{2A}$ receptor from cell lysates, along with any associated proteins. Non-immune mouse IgG was used as a control. Proteins were separated using gel electrophoresis and transferred to PVDF by Western blot. The resulting protein bands were identified by specific antibody and enhanced chemiluminescence; HA-PLD bands were detected using a rat monoclonal anti-HA HRP-conjugated antibody (clone 3F10, Roche) and sPrC-5-HT$_{2A}$R was visualised with anti-PrC antibody (HPC4, Roche) and Chemicon anti-mouse secondary antibody conjugated to HRP. In Figure 3.3 HA-PLD isoform co-immunoprecipitates can be seen in the bottom panel, sPrC-5-HT$_{2A}$R immunoprecipitates in the middle panel and HA-PLD total lysate samples in the top panel (applicable to two adjacent lanes as appropriate). The band in the lower panel and first lane represents HA-PLD1 co-immunoprecipitate pulled down with anti-PrC antibody, and the adjacent band HA-PLD1 co-immunoprecipitate pulled down with control non-immune IgG. The obviously greater density of the band isolated with anti-PrC antibody indicates that HA-PLD1 specifically interacts with, and can be pulled down with the sPrC-5-HT$_{2A}$R. In contrast, lanes 3 and 4 represent anti-PrC

and non-immune pull-downs of the HA-PLD2 isoform respectively. The high non-immune HA-PLD2 pulldown seen in lane 4 suggests that co-immunoprecipitation of HA-PLD2 with the receptor lacks specificity. Both the receptor input (middle panel) and HA-PLD input (top panel) appear even.

The results of the co-immunoprecipitation experiment shown in Figure 3.3 were quantified using Image J software to obtain densitometric measurements, and normalised, taking into account background levels, PLD input and receptor input. The mean results from such PLD pulldown experiments can be seen in Figure 3.4, shown as the scan densities of HA-PLD bands in arbitrary grey scale units. The specific scan density value for PLD1 associated with anti-PrC tag immunoprecipitates was found to be significantly greater than that for the control non-immune IgG co-immunoprecipitate ($p < 0.05$, Wilcoxon test), indicating that HA-PLD1 specifically co-immunoprecipitates with the sPrC-5-HT$_{2A}$ receptor in cells. However, PLD2 control scan densities values were not found to be significantly different from the PLD2 co-immunoprecipitate values, thus not providing any indication of specific PLD2 co-immunoprecipitation with the sPrC-5-HT$_{2A}$ receptor in vitro. All plotted points represent means ± SEM, n = 5.

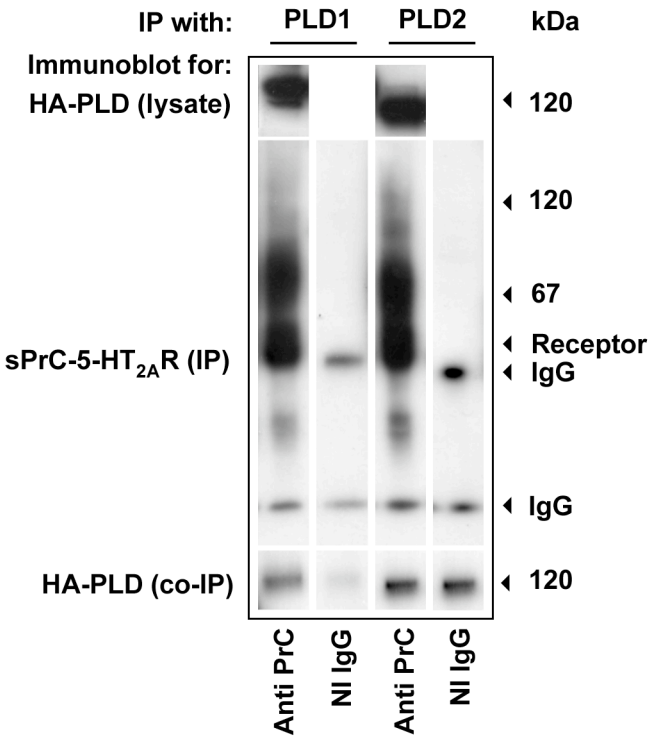


Figure 3.3 Co-immunoprecipitation of HA-PLD1 and HA-PLD2 with sPrC-5HT$_{2A}$R. Mouse monoclonal anti-PrC tag antibody (HPC4) was used to immunoprecipitate sPrC-5-HT$_{2A}$R, along with any proteins attached to the receptor. Non-immune IgG was used as a control. HPC4 was also used to visualize immunoprecipitated sPrC-5-HT$_{2A}$R. IgG bands from the immunoprecipitation are shown by arrows. HA-PLD1 and HA-PLD2 bands were visualised using rat monoclonal anti-HA HRP-conjugated antibody (clone 3F10, Roche). The levels of HA-PLD1 and 2 in co-immunoprecipitates are shown as well as PLD1 and 2 in inputs and receptor in immunoprecipitates. These observations were typical of 5 separate experiments.

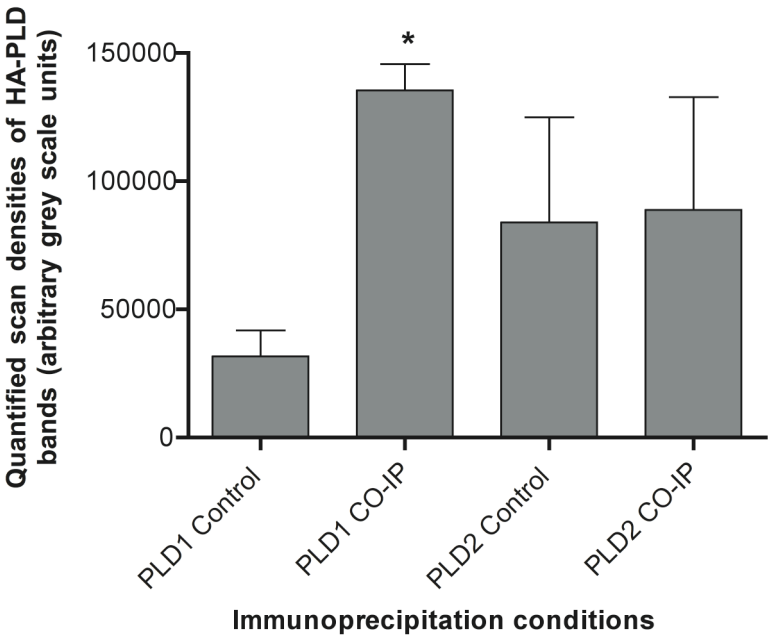


Figure 3.4 Quantification of HA-PLD1 co-immunoprecipitation with sPrC-5-HT$_{2A}$ receptor. COS7 cells were co-transfected with sPrC-5HT$_{2A}$ and HA-PLD1 or HA-PLD2, and immunoprecipitation carried out as in Figure 3.3. The graph illustrates quantified scan densities of HA-PLD1 and HA-PLD2 pulled down by anti-protein C tag antibody HPC4 and by control non-immune mouse IgG. The PLD1 pull-down obtained via immunoprecipitation with anti-protein C antibody was found to be significantly greater than that using control non-immune IgG. Conversely, the PLD2 pull-down using anti-protein C antibody was not significantly increased over levels of PLD2 immunoprecipitated with control non-immune antibody. Values were obtained from a total of 5 experiments similar to that illustrated in Figure 3.3, n = 5 (* $p < 0.05$, Wilcoxon test).

In previous experiments, the PLD-activating small G protein ARF1 has been shown to demonstrate an increased association with the 5-HT_{2A}R following exposure with 5-HT in the first 5 minutes, reducing thereafter (274). To assess whether the interaction of PLD1 with the receptor is dynamically regulated by 5-HT, HA-PLD1 was co-immunoprecipitated with sPrC-5-HT_{2A}R following increasing time periods of pre-incubation with 5-HT. COS7 cells were transfected with protein C epitope-tagged 5-HT_{2A}R, and HA-tagged PLD1, and incubated with 10 μM 5-HT for 0-60 minutes. Mouse monoclonal anti-PrC antibody was used to isolate receptor with mouse non-immune IgG for controls, and complexed PLD1 was identified by Western blot with HRP-linked rat monoclonal anti-HA antibody (clone HPC4). Figure 3.5 demonstrates the quantification of band densities (arbitrary grey scale units), which were normalised to reflect receptor and PLD1 inputs, background and non-immune HA-PLD1 pull down densities and displayed as a ratio against control HA-PLD1 co-immunoprecipitate in unstimulated cells. Normalised levels of HA-PLD1 co-immunoprecipiting with the receptor after 5-HT incubation were not found to discernably vary from that of normalised control, with values ranging from 0.75 ± 0.16 – 1.0 ± 0.02 fold of control. Values are from 3 independent experiments, results displayed as mean ± SEM.

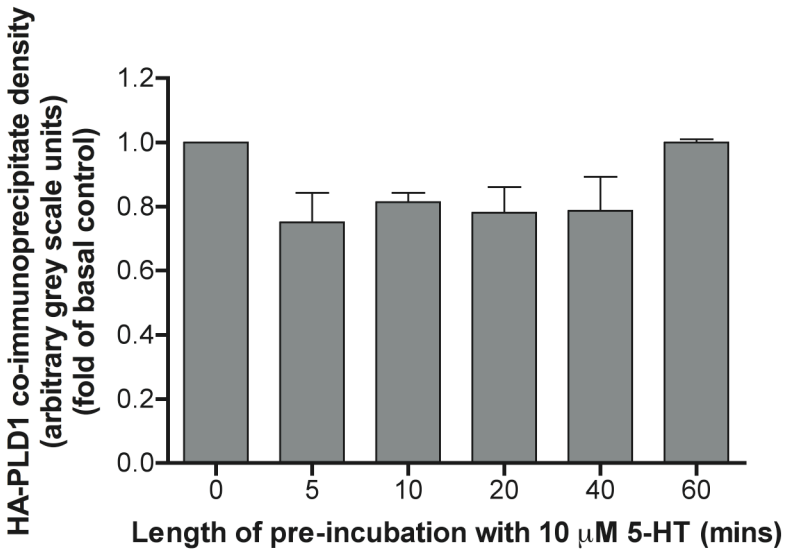


Figure 3.5 Quantification of levels of HA-PLD1 co-immunoprecipitated with wild-type 5-HT$_{2A}$R pre-incubated with 10 μM 5-HT for 0-60 minutes. Graph represents normalised levels of HA-PLD1 co-immunoprecipitated with wild-type 5-HT$_{2A}$R taking into account receptor and PLD1 inputs, background and non-immune HA-PLD1 pull down densities. HA-PLD1 co-immunoprecipitation is represented as a ratio to basal control (no pre-incubation). There were no discernable differences in quantities of HA-PLD1 pulled-down by 5-HT$_{2A}$R pre-incubated with 10 μM 5-HT for increasing periods of time between 0-60 minutes. Results displayed as mean ± SEM, n = 3.

3.2.4 PLD signalling pathways of the 5-HT$_{2A}$R-GST-fusion protein studies

To further isolate the PLD interaction site on the 5-HT$_{2A}$R, GST-fusion proteins of specific 5-HT$_{2A}$R domains were used as bait for potential interactions of PLD1/2 in enriched lysates. PLD1- or 2-expressing COS7 cell lysates were incubated with GST alone as control, or with GST-fusion proteins of the 5-HT$_{2A}$R carboxy-terminal tail (ct), third intracellular loop (i3), or a truncated variant of the carboxy-terminal tail, in which the initial nine amino acids are removed (K385ct). These initial nine amino acids (376-385), contain a conserved NPxxY motif which is implicated in ARF binding and isoform selectivity of interaction with the 5-HT$_{2A}$R (274,374). GST-receptor constructs and any complexed proteins were immobilised on Glutathione Sepharose-4B beads and any proteins captured from lysates by these constructs were isolated and separated by gel electrophoresis. HA-PLD bands were visualised using rat monoclonal anti-HA HRP-conjugated antibody (clone 3F10, Roche), and GST input detected with monoclonal mouse anti-GST antibody (Clone B-14 Santa Cruz) and HRP-linked secondary anti-mouse antibody (Chemicon).The results are illustrated in Figure 3.6a; HA-PLD1 at approximately 120 kDa (top panel) appears to demonstrate negligible interactions with the control construct (GST alone) (lane 1), and little interaction with the i3 construct (lane 4), but does appear to specifically interact with the C-terminal tail of the receptor (lane 2). Furthermore, this interaction does not appear to be inhibited in any way by the truncation of the initial 9 amino acids (lane 3); rather, PLD1 association with the K385-5-HT$_{2A}$Rct GST-fusion protein construct appears to be increased, indicating that residues 376-385 are not required for HA-PLD1 binding in vitro and may possibly act to partly restrict the interaction. Levels of HA-PLD2 captured with the GST-fusion protein constructs

(middle panel) appear to follow a similar trend, although the differences between the various bands are less pronounced. GST inputs (bottom panel) appear to be reasonably well balanced, indicating that there is no bias of the results due to varying levels of construct input. Results of the subsequent densitometric analysis and quantification of the HA-PLD1 and HA-PLD2 protein bands seen in Figure 3.6a are represented in Figures 3.6b and 3.6c respectively. Statistical analysis of the results represented in Figure 3.6b determined levels of HA-PLD1 captured with GST-K385-5-HT_{2A}Rct to be significantly greater than those captured with full length GST-5-HT_{2A}Rct (p value 0.0217), and levels of HA-PLD1 captured with GST-5-HT_{2A}Ri3 to be significantly less than those captured with GST-5-HT_{2A}Rct (p value 0.0176). Statistical analysis of the complementary HA-PLD2 data illustrated in Figure 3.6c showed there to be no significant differences in levels of HA-PLD2 pulled down with the various constructs, possibly suggesting a degree of non-specific interaction by PLD2.

The 5-HT_{2A}R binding site for ARF1 has been previously determined to lie between residues 376-385 of the C-terminal tail and thus ARF binding to the K385-5-HT_{2A}Rct variant is significantly depleted. Thus Figure 3.6 indicates that PLD1 binds to the receptor at a region distinct from the ARF binding site. To further investigate this, GST-fusion protein constructs of the 5-HT_{2A}Rct were again used to assess whether the physical association of HA-PLD1 with the receptor C-terminus would influence ARF1-HA binding, or vice versa. Figure 3.7a and b illustrate the results of experiments in which GST-5-HT_{2A}Rct constructs were incubated with either HA-PLD1 lysate/buffer (Figure 3.7a) or ARF1-HA lysate/buffer (Figure 3.7b) for 12

hours, prior to being washed and undergoing a further 12 hour incubation with ARF1-HA lysate/buffer or HA-PLD1 lysate/buffer respectively. It is evident from Figure 3.7a that prior binding of HA-PLD1 to the C-terminal tail (upper panel, lanes 1 and 3) does not appear to affect subsequent ARF1-HA binding (middle panel, lane 3; compared to lane 2 control without PLD co-immunoprecipitation). The bound ARF1-HA levels appear to be equivalent and thus unaffected by receptor-bound HA-PLD1 in vitro. Additionally, HA-PLD1 binding (top panel) did not appear to be displaced by the addition of, and subsequent GST-5-HT$_{2A}$Rct interactions with, ARF1-HA (lane 3). Furthermore, the corresponding experiment in Figure 3.7b demonstrates that HA-PLD1 binding to the 5-HT$_{2A}$Rct was not noticeably influenced by the prior binding of ARF1-HA (top panel, lane 3; compared to lane 2 control without ARF1-HA pre-incubation) and neither was ARF1-HA binding affected by the subsequent binding of HA-PLD1 (middle panel, lane 3).

To further define the potential HA-PLD1 binding site on the 5-HT$_{2A}$Rct, a series of GST-fusion proteins of sequentially truncated variants of the 5-HT$_{2A}$Rct were used to capture HA-PLD1 and ARF1-HA lysate (Figure 3.8a). Full-length GST-5-HT$_{2A}$Rct and the C-terminally-truncated 5-HT$_{2A}$Rct GST-fusion protein variants, GST-(376-470)5-HT$_{2A}$R ct, GST-(376-438)5-HT$_{2A}$R ct, and GST-(376-428)5-HT$_{2A}$Rc-terminuswere incubated with lysates from COS7 cells transfected with HA-PLD1 or ARF1-HA. GST-fusion proteins and any associated proteins were immobilised on Glutathione-Sepharose-4B beads, before being isolated via gel electrophoresis and Western blotting; HA-PLD1 (~120kDa) and ARF1-HA (~20kDa) were visualised using rat monoclonal HRP-linked anti-HA antibody (Roche). The density of the HA-

PLD1 bands indicate a decrease in HA-PLD1 binding to the C-terminal tail of the receptor as the distal region is progressively truncated. The results from 3 analogous experiments were quantified by scan densitometry; Figure 3.8b shows the mean ratio of HA-PLD1 captured by the truncated construct to that captured by the full-length GST-5-HT_{2A}Rct. Levels of HA-PLD1 captured by GST-(376-438)5-HT_{2A}Rct and GST-(376-428)5-HT_{2A}Rct were found to be significantly less than that captured by the full-length GST-5-HT_{2A}Rct ($p < 0.05$, t-test). In contrast, the density of HA-ARF1 bands shown in the lower panel of Figure 3.8a indicate that HA-ARF1 binding is unaffected by C-terminal truncations of the 5-HT_{2A}Rct. This is further corroborated by the densitometric quantification of 3 parallel experiments, represented in Figure 3.8c as the mean ratio of ARF1-HA captured by construct to that captured by GST-5-HT_{2A}Rct. Truncation of the distal region of the 5-HT_{2A}Rct was not found to result in any significant changes to ARF1-HA binding.

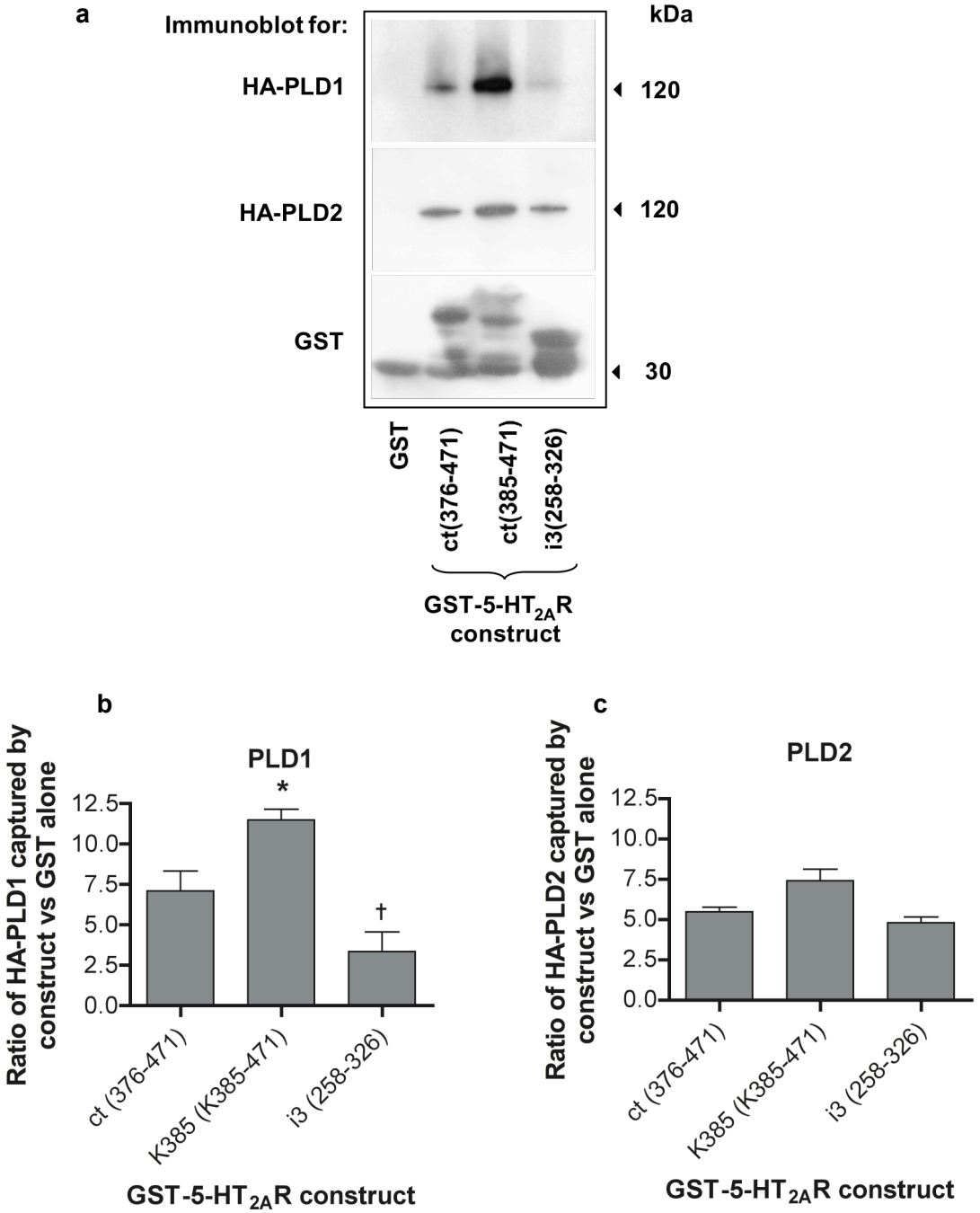


Figure 3.6 HA-PLD1 and 2 binding to GST-fusion proteins of particular domains of the 5-HT$_{2A}$R. GST-fusion proteins of the 5-HT$_{2A}$Rct (ct), a truncated variant of the 5-HT$_{2A}$Rct (K385ct) and the third intracellular loop (i3) were used as bait for potential interacting proteins in lysates of COS7 cells transfected with either HA-PLD1 or HA-PLD2. The density of HA-PLD1 and 2 bands shown in part (a) were quantified and are shown as ratios of HA-PLD1 and 2 captured by the receptor domain constructs compared to GST alone in (b) and (c), respectively. * or † denote significantly greater or lesser levels of captured HA-PLD1 when compared to that captured with GST-5-HT$_{2A}$Rct (376-471) respectively (* $p < 0.05$, t-test). All plotted points represent means ± SEM, n=8.

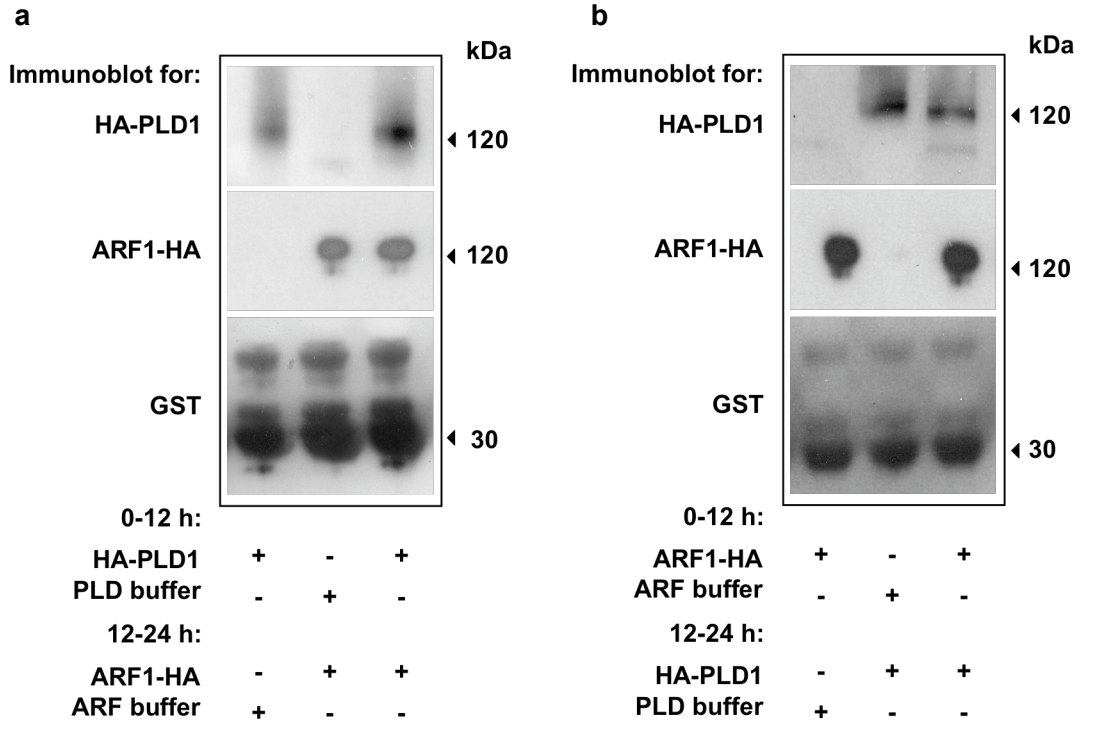


Figure 3.7 Evidence for the lack of interaction between HA-PLD1 and ARF1-HA binding to the GST-5-HT$_{2A}$Rct tail construct. In these experiments, the GST-fusion protein of the 5-HT$_{2A}$Rct was subjected to consecutive 12 hour incubations at 4°C with COS7 cell extracts enriched in HA-PLD1 or ARF1-HA or appropriate buffer controls. Bound HA-PLD1 or ARF1-HA was then detected by SDS-PAGE and blotting with 3F10 HRP-conjugated anti-HA antibody. HA-PLD1 and ARF1-HA could be clearly distinguished by their different running positions at approximately 120 and 20 kDa respectively. In the left panel (a) (PLD1 first incubation, ARF1 second incubation) it is seen that when PLD1 is already bound to the GST-5-HT$_{2A}$Rct construct the subsequent binding of ARF1 is neither facilitated nor inhibited. In the right panel (b) (ARF1 first incubation, PLD1 second incubation) it is seen that when ARF1 is already bound to the GST-5-HT$_{2A}$Rct construct the subsequent binding of PLD1 is neither facilitated nor inhibited. These results are typical of 3 independent experiments.

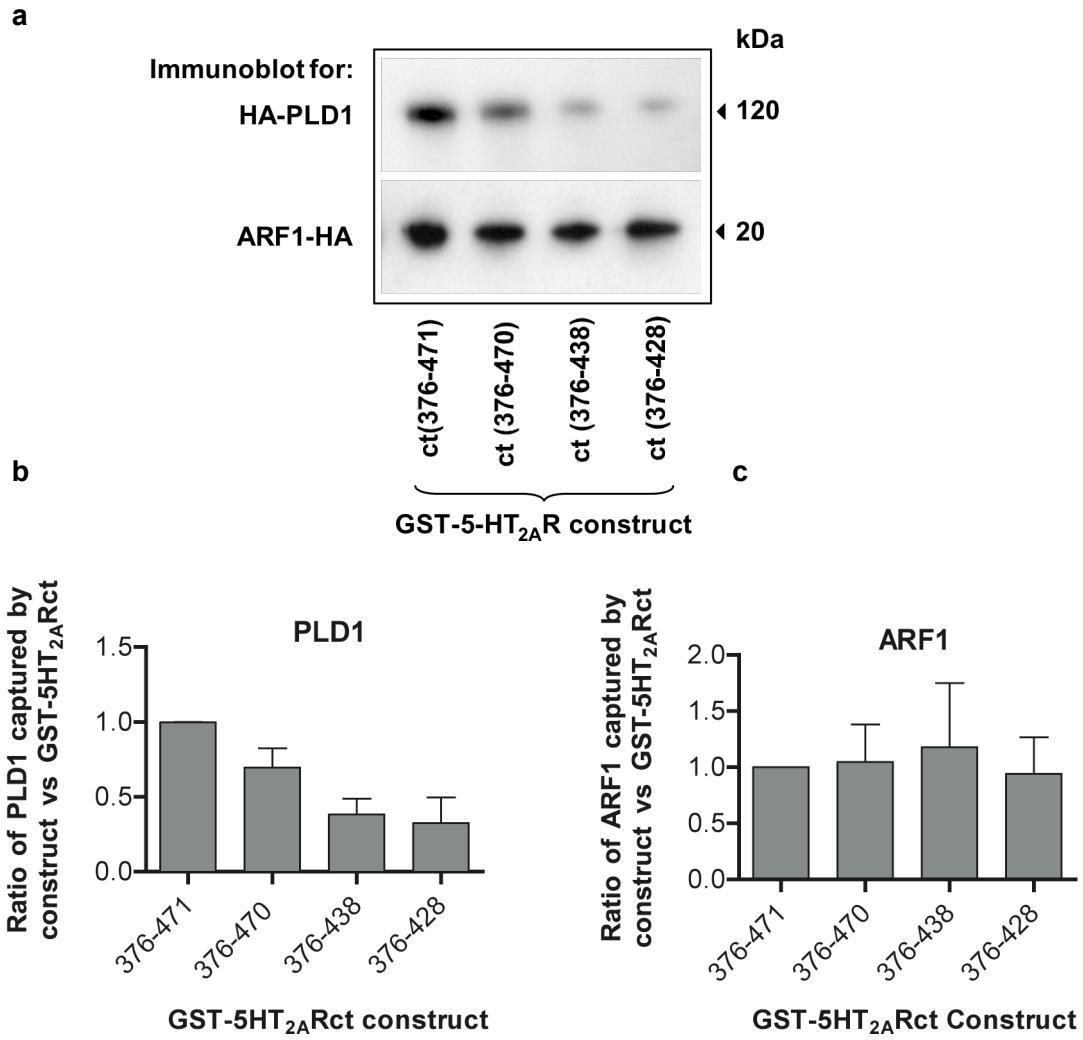


Figure 3.8 HA-PLD1 and HA-ARF1 binding to sequentially truncated GST-fusion protein constructs of the 5-HT$_{2A}$Rct. Figure (a) illustrates HA-PLD1 and ARF1-HA bound to sequentially truncated variants of the GST-5-HT$_{2A}$Rct. HA-PLD/ARF1-HA bands were scanned and quantified by densitometry, the scan densities for PLD1 and ARF1 are demonstrated as a fold of PLD1/ARF1 captured by the full-length carboxy-terminal tail, in Figures (b) and (c) respectively.

3.2.5 PLD signalling pathway of the H452Y polymorphic variant of the 5-HT$_{2A}$R – co-immunoprecipitation experiments

The results from Figure 3.8 thus indicate that PLD1 requires residues 428-470 for efficient binding to the 5-HT$_{2A}$Rct. Interestingly, there is a clinically relevant 5-HT$_{2A}$R polymorphic variant, that codes within the distal region of the C-terminal tail, in which a Tyr replaces the normal His at residue 452 (H452Y). The minor variant allele is expressed by approximately 9% of the population (301,302) and previous studies have suggested that signalling by the H452Y variant of the receptor may differ from that of the H452 receptor (308,309). Thus, it was hypothesised that the H452Y mutation may alter PLD1 binding to the 5-HT$_{2A}$R. Figure 3.9 illustrates the results of a typical co-immunoprecipitation experiment designed to identify any differences in the binding of PLD1 to the H452Y variant of the 5-HT$_{2A}$R when compared to the wild-type form. COS7 cell lysates expressing either wild-type sPrC-5-HT$_{2A}$ receptor and HA-PLD1, or H452Y variant sPrC-5-HT$_{2A}$R and HA-PLD1, were immunoprecipitated with mouse monoclonal anti-PrC tag antibody (Roche, HPC4), or with non-immune mouse IgG, as a control. Following the separation of immunoprecipitated proteins by gel electrophoresis, HA-PLD1 associated with the receptor pull-down was assessed using rat monoclonal HRP-linked anti-HA (Roche, clone 3F10) and receptor input was determined with the use of mouse monoclonal anti-PrC tag antibody (HPC4, Roche) and anti-mouse HRP-linked antibody (Chemicon). It can be seen that despite equivalent receptor (middle panel) and HA-PLD1 inputs (top panel), noticeably less HA-PLD1 is co-immunoprecipitated with the H452Y-5-HT$_{2A}$R variant (bottom panel, lane 2) than with the wild-type receptor (bottom panel, lane 1). Minimal HA-PLD1 immunoreactivity is seen in the control

lanes (bottom panel, lanes 3 and 4), indicating that the result is specific to the sPrC-5-HT$_{2A}$R co-immunoprecipitation. The densitometric readings from three identical experiments were normalised, taking into account the values for specific co-immunoprecipitated PLD1, receptor input and PLD1 input. The mean normalised value for PLD1 co-immunoprecipitated with the H452Y variant of the receptor was significantly lower, at just 44% of that for the wild-type sPrC-5-HT$_{2A}$R (p value = 0.0388, t-test).

The equivalent experiment was performed with the HA-PLD2 isoform (Figure 3.10). HA-PLD2 appears to be pulled down with both receptor variants (bottom panel, lanes 1 and 2) although similar observations were made in the control pull-downs performed with non-immune mouse IgG (lanes 3 and 4) suggesting that any interaction of HA-PLD2 here was largely non-specific. This result was typical of three independent experiments. In the example illustrated the slightly greater apparent pulldown of HA-PLD2 in cells expressing the H452Y variant 5-HT$_{2A}$R is probably due to greater input levels of receptor and HA-PLD2 inputs (middle and top panels, respectively).

3.2.6 PLD signalling pathway of the H452Y polymorphic variant 5-HT$_{2A}$R – GST-fusion protein experiments

The results from the co-immunoprecipitation experiment illustrated in Figure 3.9 were further validated with the use of GST-fusion proteins of the wild-type H452 and H452Y variants of the C-terminal tail of the 5-HT$_{2A}$R. Either GST alone, GST-5-HT$_{2A}$Rct, or GST-H452Y-5-HT$_{2A}$Rct constructs were incubated with lysate from

COS7 cells expressing either HA-PLD1 or ARF1-HA, following immobilisation on glutathione Sepharose-4B beads. GST fusion proteins and any complexed proteins were then separated by gel electrophoresis and PLD1/ARF1 was detected with the use of rat monoclonal HRP-linked anti-HA antibody (Roche, clone 3F10). Figure 3.11a shows levels of HA-PLD1 captured; it is evident that the introduction of the H452Y mutation to the GST-5-HT_{2A}Rct (lane 3) results in a dramatic decrease in HA-PLD1 binding in vitro, despite similar levels of construct input (lower panel). To establish the specificity of the result, the same constructs were used to assess binding of ARF1-HA lysate (Figure 3.11b). There was no observable difference between ARF1-HA binding to the wild-type GST-5-HT_{2A}Rct and the GST-H452Y-5-HT_{2A}Rct.

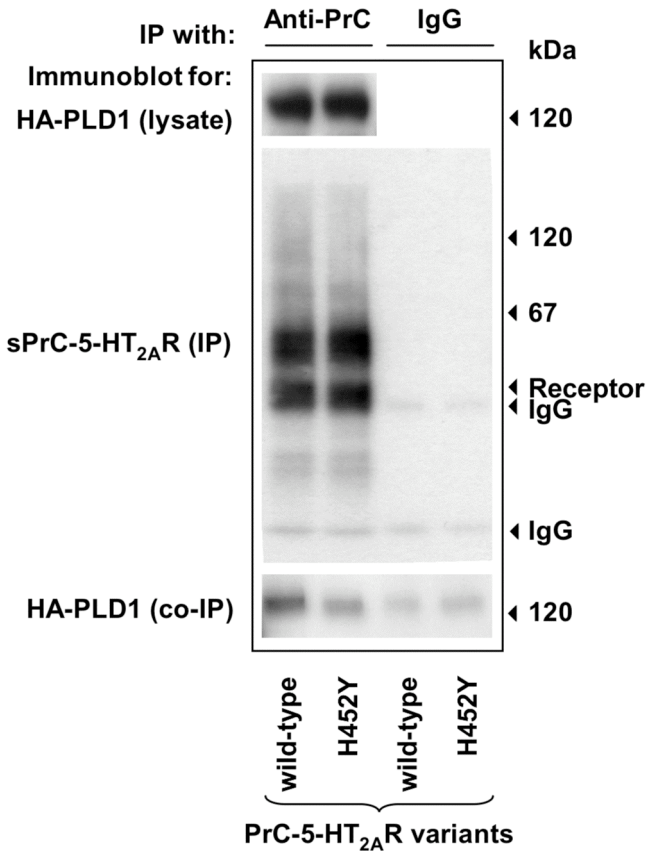


Figure 3.9 Co-immunoprecipitation of HA-PLD1 with the wild-type sPrC-5-HT$_{2A}$ and the H452Y mutant form of the sPrC-5-HT$_{2A}$R. COS7 cells were co-transfected with protein C-tagged wild-type or H452Y variant 5-HT$_{2A}$R and HA-PLD1. Anti-protein C tag antibody was used to immunoprecipitate the receptor. Non-immune mouse IgG was used as a control. The Figure shows levels of HA-PLD1 pulled down with both variants of receptor (co-IP), levels of sPrC-5HT$_{2A}$R recovered in the immunoprecipitates (IP) and HA-PLD1 inputs (lysate). These results are typical of 3 independent experiments.

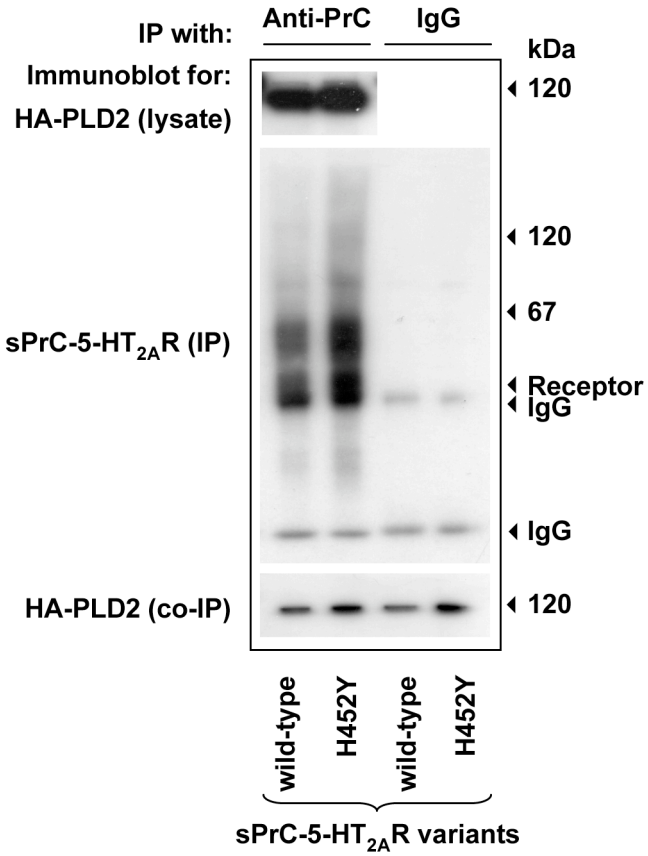


Figure 3.10 Co-immunoprecipitation of HA-PLD2 with the wild-type sPrC-5-HT$_{2A}$ and the H452Y mutant form of the sPrC-5-HT$_{2A}$ receptor. COS7 cells were co-transfected with protein C-epitope tagged wild-type or H452Y variant 5-HT$_{2A}$R and HA-PLD2. Anti-PrC tag antibody was used to immunoprecipitate the receptor. Non-immune mouse IgG was used as a control. The Figure shows levels of HA-PLD2 pulled down with both variants of receptor (co-IP), levels of sPrC-5HT$_{2A}$R forms recovered in the immunoprecipitates (IP) and HA-PLD2 inputs (lysate). These results are typical of 3 independent experiments.

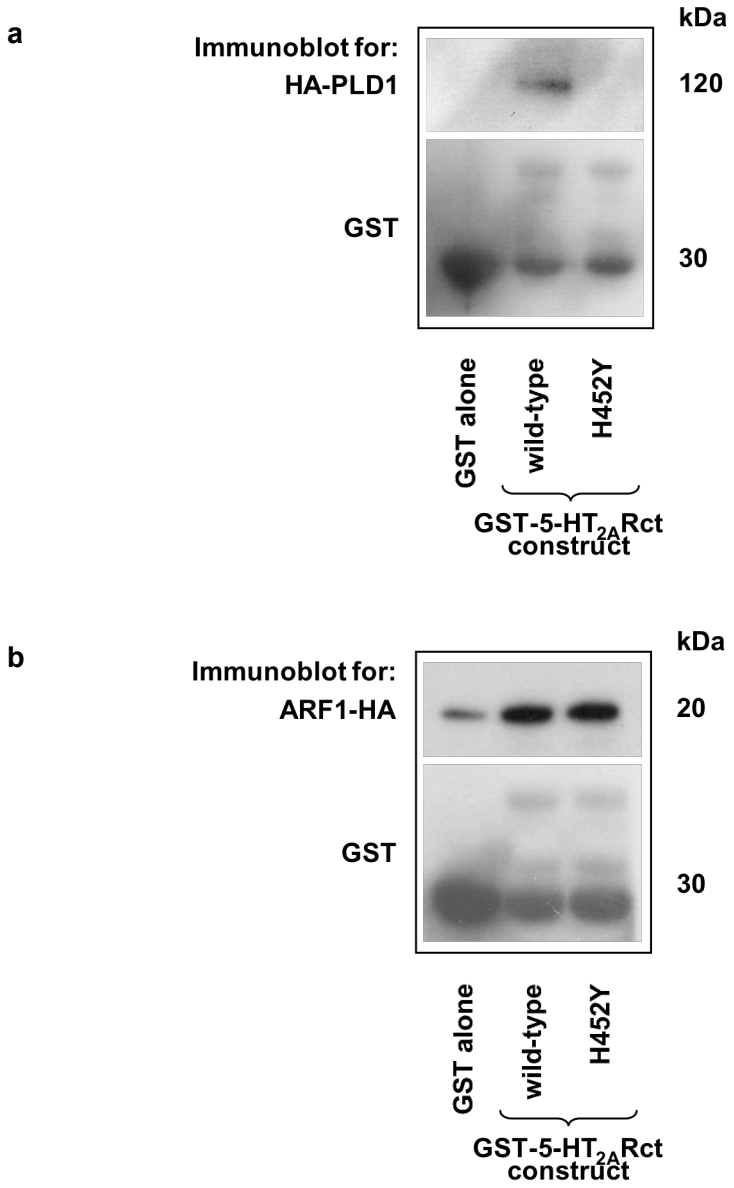


Figure 3.11 HA-PLD1 and ARF1-HA binding to GST fusion protein constructs of the C-terminal tail of the wild-type 5-HT$_{2A}$R and the H452Y 5-HT$_{2A}$R variant. HA-PLD1 or ARF1-HA-enriched COS7 cell lysates were incubated with either GST alone, wild-type 5-HT$_{2A}$R or H452Y-5-HT$_{2A}$Rct GST-fusion protein constructs. GST-fusion proteins, along with any further complexed proteins were isolated using glutathione beads. HA-PLD1 and ARF1-HA were separated and identified by Western blot and anti-HA HRP-conjugated antibody analysis. These results are typical of 3 independent experiments.

3.2.7 PLD signalling pathway of the [H452Y] polymorphic variant 5-HT_{2A}R – functional signalling studies

To assess any functional implications of decreased PLD1 binding by the H452Y 5-HT_{2A}R, COS7 cells co-transfected with either wild-type PrC epitope-tagged 5-HT_{2A}R and empty $pcDNA_{3.1}$ vector alone as a control, PLD1 or 2, or the H452Y variant of the PrC tagged 5-HT_{2A}R with empty $pcDNA_{3.1}$ vector alone as a control, PLD1 or 2, were stimulated with increasing concentrations of 5-HT and assayed for evoked PLD activity. Responses in Figure 3.12 are shown as 5-HT-induced [^{3}H]PtdBut production as a fold increment over basal unstimulated control. Control responses of wild-type and H452Y receptor variants appear approximately equivalent with EC_{50} values of 3.82 ± 0.23 nM and 9.75 ± 0.31 nM and maximum response values of 2.14 ± 0.20 and 2.01 ± 0.20 fold increment over basal, respectively. In cells additionally expressing PLD2, 5-HT-induced PLD activation mediated by the wild-type 5-HT_{2A}R, or the H452Y 5-HT_{2A}R, was similar to that in control cells with EC_{50} values of 5.4 ± 0.36 nM and 12.67 ± 0.16 nM and maximum response values of 1.65 ± 0.26 and 1.76 ± 0.29 fold increment over basal, respectively. In COS7 cells expressing wild-type sPrc-5-HT_{2A}R and PLD1, 5-HT induced significantly increased PLD responses over control, at 5-HT concentrations 3-3000 nM (*$p < 0.05$, Wilcoxon test). 5-HT-induced PLD responses mediated by the H452Y-5-HT_{2A}R in cells additionally expressing PLD1 were also significantly increased above control at 5-HT concentration of 30-3000 nM (*$p < 0.05$, Wilcoxon test). However, these responses were significantly lower than PLD responses evoked by the wild-type 5-HT_{2A}R and PLD1 expressing cells, at 5-HT concentrations 3-3000 nM († $p < 0.05$, Wilcoxon test), with maximum wild-type receptor responses in the presence of

additional PLD1 reaching 7.02 ± 0.59 fold increment over basal, whereas corresponding H452Y-5-HT_{2A}R responses only reached a maximum of 4.39 ± 0.30 fold increment over basal.

Complementary PLC assays (Figure 3.13) were performed in order to verify that the amplification of 5-HT-induced PLD activity seen in the presence of additional PLD1 (Figure 3.12) was specific for that signalling pathway. COS7 cells were co-transfected with either wild-type PrC-tagged 5-HT_{2A}R and empty $pcDNA_{3.1}$ vector alone as a control, PLD1 or 2, or the H452Y variant of the sPrC-5-HT_{2A}R with empty $pcDNA_{3.1}$ vector alone as a control, PLD1 or 2. It is evident that transfection of COS7 cells with either PLD isoform fails to induce a significant increase in 5-HT-induced PLC activity mediated by either the wild-type 5-HT_{2A}R (Figure 3.13a), or the H452Y-5-HT_{2A}R (Figure 3.13b).

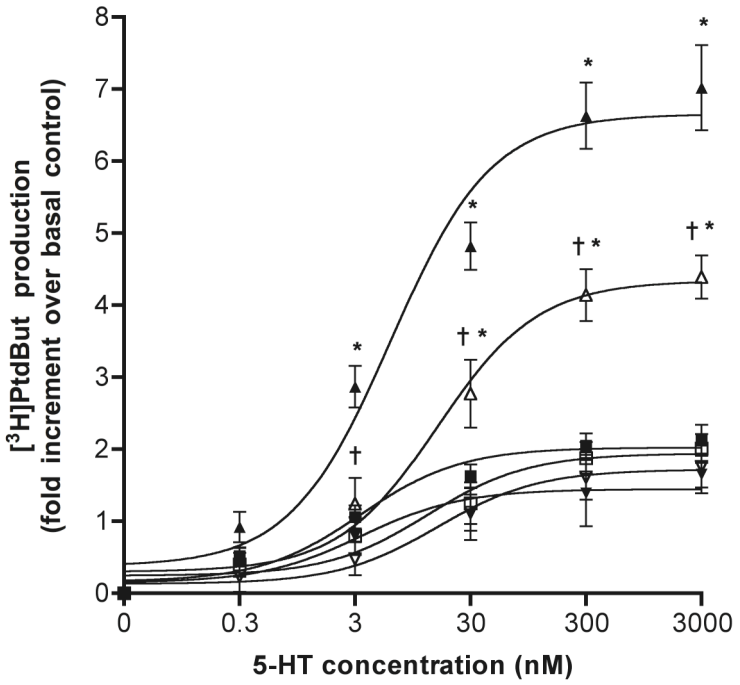


Figure 3.12 5-HT-induced PLD responses of COS7 cells co-transfected with the wild-type or H452Y mutant form of the 5-HT$_{2A}$R and PLD1 or PLD2. COS7 cells were co-transfected with either wild-type 5-HT$_{2A}$R and vector alone as a control (■), PLD1 (▲) or PLD2 (▼), or the H452Y variant of the 5-HT$_{2A}$R with vector alone as a control (□), PLD1(△) or PLD2 (▽). Cells were stimulated with increasing concentrations of 5-HT for 20 min. [^{3}H]PtdBut production was assayed to measure PLD activity. The points plotted represent means ± SEM, n = 4. Values for 5-HT-induced [^{3}H]PtdBut production were significantly greater in the presence of PLD1 than vector control at 3-3000 nM 5-HT for the wild-type receptor and at 30-3000 nM 5-HT for the H452Y variant form (*p <0.05, Wilcoxon test). The 5-HT-induced fold increment values for the H452Y variant in the presence of PLD1 were significantly less than corresponding values for the wild-type receptor at 3-3000 nM 5-HT († p <0.05, Wilcoxon test).

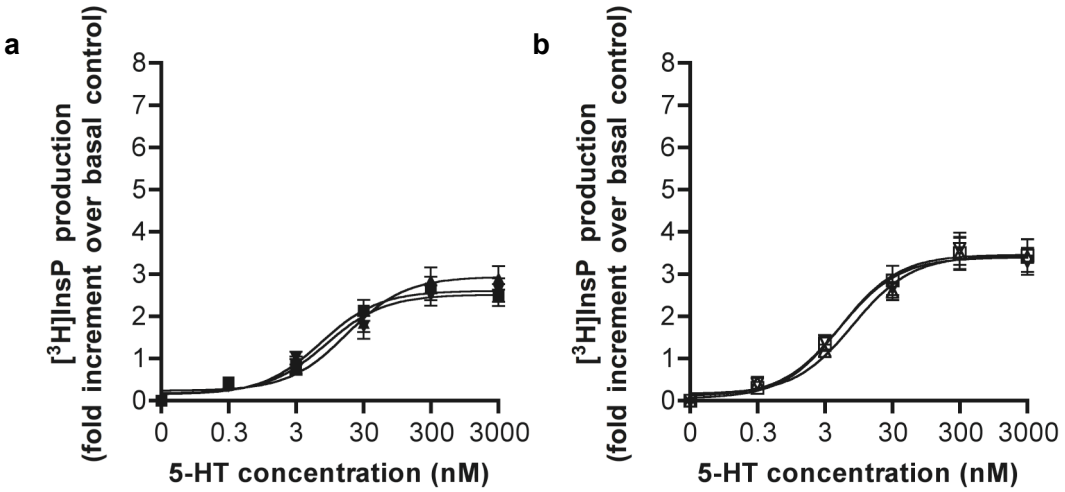


Figure 3.13 5-HT-induced PLC responses of COS7 cells co-transfected with the wild-type or H452Y mutant form of the 5-HT$_{2A}$R and PLD1 or PLD2. COS7 cells were co-transfected in (a) with the wild-type 5-HT$_{2A}$ receptor and vector alone as a control (■), PLD1 (▲) or PLD2 (▼), or in (b) with the H452Y variant of the 5-HT$_{2A}$R with vector alone as a control (□), PLD1 (△) or PLD2 (▽). Cells were stimulated with increasing concentrations of 5-HT for 180 min. [^{3}H]InsP production was assayed to measure PLC activity. No significant differences in PLC responses were noted. The points plotted represent means ± SEM, n = 4.

3.2.8 PLD signalling pathway of the wild-type and H452Y variant 5-HT$_{2A}$R – ligand-binding studies

To determine whether the differences in PLD binding and activity could be a consequence of the uneven expression of wild-type and H452Y mutant 5-HT$_{2A}$R or of differing ligand affinity, COS7 cells were transfected with either receptor variant and assayed by radioligand binding. Specific binding by wild-type or H452Y mutant 5-HT$_{2A}$R was determined by the incubation of COS7 cell membranes (microsomal fraction following removal of nuclei by low speed centrifugation, see section 2.4) with 0.8 nM of the selective 5-HT$_{2A}$R ligand [^{3}H]ketanserin (72.2 Ci/mmol), in the presence of increasing concentrations of cold ketanserin (0.1-300 nM) and in the presence/absence of excess 10 μM mianserin to define non-specific binding. COS7 cell membrane homogenates were additionally assayed for total protein concentration with the use of a Pierce BCA Protein Assay Kit. The K_D (nM) and Bmax (pmol/mg protein) displayed in Figure 3.14 are calculated from a homologous displacement protocol with non-linear curve fitting to mean values from four separate determinations, using GraphPad Prism 4. The Bmax values calculated do not appear to be discernably different between the two receptor variants, indicating that the receptors demonstrate approximately equivalent maximal numbers of ligand binding sites in our experiments. Furthermore, the similar K_D values represented in Figure 3.14 indicate that there are no marked differences in ligand affinity between the wild-type and H452Y variant 5-HT$_{2A}$R.

To assess whether the wild-type and H452Y variant receptors might differ in the extent to which they were expressed at the plasma membrane, receptors localised at

the cell surface were isolated via extracellular biotinylation, solubilisation and reversible capture on monomeric avidin beads, prior to quantification by radio-ligand binding. COS7 cells were transfected with either wild-type or H452Y 5-HT_{2A}R. Prior to lysis, cells were incubated with membrane-impermeant sulpho-NHS-biotin, which efficiently forms covalent bonds to exposed primary amine groups, such as those on Lys residues. Biotinylated proteins were captured with the use of monomeric avidin beads and eluted with 2mM biotin. Ligand binding on the solubilised receptor (both the eluate from monomeric avidin and samples of the initial lysate to assess total input of solubilised cellular receptor) was then carried out as described in section 2.6, with capture using a polyethylene glycol/bovine gamma globulin precipitation/centrifugation protocol. Total and non-specific cell-surface receptor expression were determined for both receptor variants and are displayed in Figure 3.14. There was found to be no discernable difference in the cell-surface expression of the two receptor variants, indicating that differences in the proportionate cell surface expression do not contribute to the changes in cell signalling evident between the wild-type and H452Y variant 5-HT_{2A}R.

a

Receptor construct	K_D (nM)	Bmax (pmol/mg pr)
sPrC-5-HT$_{2A}$ R	0.58 ± 0.05	0.90 ± 0.06
sPrC-[H452Y]5-HT$_{2A}$ R	0.70 ± 0.08	0.96 ± 0.08

b

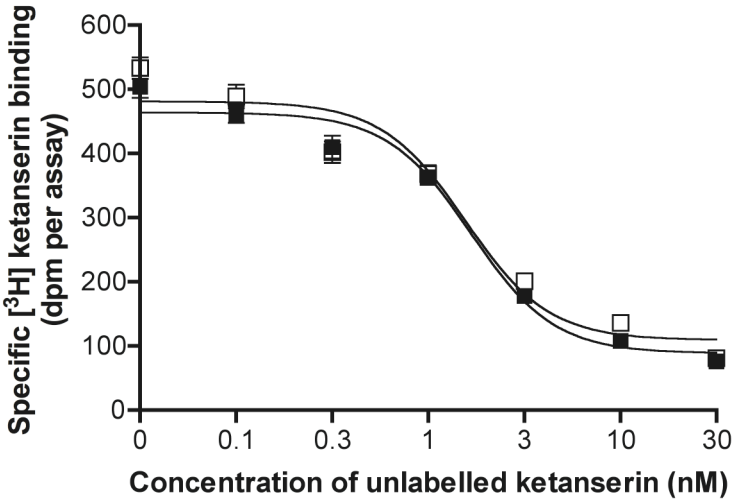


Figure 3.14 Ligand binding to wild-type and H452Y mutant constructs of the 5-HT$_{2A}$R. Levels of expression of wild-type and H452Y-5-HT$_{2A}$R were assessed by binding of the selective 5-HT$_{2A}$R ligand [^{3}H]ketanserin in the absence/presence of 10 μM mianserin, to define non-specific binding. Protein levels in COS7 cell homogenates were determined using a Pierce BCA Protein Assay Kit. A homologous displacement protocol was used (unlabelled ketanserin concentrations from 0.1-300 nM) with non-linear curve fitting to the data using GraphPad Prism 4. Curves were fitted to mean values from four separate determinations.

	Specific [^{3}H] ketanserin binding (dpm/assay)	
	Wild-type -5-HT$_{2A}$R	**H452Y-5-HT$_{2A}$R**
Cleared lysate (input)	372 ± 31	336 ± 26
Eluate from monomeric avidin	135 ± 32	154 ± 30
Mean recovered % of solubilised receptor that was cell-surface biotinylated	36.3%	45.8%

Figure 3.15 Ligand binding to biotinylated wild-type and H452Y-5-HT$_{2A}$R. Levels of plasma membrane expression of wild-type and H452Y-5-HT$_{2A}$R were assessed by binding of the selective 5-HT$_{2A}$R ligand [^{3}H]ketanserin in the absence/presence of 10 μM mianserin, to define non-specific binding at biotinylated receptors captured with avidin beads.

3.3 Discussion

PLD1 and PLD2 enzymes demonstrate diverse cellular localisations, modes of regulation and intracellular functions. The present findings demonstrate a significant amplification of PLD responses mediated by the 5-HT$_{2A}$R only in the presence of the PLD1 isoform, and additionally a significant decrease in PLD responses conferred by a negative mutant form of PLD1, but not PLD2. The lack of amplification of 5-HT induced PLC responses in the presence of either wild-type PLD isoform or of attenuation by corresponding negative mutant constructs demonstrates the specificity of the PLD1-evoked response. This indicates an isoform-specific functional role of PLD1 in 5-HT$_{2A}$R-mediated PLD signalling. Studies investigating 5-HT-mediated PLD signalling in pulmonary artery smooth muscle cells concluded that PLD activation was mediated by the 5-HT$_{2A}$R, and that this primarily involved activation of the PLD1 isoform (378). However, it is likely that the mode of 5-HT$_{2A}$R activation is also cell-type dependent, thus it should not be assumed that 5-HT$_{2A}$R-mediated PLD activation is always PLD1-selective. PLD1 activation has previously been demonstrated to induce cellular effects, such as such as agonist-induced secretion, actin reorganisation, cell adhesion and migration (199,245-247), whereas PLD2 activation downstream of the μ-opioid and glutamate receptors has been demonstrated to result in receptor endocytosis, a function apparently specific to the PLD2 isoform of the enzyme (248,379). Thus PLD1-selective activation could result in specific cellular responses, in addition to specific modes of regulation. 5-HT$_{2A}$R-induced PLD responses were previously demonstrated to be primarily ARF1-mediated in COS7 cells, with ~ 50% reduction of PLD responses seen with the introduction of a dominant negative [T31N]ARF1 mutant (50,274,374). However, as

previously discussed, the GPCR-mediated activation of PLD can be brought about via a range of signalling events, including the activation of protein kinase C, tyrosine kinases, PI 3-kinase, Gq or Gi proteins, RhoA and putatively following increasing intracellular Ca^{2+} levels (244). Thus various dominant negative constructs, drugs and toxins with inhibitory effects on these pathways were used to assess alternative influences upon 5-HT$_{2A}$R-mediated PLD responses. However, none of the inhibitory agents produced significant reductions in PLD activity: the dominant negative Q209L/D277N-Gαq construct caused only a minor (~ 15%) decrease in 5-HT$_{2A}$R-mediated PLD responses despite evoking a significant (~ 50%) reduction in GnRH receptor-mediated PLD activity as a control; the PLC inhibitor U 73122 and tyrosine kinase inhibitor AG 213 did not induce any reduction in PLD responses; the protein kinase C inhibitors CGP 41251 and bisindolylmaleimide 1 had little or no inhibitory effects upon 5-HT-induced PLD responses; inhibition of Gi/o with pertussis toxin or inhibition of RhoA-mediated pathways with the dominant negative [T19N]RhoA mutant induced only minimal reductions in PLD response; and finally, synergistic inhibition of RhoA and PKC-mediated PLD1 activation via the use of the PIM87/IR-PLD1 construct attenuated the amplification of 5-HT$_{2A}$R PLD responses by only around 15% compared to that achieved by wild-type PLD1. Therefore, the activation of PLD1 by 5-HT$_{2A}$R appears to be primarily ARF-mediated, however, PKC and RhoA-mediated PLD activity may contribute to the residual 5-HT$_{2A}$R-mediated PLD activity demonstrated by Johnson et al in COS7 cells expressing dominant negative ARF1 (374). Alternatively, the remaining PLD response could be a result of cellular adaptations resulting from the selective inhibition of ARF1 in 5-HT$_{2A}$R-expressing COS7 cells. Although both PLD isoforms have previously been shown to to be

capable of undergoing ARF-mediated activation, PLD1 is the primary PLD isoform involved in ARF-mediated PLD activation (199,247,332,380). There is evidence to suggest that PLD1 undergoes ARF-mediated activation by various GPCRs, including the M_3 receptor (362) and the somatostatin receptor (364). However, PLD2 has also been reported to undergo GPCR-induced ARF-mediated activation, such as that mediated by the μ-opioid receptor (372).

To further investigate the molecular arrangements underlying 5-HT$_{2A}$R-mediated PLD activation, the potential physical interaction of the two PLD isoforms with the 5-HT$_{2A}$R was assessed by co-immunoprecipitation. Quantification of PLD2 co-immunoprecipitation with sPrC-5-HT$_{2A}$R demonstrated that the non-specific pulldown identified in non-immune IgG controls contributed approximately 95% of the levels of PLD2 that apparently co-immunoprecipitated with the receptor. Presumably, any interactions contributing to the control pull-down of PLD2, such as direct interactions with antibody or Protein G-Sepharose beads would also be a factor contributing to the apparent PLD2 co-immunoprecipitation with the receptor. In contrast, PLD1 co-immunoprecipitated specifically with the 5-HT$_{2A}$R specific PrC tag antibody pulldowns, but not non-immune IgG controls. Thus, the specific co-immunoprecipitation of PLD1 but not PLD2 with the sPrC-5-HT$_{2A}$ receptor further corroborates the selective involvement of the PLD1 receptor in 5-HT$_{2A}$R-mediated PLD activity. Further evidence of PLD isoforms co-immunoprecipitating with GPCRs is limited, however, PLD2 has been co-immunoprecipitated with both the metabotropic glutamate receptors 1a and 5a (367) and the μ-opioid receptor, which

was found to involve interactions with the N-terminal region of PLD and the C-terminus of the μ-opioid receptor (372).

sPrC-5-HT$_{2A}$R association with ARF1-HA has been shown to increase with 5-HT stimulation in a time- and concentration-dependent manner (274). Thus co-immunoprecipitation experiments were used to characterize any dynamic changes in the binding of HA-PLD1 to sPrC-5-HT$_{2A}$ receptor. Stimulation with 1 μM 5-HT to a maximum of 1 hr did not, however, induce any significant changes in HA-PLD1 association with the receptor, indicating that, in contrast to ARF1-HA, HA-PLD1 does not appear to form a dynamic association with sPrC-5-HT$_{2A}$R. However, there are various reports indicating that PLD1 activation can involve, or be subsequent to, PLD1 relocalisation: carbachol stimulation of COS7 cells overexpressing M$_3$ muscarinic receptor was found to induce the BFA-insensitive translocation of both ARF1 and PLD1 to the plasma membrane (244); antigen-stimulation of RBL-2H3 cells reportedly induces the co-localisation of PLD1b with Rac1, ARF6 and PKCα (381); and PLD1, but not PLD2 was found to translocate to the plasma membrane of cells incubated with the PLD1-activating cytokine TNF-α, in a time-dependent manner (382). Thus PLD1 availability in the plasma membrane may be dynamically regulated. It is possible that a sufficient pool may already be present to maintain PLD:5-HT$_{2A}$R interactions, or that the binding or dissociation rate may be slow or limited by other factors.

GST-fusion proteins of 5-HT$_{2A}$R domains (ct, K385ct and i3) further defined the locus of PLD1 binding to the C-terminal tail domain of the receptor, at a point distal

to the initial 9 amino acids. PLD2 binding was not detectably altered between any of the different receptor domains, further confirming the unspecificity of PLD2 binding by the 5-HT$_{2A}$R. The 5-HT$_{2A}$R third intracellular loop, which has been reported to bind arrestin isoforms and G-proteins (168) bound < 50% the amount of PLD1 that bound to the full-length receptor C-terminal tail. Removal of the initial 9 residues of the C-terminal domain appeared to increase PLD1 binding by approximately >50% of that seen with the full-length C-terminus. These initial 9 amino acids form part of an NPxxY motif (376-380) that has been implicated as a critical determinant of ARF-dependent signalling in rhodopsin family GPCRs (50). Residues 376-385 were further demonstrated to be essential for both the co-immunoprecipitation and isoform-selectivity of ARF isoforms by the 5-HT$_{2A}$R. Thus these studies indicate that PLD1 binds at a distinct region from ARF, at a site more distal on the C-terminal tail. It has been suggested that residues Thr 381-Lys 385 of the 5-HT$_{2A}$R may form a flexible hinge region to an adjacent eighth helical segment that is anchored to the membrane by a palmitoylated Cys (Cys 397) and runs parallel to the plane of the membrane (274). Increased levels of PLD1 binding introduced with the truncation of the C-terminal tail could be a result of conformational alterations of the receptor terminus subsequent to the removal of residues 376-384; for instance, changes resulting in the further exposure of sites involved in PLD interactions. However, due to the in vitro nature of the experiment and isolation of the C-terminus from the remainder of the receptor this may be unrepresentative of potential outcomes in the physiological state. Alternatively, it was considered that a reduction in ARF binding following removal of residues 376-385 (274), could be a factor of subsequent increased PLD1 binding levels. Thus GST-fusion proteins of the full-length 5-HT$_{2A}$R

C-terminal tail were used to assess the influence of prior binding of ARF1 to the receptor upon subsequent PLD1 binding to the receptor and, vice versa, the effects of prior binding of PLD1 to the receptor upon subsequent ARF1 binding. The lack of evident interruption or conversely, enhancement of the binding of ARF/PLD1 by prior occupancy with the other binding partner indicates that receptor-bound ARF1 neither precludes nor is required for PLD1 binding. Furthermore, the apparent inability of either construct (ARF1/PLD1) to induce a dissociation of pre-bound construct (PLD1/ARF1 respectively) from the receptor further indicates that these constructs are binding to distinct sites of the receptor C-terminus. Thus these findings indicate that the binding of PLD1 and ARF1 by the 5-HT$_{2A}$R are independent of one another.

The PLD1 binding site on the 5-HT$_{2A}$R was further defined with the use of sequential C-terminally-truncated GST-fusion proteins of the C-terminal tail. The truncations, (376-470, 376-438, 376-428) and the full-length C-terminal tail were found to bind HA-ARF1 with an approximately equivalent effectiveness, indicating that residues 428-471 of the 5-HT$_{2A}$Rct are not a critical determinant of HA-ARF1 binding. Conversely, the loss of the terminal residue, Val 471 resulted in a small (approximately 30%) decrease in HA-PLD1 binding. Val 471 forms part of a VSCV motif that acts as a recognition site for PDZ domain proteins in the human 5-HT$_{2A}$ receptor. This canonical type I PDZ ligand has been demonstrated to bind a specific set of Post-synaptic density protein, Drosophila disc large tumor suppressor, and zonula occludens-1 protein (PDZ) proteins, including the multi-PDZ domain protein I (MUPP1), SAP97, channel interacting PDZ protein (CIPP) and post synaptic

density-95 (PSD-95) (190,191). PDZ domain proteins often contain multiple PDZ domains, potentially enabling them to simultaneously interact with more than one binding partner, thereby facilitating a variety of protein-protein interactions and larger signalling complexes (383). Association of the PDZ domain protein PSD-95 with the 5-HT$_{2A}$R is reported to result in an increase in 5-HT-induced-PLC activation, which is ablated with the truncation of the 3 extreme 5-HT$_{2A}$Rct residues (Ser 469-Val 471) and corresponds to enhanced receptor retention at the cell surface (192). It is possible that a 5-HT$_{2A}$R PDZ domain protein scaffolding complex could contribute to PLD assocation, and that this interaction is interrupted with the removal of the extreme C-terminal Val 471 residue, thus explaining the partial reduction in PLD association introduced with this truncation. The removal of a further 32 residues to yield 376-438 5-HT$_{2A}$Rct however was found to result in a much more profound, approximately 70% loss of PLD association. The further loss of another 10 residues with the 376-428 5-HT$_{2A}$Rct construct did not induce a discernably greater loss in PLD association than that seen with the GST-T438 5-HT$_{2A}$Rct construct, thus implying that key residues involved in PLD binding may be located between residues T438 and C470. A more precise definition of the binding site would require additional truncation constructs and an extensive array of single residue mutations.

A clinically relevant single nucleotide polymorphism (SNP) exists at residue 452, located between residues 438-470, in the distal region of the 5-HT$_{2A}$Rct. This naturally occurring minor allele, involves the substitution of His 452 by Tyr (H452Y), and is known to be expressed by approximately 9% of the population (301). Meta-analysis studies have concluded that there is a significant association

between the heterozygous expression of the H452Y minor allele and a resistance to clozapine, an atypical antipsychotic with high affinity for the 5-HT$_{2A}$R (301). In addition, the H452Y SNP has been linked to poor memory, attention deficit hyperactivity disorder and biopolar disorder (307,384,385). Studies investigating signalling pathways downstream of the H452Y receptor have also concluded that expression of the minor allele results in a decrease in the speed and amplitude of agonist-stimulated intracellular calcium mobilisation in platelets (308). A subsequent study has suggested that both PLC and PLD activation may be decreased downstream of the H452Y receptor in association with enhanced desensitisation of the receptor (309). Due to the location of the coding substitution, which is positioned within the distal region of the 5-HT$_{2A}$Rct, it was hypothesised that the minor variant of the receptor might influence PLD interactions and signalling pathways. Co-immunoprecipitation of HA-PLD1 with the H452Y variant of the sPrC-5-HT$_{2A}$R showed clearly decreased interactions between PLD1 and the minor variant of the receptor when compared to the wild-type receptor. This was further confirmed in experiments to assess the binding of PLD1 to GST-fusion proteins of the wild-type 5-HT$_{2A}$Rct and the H452Y variant. Again, levels of PLD1 captured with the H452Y receptor variant were greatly decreased. These data suggest that His 452 is a critical determinant of PLD binding, which is interrupted when it is substituted by Tyr. It is possible that the minor allele of the receptor adopts an alternative conformation, which obscures regions of the receptor necessary for the efficient binding of PLD. It is not obvious that the substitution of His, a polar positively charged amino acid with a hydropathy index of -3.2, by Tyrosine, a polar but neutrally charged amino acid with a hygropathy index of -1.3, would necessarily cause an altered conformational

state in both the full-length receptor and in the truncated GST-fused C-terminal tail of the receptor, although unknown binding interactions with other residues could occur and be of importance here. Alternatively, His 452 could be directly involved in molecular interactions with PLD1, whereby the presence of the H452Y minor allele could result in a direct decrease in PLD1 interaction.

Activation of PLD1 was also significantly decreased downstream of the H452Y variant of the receptor when compared to wild-type receptor, suggesting the functional importance of reduced PLD1 binding seen with the minor variant of the receptor. Both PLD2 activation and endogenous PLD responses did not appear to be altered in the H452Y variant of the receptor. The lack of detectable differences in endogenous PLD responses of the wild-type and H452Y variant receptor may possibly be because these responses are not large enough to be able to detect a significant decrease in the H452Y variant compared to the wild-type responses. The lack of any detectable differences between PLC responses of the wild-type and H452Y receptor indicates that differences in PLD signalling between these receptor forms are specific to that pathway, which accords with the observations of diminished PLD1 docking to the C-terminal tail domain of the H452Y variant. To ensure that differences in PLD responses were not as a result of uneven expression of the two receptor variants, overall or at the cell surface, the expression of the wild-type sPrC-5-HT$_{2A}$R and H452Y-sPrC-5-HT$_{2A}$R, both within cellular microsomal membranes and at the cell surface, was quantified with radio-ligand binding and biotin-tag-selected radio-ligand binding, respectively. No significant differences were noted between expression or deployment of the two receptor forms.

In conclusion, these experiments demonstrate for the first time that the 5-HT_{2A}R directly binds PLD1 to its C-terminal tail at a locus in the distal tail importantly involving residues between 438 and 470, including residue 452, the site of a common and physiologically significant polymorphism, H452Y. The PLD1 binding site is independent of that for ARF1 in the proximal tail and the docking of the two ligands is dynamically independent. Both ARF1 and PLD1 however contribute important to an apparently heterotrimeric G protein-independent signalling pathway from the 5-HT_{2A}R, and the docking of PLD1 and amplification of functional signalling through this pathway by additional PLD1 are selectively disrupted in the H452Y. The work points to the existence of a functionally important 5-HT_{2A}R/ARF1/PLD1 signalling complex and its specific disruption in the H452Y variant receptor, a distinction with potential physiological significance, for example in psychiatric disorders where the 5-HT_{2A}R plays a role.

CHAPTER 4.0

4.0 Desensitisation of 5-HT_{2A}R-mediated PLC responses and influences of the H452Y polymorphism

4.1 Introduction

4.1.1 Arrestin structure and function

The mammalian arrestin family comprises 4 arrestin isoforms; arrestin-1, arrestin-2, arrestin-3 and arrestin-4. Arrestin-1 was the first arrestin isoform to be identified, in retinal extracts, and was originally named 48-kDa protein, or S antigen (386). Arrestin-2 and arrestin-3 are alternatively named β-arrestin 1 (β-arrestin1) and β-arrestin 2 (β-arrestin2) respectively. Arrestin-4 is also termed cone arrestin or X-arrestin. Mammalian arrestin-1 and arrestin-4, both of which are visual arrestins, are expressed within retinal rod and cone photoreceptors (387). These specialised proteins bind specifically to rhodopsin or cone cell pigments (387). Mammalian β-arrestin 1 and β-arrestin 2 are expressed ubiquitously and act as negative regulators (388), adaptor proteins (389,390) and scaffolding proteins for many GPCRs (391).

Crystallographic structural studies reveal a basic architecture that typifies arrestins, consisting of two antiparallel seven-stranded β-sheet sandwich domains (N- and C-domains), residues 8-180 and 188-362 respectively in bovine visual arrestin, linked via a 12 residue long hinge region (127,387,392,393), and flanked to either end by an N- and C-terminal domain. The N-domain is connected to a small N-terminal α-helix, and contains Src binding sites, in addition to a phosphate sensor domain thought to be essential for the selective binding to phosphorylated receptors (394). The C-domain contains an inositol hexakisphosphate $InsP_6$ recognition site, and an

RRSLHL MAP kinase recognition domain (394). Finally, the C-terminal domain of the non-visual arrestins contains regions responsible for forming interactions with the endocytic machinery (clathrin and AP-2) (387,392,393). At the centre of the structure is a polar core, composed of charged interactions of five solvent-excluded interacting charged residues of the N- and C-domains and C-terminus (Asp 30, Arg 175, Asp 296, Asp 303 and Arg 382 in bovine visual arrestin) (395) (396) (127) (387).

The mechanism by which arrestin preferentially binds activated, phosphorylated receptors is thought to require the simultaneous activation of two arrestin domains, the 'phosphate sensor' and an 'activation sensor' (394,397). The phosphate sensor is buried deep within the polar core of the protein and consists of an intra-molecular salt-bridge composed of a positively-charged residue (Arg 175 in bovine visual arrestin) of the N-domain and a negatively-charged residue (Asp 296 in bovine visual arrestin) of the C-domain (394,397). This salt-bridge is thought to lock the protein in an 'inactive' state that is unable to form high-affinity interactions with the receptor. Mutagenesis of both participating residues has been used to demonstrate that loss of the salt-bridge confers a corresponding loss of selectivity for phosphorylated receptors by arrestin (398). Our understanding of arrestin structure without any stimulation is additionally supported by further intra-molecular interactions, including a 'three-element interaction', that exists between β-strand I and α-helix I in the N-domain and the C-terminal tail, and hydrophobic interactions between the N- and C-domains (394,397). The current model of arrestin activation (described using residue numbers of bovine visual arrestin) is thought to be a multi-step process involving various interaction sites: the arrestin 'activation sensor' is conjectured to

bind regions of the receptor that change conformation upon activation, probably resulting in an inhibition of interactions existing between the two arrestin domains; additionally, Lys 14/15 of β-strand I reportedly associate with negatively charged receptor-bound phosphates, which acts to destabilize the three-element interaction, resulting in the dissociation and movement of the C-terminal tail and subsequent loss of Arg 382 from the polar core; meanwhile, Lys 14/15 are thought to facilitate the entry of the phosphates into the polar core, thereby allowing the association of Arg 175 with receptor-bound phosphates (394,397). Thus, the negatively-charged phosphates act to neutralise the positive charge of Arg 175, the salt-bridge breaks and the polar core is disrupted (394,397). Due to the additional loss of Arg 382 from the polar core, the remaining residues (Asp 30, Asp 296 and Asp 303) all become unopposed negatively-charged residues and thus repel each other, resulting in a conformational change in the orientation of the arrestin domains with relation to one another (394,397). This structural change introduces further receptor-binding sites to the active phosphorylated receptor, that engage in high-affinity semi-irreversible interactions with the receptor (394,397).

4.1.2 GRK family kinases

GPCR kinases (GRKs) act to phosphorylate Ser/Thr GPCR residues. The mammalian GRK family comprises 7 GRK isoforms that are categorised into 3 subfamilies (399). GRK1 and 7, both of which are expressed exclusively within retinal cells form one sub-group - the visual GRK subfamily. The non-visual GRKs are divided into the remaining two subfamilies: the β-adrenergic receptor kinases subfamily, comprising GRK2 and GRK3; and the GRK4 subfamily, consisting of

GRK4, GRK5 and GRK6 (399,400). GRK2, 3, 5 and 6 are all expressed ubiquitously, whilst GRK4 is primarily expressed in the testes (401). The non-visual GRKs have a common basic architecture, displaying a highly conserved N-terminus of approximately 185 residues, connected to a central, well-conserved catalytic domain of approximately 270 amino acid residues, and terminating in a C-terminal tail of variable length (105-230 amino acid residues) (399). The N-terminal domain is thought to be involved in receptor recognition and exhibits a Regulator of G protein Signalling (RGS) homology domain, which in the case of GRK2 and GRK3, is thought to be involved in the regulation of G protein coupling (400,402-404). Other N-terminal regions are reported to be involved in membrane targeting and where the GRK4 subgroup is concerned, binding phosphatidylinositol (4, 5)-bisphosphate (399,405,406), which is thought to result in an increase in the catalytic kinase activity of the GRK (399,405,406). Specific binding sites expressed on the GRK C-terminus are thought to be important for both GRK localisation and agonist-dependent translocation, thus influencing GRK function (127,399,407-410). GRK2 and 3 possess both PH domains and Gβγ subunit binding domains, which interact with phosphoinositides and βγ subunits of heterotrimeric G proteins respectively, thereby inducing GRK translocation to the plasma membrane (127). Both GRK4 and 6 contain C-terminal cysteine residues that undergo palmitoylation and anchor the GRK to the plasma membrane, whereas GRK5 contains a C-terminal sequence of 46 basic amino acids that interact with phospholipids at the membrane, again localising the protein to the plasma membrane (127).

In addition to acting as receptor kinases, GRKs have been demonstrated to interact with a diverse array of signalling and trafficking proteins, including PI-3 kinase, G protein-coupled receptor kinase interactor (GIT) proteins, caveolin, clathrin, Hsp90, calcium-binding proteins and Gαq and Gβγ subunits (399). Thus, in addition to being regulated by binding partners such as caveolin, calmodulin, actin and Akt, GRKs may also be directly involved in alternative cellular processes such as inhibition of ERK and PLC-β activation via interactions with MEK1 and Gq/11, respectively (399).

4.1.3 GRKs and arrestins in GPCR desensitisation

The classical mode of homologous desensitisation follows GPCR agonist-activation, which yields free Gβγ subunits. It is thought that these βγ subunits interact with GRK2/3 proteins, which results in GRK2/3 translocation to membrane-bound receptors (411). Once localised at the receptor, GRK mediates the phosphorylation of the agonist-activated receptor at Ser/Thr residues generally of the C-terminal tail or 3rd intracellular loop (412). GRK2 and 3 have been identified as the isoforms primarily involved in agonist-dependent receptor phosphorylation (413-415): Ren et al used small interfering RNA sequences (siRNAs) specific to the only widely expressed GRKs, GRK2, -3, -5, and -6 to investigate GRK isoform-specific regulation of the vasopressin-2 receptor (V2R), GRK2 and -3 were determined to be primarily responsible for V2R phosphorylation and inhibition of Gs-mediated cAMP responses (415). Furthermore, H1 histamine receptor desensitisation has been demonstrated to be GRK2-, but not GRK5-dependent (416). However, there is growing evidence to implicate members of the GRK4 subfamily in regulating events

that moderate GPCR signalling. GRK4 is implicated in the regulation of various GPCRs in specific tissues at which it is expressed relatively highly, such as desensitisation of the type 1a metabotropic glutamate receptor in Purkinje cells (417). GRK5 is now known to be able to phosphorylate numerous GPCRs when coexpressed with receptor (401), but is also demonstrated to regulate endogenously expressed vasoactive intestinal polypeptide type I (VPAC1) receptors (418). Endogenously expressed GRK5 is further reported to regulate thrombin-induced signalling in endothelial cells (419). Additionally, GRK6 is implicated in the regulation of various GPCRs; it is thought to induce β-adrenoceptor desensitisation in uterine smooth muscle cells (420), and its overexpression has been shown to result in increased phosphorylation of the M_3 muscarinic receptor in SH-SY5Y neuroblastoma cells (421).

Receptor activation and subsequent GRK-mediated phosphorylation results in the recruitment of β-arrestin isoforms to the plasma membrane (412). The subsequent binding of β-arrestin by agonist-activated receptor induces a conformational change in the β-arrestin (422). Phosphate moieties of the receptor disrupt the delicate ionic equilibrium of the β-arrestin central polar core (387,412,414,422) and subsequently, interactions between the phosphate sensor region and the C-terminus are broken, resulting in the dissociation of the N- and C-termini and a likely rearrangement of the N- and C-domains (387,412,414,422). This yields the 'active' β-arrestin conformation, which binds the receptor with high affinity. β-arrestin is conjectured to bind at such a position as to sterically hinder receptor:G protein interactions, thereby

preventing G protein re-association and resulting in the termination of G protein-mediated signalling (394).

Heterologous desensitisation occurs downstream of GPCR phosphorylation by second-messenger-dependent kinases such as cyclic-AMP dependent kinase (PKA) and protein kinase C (PKC) (127,423). These kinases also phosphorylate GPCRs at Ser/Thr residues of the intracellular loops and C-terminal tail (127,423). Conversely, agonist-activation of the receptor is not a prerequisite for receptor phosphorylation in this system (127,423). Thus, a receptor may become desensitized as the result of PKA/PKC activation by an alternative receptor, hence the use of the term 'heterologous desensitisation' (127,423). Various GPCR sub-types, such as the D2 dopamine receptor (424) and the δ-opioid receptor (425) have been demonstrated to associate with β-arrestins following phosphorylation by PKC (426). PKC and PKA have also been demonstrated to phosphorylate and activate GRK2, thereby increasing its ability to phosphorylate and desensitize specific receptors (426).

4.1.4 Arrestins in GPCR internalisation

β-arrestin isoforms are additionally implicated as mediators of the internalisation of many GPCRs, by facilitating receptor linkage to clathrin-coated vesicles (390). Following the binding of agonist-activated receptor by β-arrestin isoforms and the subsequent dissociation of the β-arrestin C-termini from the polar core, binding sites for AP2 and clathrin situated on the β-arrestin C-terminal tail are revealed (387,412,414,422). Clathrin, a trimeric protein and the primary constituent of clathrin-coated pits, is able to self-polymerise into a lattice to surround the receptor-

containing vesicle via a cage-like structure. AP-2, an adaptor protein, forms a complex by linking to the clathrin molecules and associating with accessory proteins, such as dynamin (which facilitates the fission of vesicles from a membrane) and EPS-15 (which is thought to facilitate the AP180 adaptor protein-mediated assembly of clathrin) (427)) to facilitate internalisation (428). Once the clathrin-coated pit is assembled, it pinches off from the membrane, mediated by dynamin, and the clathrin coat disassembles to leave the vesicle (429).

β-arrestin isoforms bind, via an LIEF sequence within residues 374-377 of the carboxy-terminal tail, to residues 89-100 of the N-terminal domain of the clathrin heavy chain (127,430,431). The interaction with clathrin can be regulated via phosphorylation of the β-arrestin 1 Ser 412 residue by ERK MAP kinase (430,432). Phospho-Ser 412 β-arrestin 1, although still capable of binding receptor is unable to bind clathrin. However, upon β-arrestin 1 recruitment to the membrane, a phosphatase catalyses the dephosphorylation of the protein, thereby promoting clathrin-mediated receptor internalisation (430,432). Interestingly, no equivalent residue is present in β-arrestin 2, exemplifying differential regulatory potential of β-arrestin 1 and β-arrestin 2.

β-arrestin isoforms have also been demonstrated to bind, via an RxR sequence, to the β2 adaptin subunit of the AP-2 complex (127), an interaction that has been shown to be independent of clathrin binding (430). A further factor that may promote arrestin:AP-2 mediated events may be phosphoinositide 3-kinase (PI 3-kinase); GRK isoforms have been demonstrated to form cytosolic complexes with PI 3-kinase upon

agonist stimulation of the β-adrenergic receptor, resulting in the GRK-mediated agonist-dependent translocation of PI 3-kinase to the plasma membrane (433-435). Subsequently, phosphatidylinositol 3,4,5-phosphate species are generated in a PI 3-kinase-dependent manner at the plasma membrane, that in turn are thought to increase AP-2 recruitment to the membrane (433-435).

Following internalisation, GPCRs demonstrate two distinct intracellular trafficking patterns dependent upon their interactions with β-arrestin isoforms (436). GPCRs have been classified as class A or class B receptors, based upon their β-arrestin binding characteristics (437). Class A receptors include the μ-opioid, endothelin A, dopamine D1A, β_2 adrenergic and α1B adrenergic receptors (127,435). Class A receptors form transient interactions with β-arrestin isoforms, but bind β-arrestin 2 with higher affinity than β-arrestin 1 (127). This relatively weak interaction putatively occurs as a result of the transient ubiquitination state of β-arrestins that are complexed to class A receptors (435,437,438). Following internalisation of class A GPCRs, the receptor:β-arrestin complex dissociates and the receptor is thought to be rapidly recycled back to the plasma membrane representing resensitisation (127,436). However, class B receptors, such as neurotensin 1, vasopressin type-2, angiotensin II type-1a, thyrotropin-releasing hormone and neurokinin NK-1 receptors, demonstrate a more stable, prolonged interaction with β-arrestin isoforms and bind with approximately equal affinities to β-arrestin 1 and β-arrestin 2 (127). Following internalisation, β-arrestin does not dissociate from class B receptors and the complex is targeted to endocytic vesicles (127,436). The recycling of class B receptors back to the plasma membrane is much slower and it has been suggested that stable GPCR:β-

arrestin interactions may promote degradation of the receptor (127,436). It has been demonstrated, using the dopamine D_2 and D_3 receptors, that both the DRY motif (within the second intracellular loop) and the C-terminal tail of GPCRs determine the strength of β-arrestin interactions (439).

4.1.5 Arrestin-dependent signalling

In addition to facilitating the scaffolding of complexes involved in receptor endocytosis, β-arrestin isoforms have also been shown to act as scaffolds for multi-component signalling complexes (132,133,137,440-443). It has been proposed that upon activation, β-arrestin isoforms may adopt diverse conformations, depending upon the phosphorylation profile of the bound receptor (444). Thus, the array of potential β-arrestin binding partners and hence, the resulting functional output, may be influenced by the initial pattern of phosphorylated sites on the GPCR (444). β-arrestin-dependent signal transduction was first demonstrated in β2-adrenergic receptor-mediated MAP kinase activation, by means of its inhibition following the expression of dominant negative mutant β-arrestin constructs in HEK293 cells (443). Subsequent studies have led to the identification of many GPCRs that can signal in a β-arrestin-dependent manner, including the gonadotrophin-releasing hormone, angiotensin II type I, vasopressin 2, parathyroid hormone receptors and 5-HT_{2A}R (132,133,137,440-442,445). β-arrestins have been demonstrated to link GPCRs to various signalling complexes involving Src family tyrosine kinases, ERK1/2 and JNK3 (446,447) . The scaffolding of Src by β-arrestins is thought to be important for several processes: it has been demonstrated to be involved in the tyrosine phosphorylation of dynamin, which is potentially a prerequisite for receptor

endocytosis (127,448,449); it is implicated in the stimulation of neutrophil degranulation (127); and it is additionally thought to be one route for activation of the ERK MAP kinase cascade (127,448,449). MAP kinase cascades are thought to be activated via the scaffolding and subsequent activation of upstream enzymes required for MAP kinase activation (435). Arrestin-dependent ERK 1/2 activation, downstream of both ligand activated β_2-adrenergic receptor and gonadotrophin-releasing hormone receptors, appears to result in a delayed, sustained response, when compared to G protein-dependent phospho-ERK signals (133,450). The strength of the arrestin:GPCR interaction is thought to have a significant influence upon the efficiency of ERK 1/2 phosphorylation and thus ERK 1/2 activation (131). Thus, it follows that stably interfaced arrestin:Class-B GPCR complexes induce greater ERK 1/2 phosphorylation than weaker, more transient arrestin:Class-A GPCR complexes (131).

β-arrestin 1 and β-arrestin 2 have also been demonstrated to promote the desensitisation of particular GPCRs by acting as scaffolds for enzymes that inhibit specific signalling pathways. β-arrestin isoforms have been implicated in the translocation and scaffolding of phosphodiesterases (PDEs) to activated β_2-adrenergic receptors at the plasma membrane (435,451). The localization of PDEs at the receptor results in the degradation of the second messenger cAMP, which further results in the inhibition of cAMP dependent-PKA activation (435). Furthermore, subsequent to the binding of activated M_1 muscarinic receptors, β-arrestin isoforms have been demonstrated to interact with diacylglycerol kinases (DGKs) (435,452). DGKs act to catalyze the ATP-dependent conversion of diacylglycerol (DAG), to

phosphatidic acid (PA) (452). Thus the β-arrestin mediated recruitment of DGK to the activated receptor results in a decrease in DAG signalling, and yet promotes signal transduction downstream of the signalling molecule PA (452).

4.1.6 Involvement of GRKs and arrestins in 5-HT$_{2A}$R regulation

Various studies have investigated regulation of 5-HT$_{2A}$R signalling. Desensitisation mechanisms are thought to be cell-specific, ligand-specific and receptor-specific (130,170,174,292,453,454), thus there is no standard pattern of 5-HT$_{2A}$R desensitisation. Putative desensitisation-related phosphorylation sites were identified by the sequential mutagenesis of Ser/Thr residues of the intracellular regions of the 5-HT$_{2A}$R expressed in HEK293 cells (455). Only the mutagenesis of Ser 188 in the second intracellular loop and Ser 421 of the C-terminal tail was found to inhibit agonist-mediated desensitisation, thus suggesting that these Ser residues may undergo phosphorylation by kinases, potentially including GRKs (455). However, when HEK293 cells were induced to overexpress GRK2 and GRK5, or catalytically inactive GRK2 (GRK2-K220R) together with the 5-HT$_{2A}$R, and stimulated with quipazine, desensitisation was unaffected, indicating that GRK2 and GRK5 appear not to be involved in quipazine-induced 5-HT$_{2A}$R desensitisation in HEK293 cells (130). GFP-tagged 5-HT$_{2A}$R expressed in HEK293 cells has also been demonstrated to internalise independently of β-arrestin 1 and β-arrestin 2 (456). Furthermore, the systematic mutagenesis of protein kinase C consensus phosphorylation sites within the cytoplasmic domains of the 5-HT$_{2A}$R was shown to have no effect upon 5-HT-induced desensitisation (455). However, PKC activation is reportedly involved in 5-

HT_{2A}R desensitisation and internalisation (457,458) (459), although the effects of PKC activation are thought to be cell-dependent (292,454,458).

In an attempt to elucidate arrestin binding sites in the 5-HT_{2A}R, Gelber et al used GST-fusion proteins of the 5-HT_{2A}R third intracellular loop to evaluate binding of arrestin isoforms (186). Visual arrestin, β-arrestin 1 and β-arrestin 2 were found to interact with i3 of the 5-HT_{2A}R with seemingly equivalent affinities (186). The 5-HT_{2A}R has surprisingly been demonstrated to undergo internalisation via an endosomal pathway in response to incubation with both agonists and antagonists, in vitro and in vivo (299,460). It has been suggested that the therapeutic effects of some typical and atypical antipsychotics may in part be due to the potentiation of 5-HT_{2A}R internalisation and redistribution (172,292,299,460). It appears that the 5-HT_{2A}R is capable of undergoing desensitisation and internalisation via diverse mechanisms, potentially dictated by cell-type or agonist-type (130,170,174,292,453,454). A study by Bhatnagar et al suggested that the 5-HT_{2A}R internalises in an arrestin-independent, but dynamin-dependent manner in HEK293 cells (456). This study was corroborated by further observations that the overexpression of a truncation mutant of β-arrestin 1 (319-418), that blocks 5-HT_{2A}R endocytosis in HEK293 cells, had no discernable effect upon agonist-mediated 5-HT_{2A}R desensitisation, although overexpression of a dominant-negative mutant of dynamin (DynK44A) was also determined not to influence desensitisation of the receptor (130). However, overexpression of the same arrestin and dynamin mutant constructs in C6 glioma cells acted to potentiate desensitisation, putatively by inhibiting the resensitisation of receptors, thus implying that both dynamin and β-arrestin 1 are required for 5-HT_{2A}R internalisation in C6

glioma cells (130). Furthermore, the expression of a constitutively active β-arrestin 1 mutant (R169E) in HEK293 cells co-transfected with 5-HT_{2A}R resulted in the agonist-independent desensitisation and internalisation of the receptor, suggesting that arrestins are, at least in principle, capable of interacting with the 5-HT_{2A}R (453,461).

4.2 Results

4.2.1 Desensitisation of 5-HT_{2A}R PLC signalling and the influence of arrestins

Previous studies have suggested that the H452Y-5HT_{2A}R may exist in a partially pre-desensitised state, demonstrating PLC responses that resemble the reduced agonist-induced PLC activity seen with wild-type receptors presensitised with 10- or 30-minute exposures to 5-HT in NIH3T3 cells (309). Additionally, platelets of H452Y heterozygotes were found to demonstrate reduced Ca^{2+} mobilisation (308). To further investigate this possibility here, COS7 cells were transfected with either 5-HT_{2A}R or H452Y-5-HT_{2A}R and assayed to assess whether the H452Y mutation confers any alteration to the time-course of 5-HT-induced PLC activation responses (Figure 4.1a). The H452Y mutation was not found to cause any significant time-dependent alteration in 10 μM 5-HT-induced PLC activation represented as [^{3}H]InsP production shown as a fold of basal; 5-HT_{2A}R-mediated PLC responses reached a maximum of ~ 6.2 ± 0.39 fold of basal control, whilst H452Y-5-HT_{2A}R-mediated PLC responses reached a maximum of ~ 6.6 ± 0.57 fold of basal control. Basal values were not discernably different between cells expressing the two variants of the 5-HT_{2A}R. PLC responses for both receptor variants were found to desensitise in these

experiments between approximately 140 and 180 min of agonist incubation with no detectable difference between the receptor variants. Pilot experiments indicated that [^{3}H]InsP values in unstimulated COS7 cells transfected with the wild-type-5-HT_{2A}R, H452Y-5-HT_{2A}R or empty vector were not discernably increased through the duration of the assays.

To further investigate 5-HT_{2A}R desensitisation in a different cell type that may contain additional relevant proteins, C6 glioma cells (which endogenously express 5-HT_{2A}R at low levels), were transfected with either $pcDNA_{3.1}$ vector as a control, wild-type 5-HT_{2A}R, or H452Y-5-HT_{2A}R, stimulated with 10 μM 5-HT for between 0-180 min and assayed for PLC responses (Figure 4.1b). The endogenous 5-HT_{2A}R produced a maximum response of approximately 3.8 ± 0.13 fold of basal; this was increased to approximately 5.6 ± 0.31 and 6.1 ± 0.43 fold of basal with additional expression of wild-type 5-HT_{2A}R and H452Y-5-HT_{2A}R, respectively. Evidently, the H452Y substitution also did not appear to confer any alterations to agonist-stimulated PLC activity compared to the wild-type receptor in C6 glioma cells. Although PLC responses in the control cells appeared to undergo desensitisation between 140-180 min of 5-HT-stimulation, there was no apparent reduction in the rate of either wild-type 5-HT_{2A}R or H452Y-5-HT_{2A}R-mediated PLC responses within the maximum 180 minute 5-HT incubation.

To further investigate the desensitisation of wild-type- and H452Y-5-HT_{2A}R-mediated PLC activity by means of an alternative protocol, COS7 cells were transfected with either 5-HT_{2A}R (Figure 4.2a) or H452Y-5HT_{2A}R (Figure 4.2b) and

pre-treated with 10 μM 5-HT plus 10 μM pargyline (monoamine oxidase inhibitor; to prevent 5-HT breakdown) for increasing periods of time between 0–30 min. After washing twice and addition of lithium to prevent inositol phosphate recycling, cells were then either stimulated for 30 min with 10 μM 5-HT, 1 μM ritanserin (a 5HT$_{2A}$R antagonist, to expose any potential effect of residual un-eluted 5-HT), or not stimulated at all, with 10 μM pargyline present in each case. Pre-incubation for up to 30 min with 10 μM 5-HT did not induce any significant changes to PLC responses mediated by either the 5-HT$_{2A}$R or H452Y-5HT$_{2A}$R in COS7 cells under any of the three conditions, when compared to cells that had not been pre-stimulated.

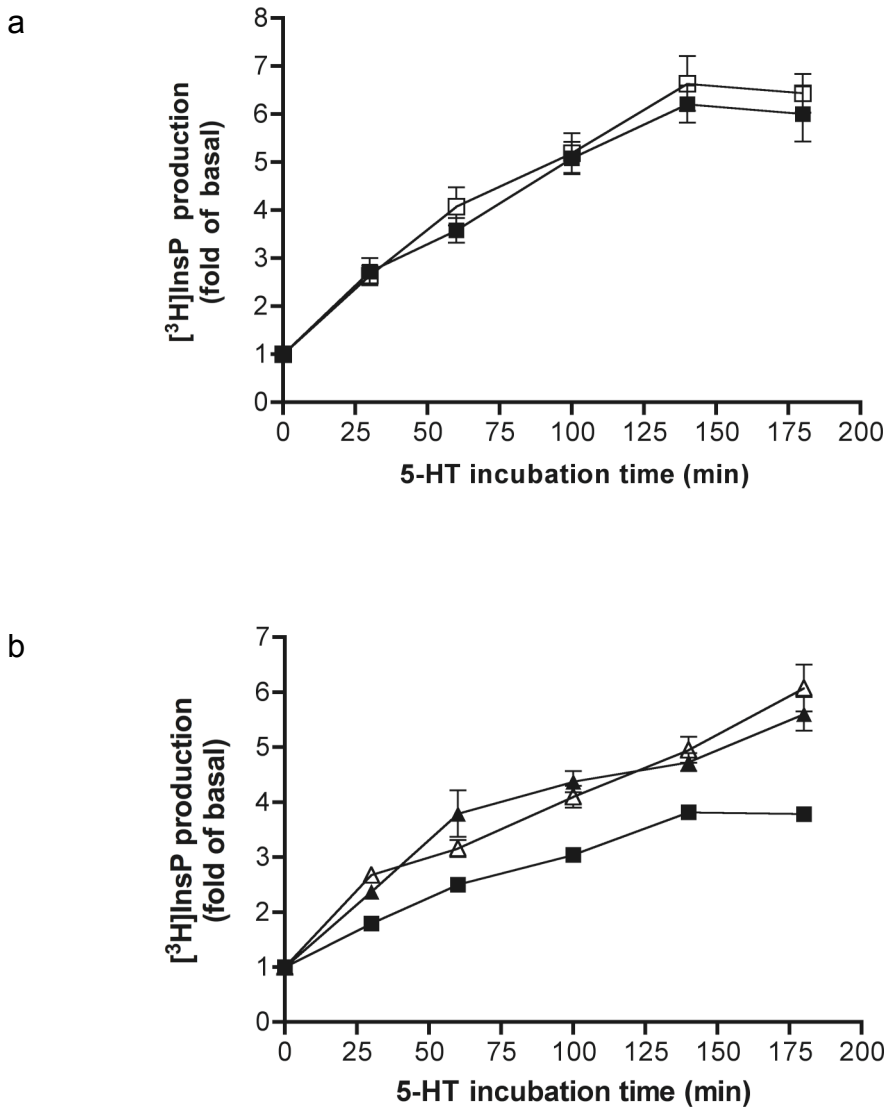


Figure 4.1 Time-dependent 5-HT-stimulated PLC responses of (a) COS7 cells and (b) C6 glioma cells transfected with wild-type HA-5-HT_{2A} R or H452Y HA-5-HT_{2A} R. Graphs illustrate the 0-180 minute time-course of 10 μM 5-HT-stimulated PLC responses. (a) shows COS7 cells transfected with HA-tagged wild-type-5-HT_{2A}R (■) or HA-tagged H452Y-5-HT_{2A}R (□). (b) shows C6 glioma cells (which endogenously express 5-HT_{2A}R), transfected with $pcDNA_{3.1}$ vector (■) (as control), wild-type 5-HT_{2A}R (▲), or H452Y variant 5-HT_{2A}R (▽). PLC activity is measured as [^{3}H]inositol phosphate ([^{3}H]InsP) production. Values are means ± SEM, n = 3 and 4, in (a) and (b) respectively. No significant differences between responses of the wild-type and H452Y variants were detected by Wilcoxon test.

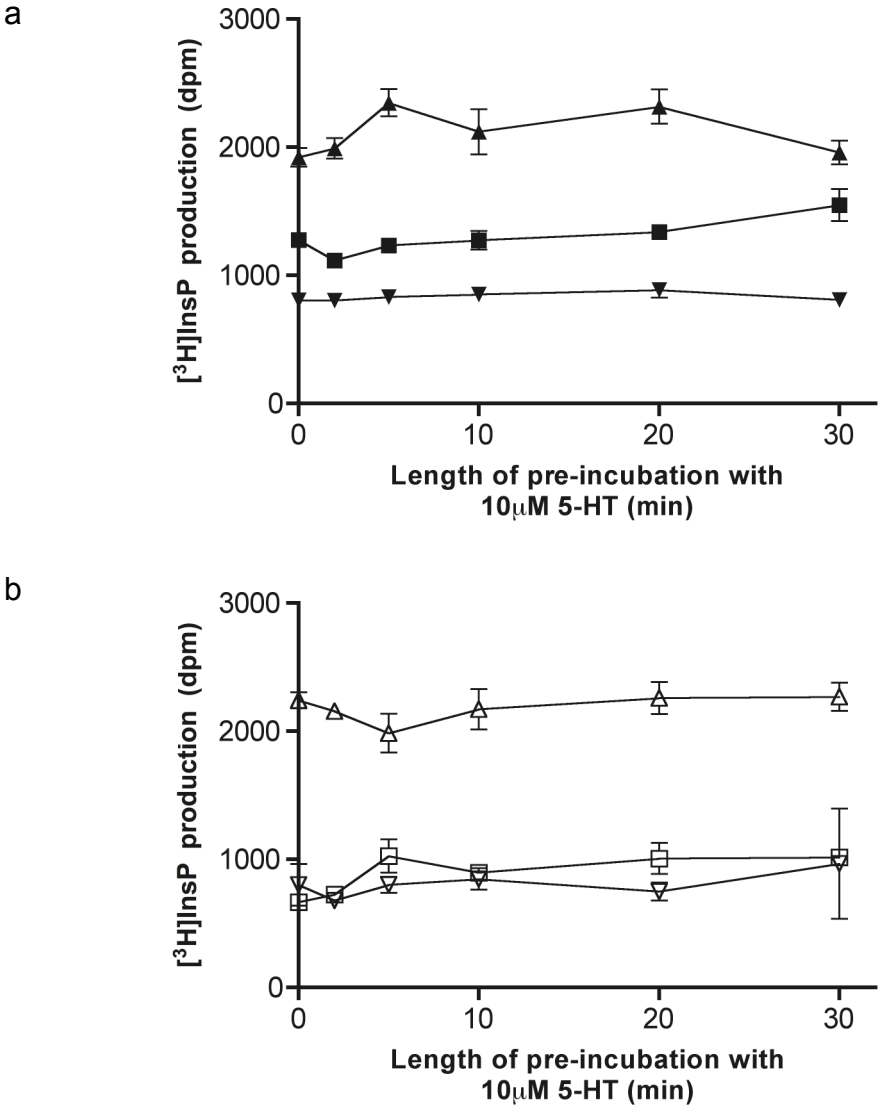


Figure 4.2 PLC responses of COS7 cells transfected with HA-5-HT$_{2A}$R or H452Y HA-5-HT$_{2A}$R that had been pre-stimulated with 10 μM 5-HT for up to 30 min. PLC responses are shown of COS7 cells transfected with either (a) wild-type or (b) H452Y variant 5-HT$_{2A}$R. Prior to assay, cells were pre-incubated for 0-30 min with 10 μM 5-HT in addition to 10 μM pargyline (which inhibits 5-HT breakdown). Subsequently, after washing and addition of lithium, cells were incubated for 30 min with 10 μM 5-HT (▲ or △ respectively), 1 μM ritanserin (5-HT$_{2A}$R antagonist) for 30 min (▼ or ▽ respectively), or not stimulated at all (■ or □ respectively) with 10 μM pargyline present in each case. PLC activity is measured as [^{3}H]InsP production (dpm per sample). Values are means ± SEM, n = 4.

To further investigate the desensitisation profiles of wild-type- and H452Y-5-HT_{2A}R-mediated PLC responses with respect to potential involvement of arrestins, COS7 cells were co-transfected with complements of wild-type-5-HT_{2A}R plus $pcDNA_{3.1}$ or β-arrestin 1 (Figure 4.3a) and H452Y-5-HT_{2A}R plus $pcDNA_{3.1}$ or β-arrestin 1 (Figure 4.3b). The time-course of PLC activation was investigated to a maximum 180 min 5-HT-incubation. β-arrestin 1 was not found to induce any significant alteration in the time-course of wild-type- or H452Y-5-HT_{2A}R-mediated PLC responses, with all maximum responses (at 180 min time point) being very similar and lying within the range of 5.54 ± 0.26 - 6.47 ± 0.06 fold of basal responses.

COS7 cells were additionally assayed to determine the potential effects of the β-arrestin 2 isoform on wild-type- and H452Y-5-HT_{2A}R-mediated PLC responses. COS7 cells were co-transfected with complements of wild type 5-HT_{2A}R plus $pcDNA_{3.1}$ or β-arrestin 2 (Figure 4.4a), and H452Y-5-HT_{2A}R plus $pcDNA_{3.1}$ or β-arrestin 2 (Figure 4.4b). Again, the time course of PLC activation was investigated to a maximum 180 min 5-HT incubation. β-arrestin 2 was not found to induce any significant alteration in wild-type 5-HT_{2A}R-mediated PLC responses from 0-180 min with maximum values lying between 6.1 ± 0.36 and 6.5 ± 0.39 fold of basal. However, the addition of β-arrestin 2 was found to evoke a significant decrease in H452Y-5-HT_{2A}R-mediated PLC responses at 100-180 min 5-HT stimulation ($p<0.05$ by Wilcoxon test), with maximum wild-type- and H452Y-5-HT_{2A}R-mediated PLC responses reaching 6.83 ± 0.22 and 4.67 ± 0.07 fold of basal respectively.

COS7 cells expressing wild-type-5-HT_{2A}R plus control $pcDNA_{3.1}$ vector, or β-arrestin 2 (Figure 4.5a), and H452Y-5-HT_{2A}R plus control $pcDNA_{3.1}$ vector or β-arrestin 2 (Figure 4.5b), were further investigated to assess PLC responses throughout a range of 5-HT concentrations, from 1-10,000 nM. β-arrestin 2 did not evoke any discernable change in the 5-HT-induced PLC responses of wild type 5-HT_{2A}R-expressing COS7 cells, with EC_{50} values for 5-HT_{2A}R plus $pcDNA_{3.1}$ transfected cells and 5-HT_{2A}R plus β-arrestin 2 transfected cells of 8.36 ± 0.44 nM and 10.02 ± 0.98 nM respectively and corresponding Emax values of 3.89 ± 0.79 fold of basal and 3.66 ± 0.38 fold of basal respectively. However, H452Y-5-HT_{2A}R-expressing COS7 cells co-transfected with β-arrestin 2 demonstrated significantly decreased levels of 5-HT-induced PLC activity compared to $pcDNA_{3.1}$ controls. Levels of PLC activity elicited by 5-HT concentrations 100-10,000 nM from H452Y-5-HT_{2A}R plus β-arrestin2-expressing cells were found to be significantly less than control, (* $p<0.05$ by Wilcoxon test), with maximum wild-type and H452Y-5-HT_{2A}R-mediated [^{3}H]InsP production reaching 4.07 ± 0.54 and 2.83 ± 0.32 fold of basal, respectively. EC_{50} values for H452Y-5-HT_{2A}R plus $pcDNA_{3.1}$ and H452Y-5-HT_{2A}R plus β-arrestin 2-expressing cells were not significantly different, being 9.44 ± 0.65 nM and 8.98 ± 0.68 nM respectively.

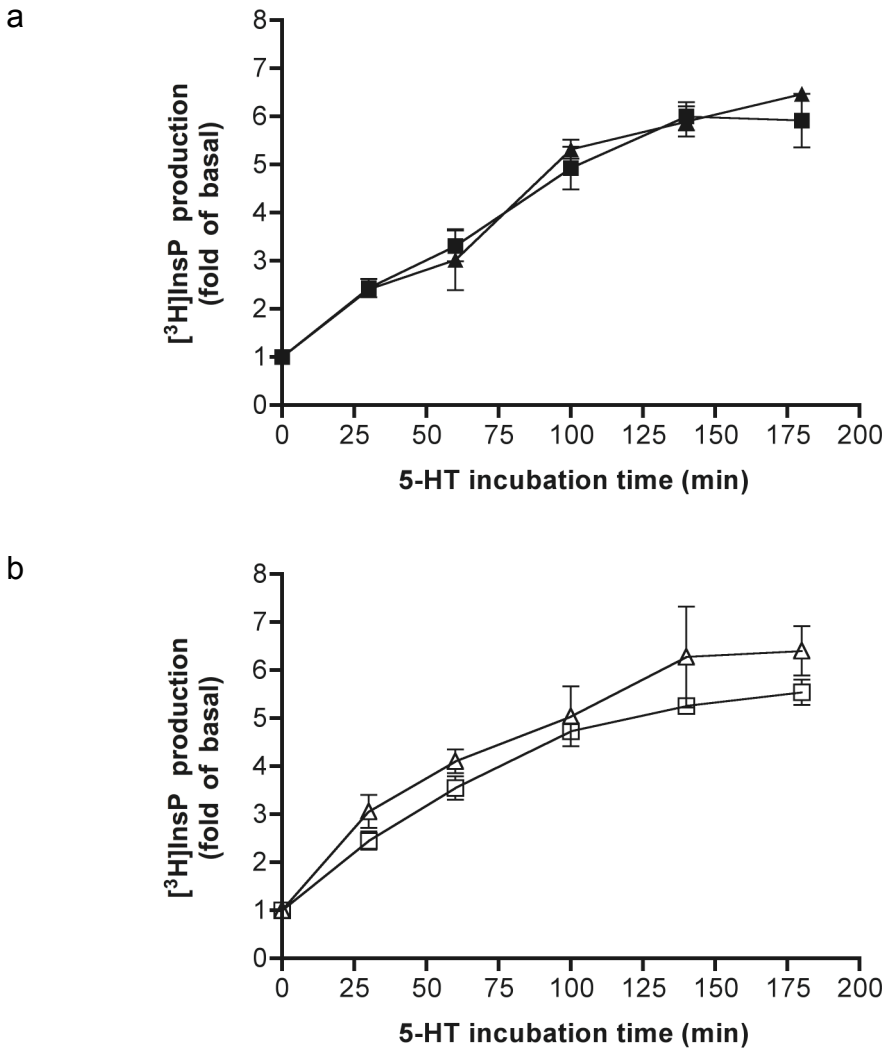


Figure 4.3 5-HT-induced PLC responses of COS7 cells co-transfected with wild type or H452Y variants of HA-5-HT$_{2A}$R and β-arrestin 1, or empty plasmid as a control. PLC responses are shown of COS7 cells transfected with HA-tagged wild-type (a) or H452Y (b) variants of the 5-HT$_{2A}$R together with β-arrestin 1 (▲ or △ respectively) or pcDNA$_{3.1}$ vector as a control (■ or □ respectively). Cells were stimulated with 10 μM 5-HT for 0-180 min. PLC activity is measured as [^{3}H]InsP production. The points plotted represent means ± SEM, n = 4. No significant effects of β-arrestin 1 were detected.

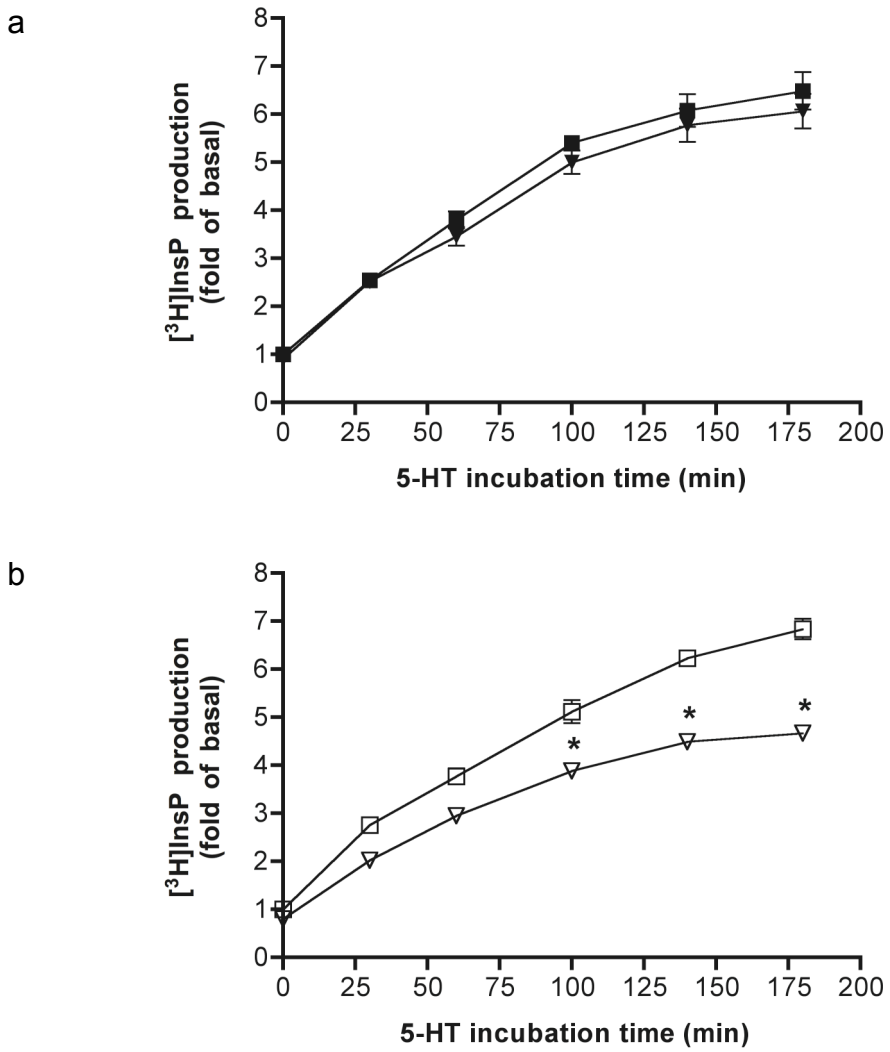


Figure 4.4 5-HT-induced PLC responses of COS7 cells co-transfected with wild type or H452Y variants of HA-5HT$_{2A}$R and β-arrestin 2, or empty plasmid as a control. PLC responses are shown of COS7 cells co-transfected with HA-tagged wild-type (a) or H452Y (b) variants of the 5-HT$_{2A}$R plus β-arrestin 2 (▼or ▽ respectively) or pcDNA$_{3.1}$ vector as a control (■ or □ respectively). Cells were stimulated with 10 μM 5-HT for 0-180 min. PLC activity is measured as [^{3}H]InsP production. β-arrestin 2 caused a significant decrease in the H452Y-5-HT$_{2A}$R signalling response between 100-180 min agonist-incubation, despite conferring no alteration to wild-type 5-HT$_{2A}$R-mediated PLC responses, with asterisks denoting significance (* $p < 0.05$, Wilcoxon test). The points plotted represent means ± SEM, n = 4.

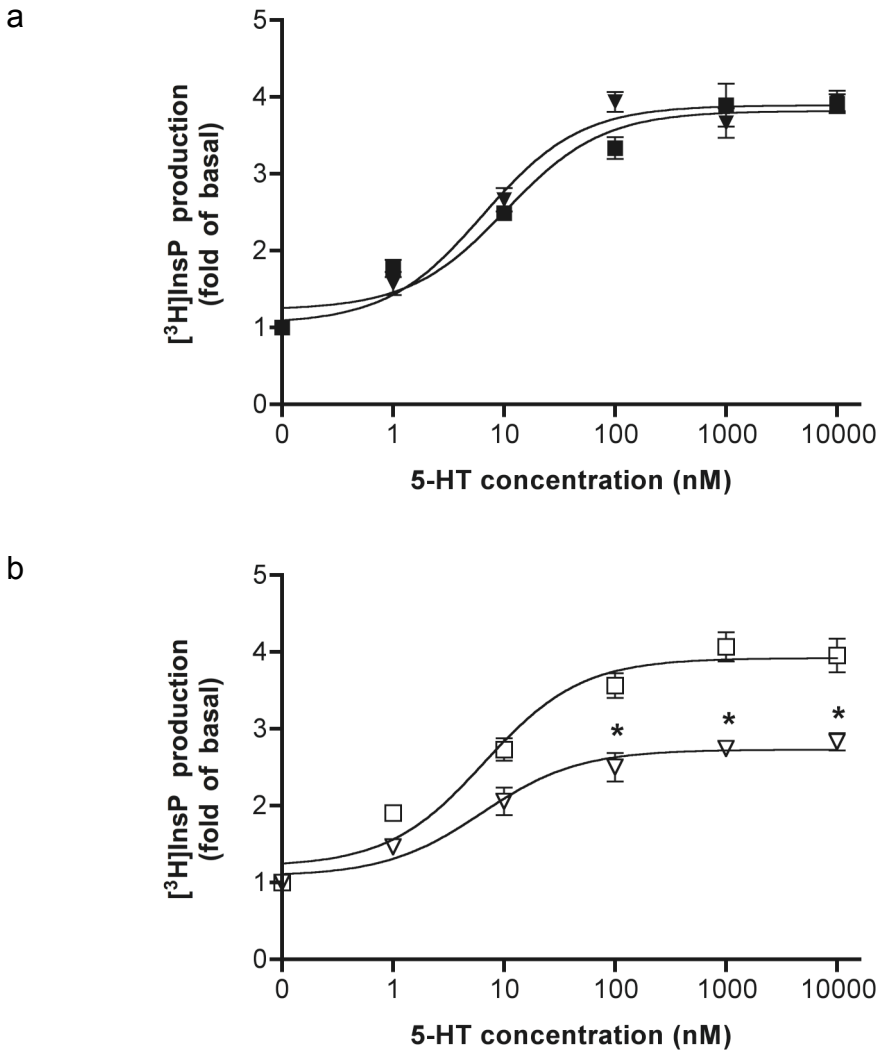


Figure 4.5 Effect of β-arrestin 2 on the concentration-dependence of 5-HT-induced PLC activation in COS7 cells transfected with wild-type or H452Y variant 5-HT$_{2A}$R. PLC responses of COS7 cells transfected with HA-tagged wild-type (a) or H452Y (b) variants of the 5-HT$_{2A}$R together with β-arrestin 2 (▼or ▽ respectively) or pcDNA$_{3.1}$ vector as a control (■ or □ respectively). Cells were stimulated with 1-10,000 nM 5-HT for 180 min. PLC activity is measured as [^{3}H]InsP production. β-arrestin 2 caused a significant decrease in the H452Y-5-HT$_{2A}$R signalling response between 100-10,000 nM despite conferring no alteration to wild-type 5-HT$_{2A}$R-mediated PLC responses, with asterisks denoting significance (* $p < 0.05$, Wilcoxon test). All values are the means ± SEM, n = 3.

4.2.2 Arrestin interaction with the 5-HT_{2A}R

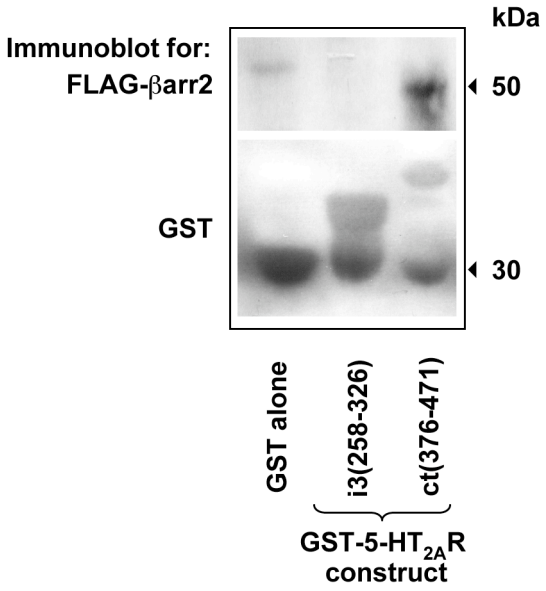
It has been previously reported that β-arrestin 2 (like β-arrestin 1 and visual arrestin) associates with the third intracellular loop of the 5-HT_{2A}R (5-HT_{2A}Ri3) (186). However, the C-terminally located H452Y polymorphism was observed to influence the effects of β-arrestin 2 on PLC signalling. We thus investigated potential physical interactions of FLAG-tagged β-arrestin 2 with GST-fusion proteins of both the third intracellular loop of the wild-type receptor (GST-5-HT_{2A}Ri3) and the carboxy terminal tail of the wild-type receptor (GST-5-HT_{2A}Rct) (Figure 4.6a). In addition, we further compared interactions of FLAG-tagged β-arrestin 2 with GST-5-HT_{2A}Rct and the corresponding H452Y variant 5-HT_{2A}R construct (GST-H452Y-5-HT_{2A}Rct) (Figure 4.6b). GST-receptor constructs were incubated with lysates from β-arrestin 2 overexpressing COS7 cells, before being immobilized on glutathione Sepharose-4B beads and the captured proteins separated by gel electrophoresis. FLAG-β-arrestin 2 bands were visualised using rabbit polyclonal anti-FLAG (Affinity Bioreagents) and HRP-linked anti-rabbit secondary antibody (Chemicon), and GST inputs were detected with mouse monoclonal anti-GST antibody (Clone B-14, Santa Cruz) and HRP-linked anti-mouse secondary antibody (Chemicon). The results of Figure 4.6a indicate that FLAG-β-arrestin 2 binds with a greater affinity to the C-terminal tail of the receptor construct (GST-5-HT_{2A}Rct) (third lane), when compared to the third intracellular loop of the receptor construct (GST-5-HT_{2A}Ri3) (middle lane), despite similar or lower input levels of the c-terminus construct (running at approximately 40 kDa and 36 kDa respectively for c-terminal and i3 constructs). Furthermore, the introduction of the H452Y substitution to the GST-fusion protein construct of the 5-HT_{2A}Rct appears to confer a marked increase in binding of FLAG-β-arrestin2

(Figure 4.6b), despite similar input levels of constructs, which is consistent with the functional studies descried above.

In an attempt to further investigate the cellular association of β-arrestin 2 with the 5-HT_{2A}R and H452Y-5-HT_{2A}R, COS7 cells were co-transfected with either wild-type HA-tagged 5-HT_{2A}R or HA-H452Y-5-HT_{2A}R and FLAG-β-arrestin 2, stimulated with 10 μM 5-HT for 30 min, or not stimulated at all, before receptor immunoprecipitation with mouse monoclonal anti-HA antibody (12CA5) (2.4 μg/μl), or non-immune mouse IgG (2.4 μg/μl) as control (Figure 4.7). Following the separation of immunoprecipitated proteins by gel electrophoresis, the amount of FLAG-β-arrestin 2 associated with the receptor pull-downs was assessed using rabbit polyclonal anti-FLAG (Affinity Bioreagents) and HRP-linked anti-rabbit antibody (Chemicon). Receptor input was determined with the use of rat monoclonal HRP-linked anti-HA antibody (Roche, clone 3F10). FLAG-β-arrestin 2 bands (lower panel in Figure 4.7) could not be visualised due to the presence of heavy IgG antibody fragment bands which often appear at both ~25kDa and ~50kDa due to non-specific interactions encountered with particular detection antibodies. These results were typical of 2 independent experiments. Thus in an attempt to eliminate the non-specific IgG bands, the same co-immunoprecipitation was performed, but using anti-HA antibody that was previously covalently conjugated to the Protein G Sepharose beads, as outlined in section 2.9 (Figure 4.8). Again, despite an obvious decrease in bands at ~50kDa when using similarly conjugated non-immune IgG as a control, FLAG-β-arrestin 2 could not be clearly visualised. It was considered likely that available concentrations of pull-down antibody were decreased as a result of

inefficient conjugation to the Protein G beads and thus did not capture sufficient HA-5-HT_{2A}R or HA-H452Y-5-HT_{2A}R to provide detectable levels of associated FLAG-β-arrestin 2.

a



b

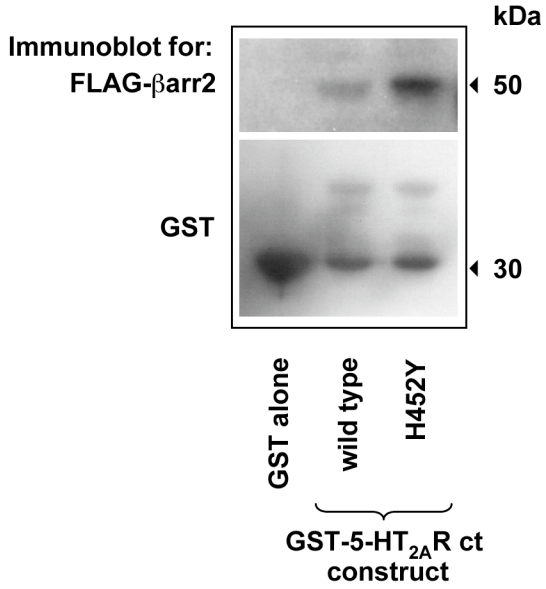


Figure 4.6 β-arrestin 2 binding to GST-fusion proteins of wild-type- and H452Y-5-HT$_{2A}$R domains. In (a) GST-fusion proteins of the 5-HT$_{2A}$R carboxy terminal tail (ct) and the third intracellular loop (i3) as well as unconjugated GST (running at approximately 40 kDa, 36 kDa and 29 kDa respectively) were used as potential bait for proteins in lysates of COS7 cells transfected with FLAG-tagged β-arrestin 2. FLAG-β-arrestin 2 was found to preferentially bind to the 5-HT$_{2A}$Rct over the 5-HT$_{2A}$Ri3. In (b) FLAG-β-arrestin 2 binding was compared between GST-fusion proteins of the wild-type 5-HT$_{2A}$Rct, the corresponding H452Y construct and GST alone. The H452Y substitution conferred a marked increase in the binding of FLAG-β-arrestin 2 by the receptor. These results were typical of 3 independent experiments.

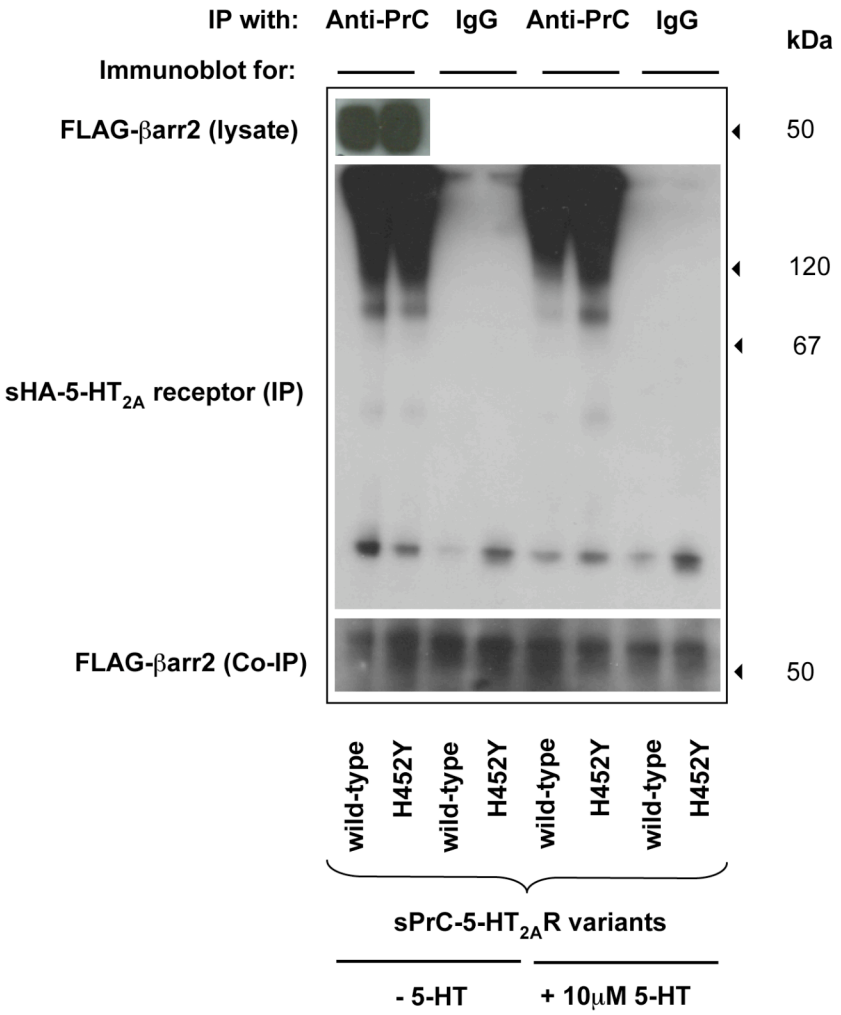


Figure 4.7 Investigation of co-immunoprepcipitation of β-arrestin 2 with wild-type HA-5-HT$_{2A}$R and HA-H452Y-5-HT$_{2A}$R. COS7 cells were co-transfected with HA-tagged wild type or H452Y variant 5-HT$_{2A}$ receptor and FLAG-tagged β-arrestin 2. Cells were either not stimulated or stimulated with 10 μM 5-HT for 30 min. Anti-HA tag antibody was used to immunoprecipitate the receptor. Non-immune mouse IgG was used as a control. The figure shows levels of FLAG-β-arrestin 2 pulled down with both variants of receptor (Co-IP), levels of HA-5HT$_{2A}$ receptors recovered in the immunoprecipitates (IP) and FLAG-β-arrestin 2 inputs (lysate). The FLAG antibody used to detect receptor-associated FLAG β-arrestin 2 unfortunately showed substantial non-specific binding, probably to an interaction with IgG heavy chain which would run close to the position expected for arrestins.

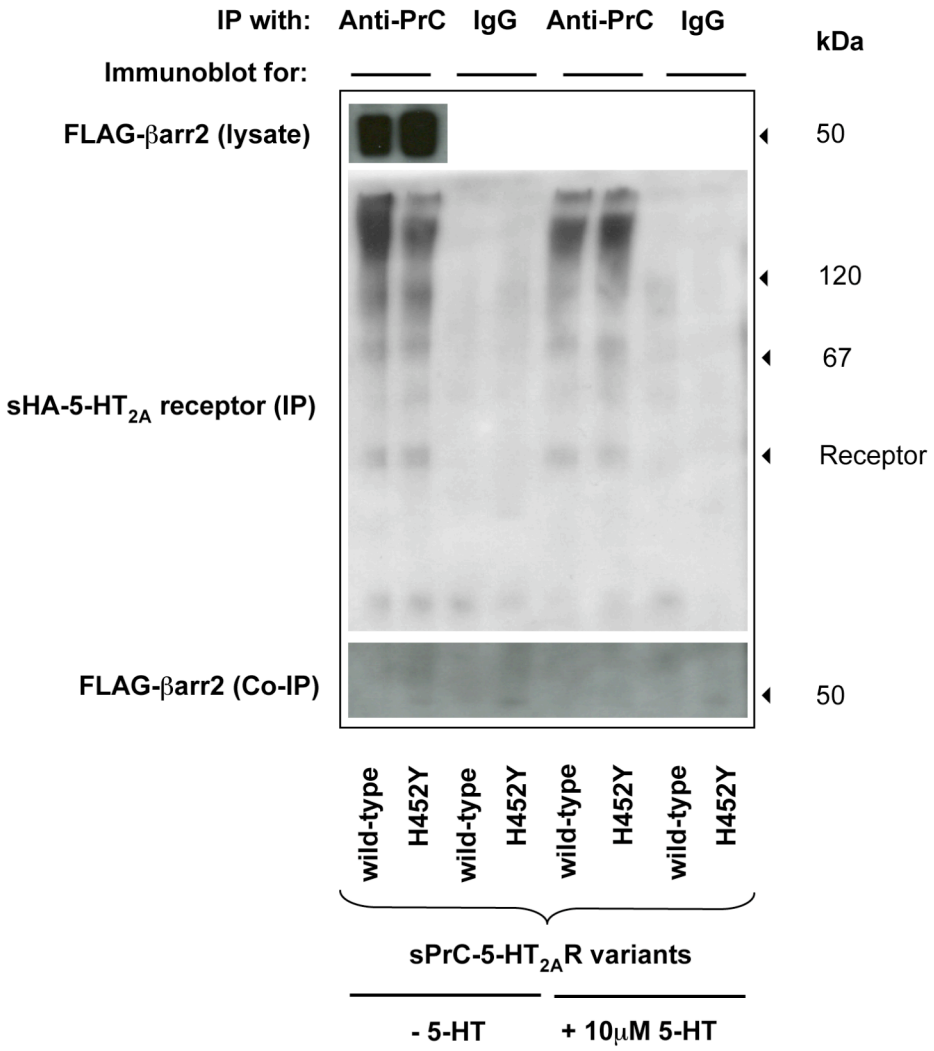


Figure 4.8 Investigation of co-immunoprepcipitation of β-arrestin 2 with wild type HA-5-HT$_{2A}$R and HA-H452Y-5-HT$_{2A}$R using HA-antibody conjugated to Protein G-Sepharose beads. COS7 cells were co-transfected with HA-tagged wild-type or H452Y variant 5-HT$_{2A}$R and FLAG-tagged β-arrestin2. Cells were either not stimulated or stimulated with 10 μM 5-HT for 30 min. Anti-HA tag antibody was conjugated to Protein G-Sepharose beads and used to immunoprecipitate the receptor. Similarly conjugated non-immune mouse IgG was used as a control. The figure shows levels of FLAG-β-arrestin 2 pulled down with both variants of receptor (Co-IP), levels of HA-5HT$_{2A}$R recovered in the immunoprecipitates (IP) and FLAG-β-arrestin 2 inputs (lysate). Unfortunately FLAG-β-arrestin 2 could not be clearly detected in the receptor pull-downs.

4.2.3 Investigation of the potential significance of residue Ser 453 in the 5-HT_{2A}R

The H452Y polymorphism is situated adjacent to Ser 453, a potential GRK phosphorylation site. To investigate whether this site is involved in the functional influence of the H452Y substitution, new mutant variants of the 5-HT_{2A}R were generated that substituted an Ala (A) for the usual Ser (S) 453, to produce S453A-5-HT_{2A}R and H452Y-S453A-5-HT_{2A}R. To assess any functional impact of the new mutation, COS7 cells were co-transfected with either wild-type -5-HT_{2A}R, S453A-5-HT_{2A}R, or H452Y-5-HT_{2A}R or H452Y-S453A-5-HT_{2A}R and the time-course of 5-HT-induced PLC activation was assessed. Figure 4.9a illustrates the PLC responses of wild-type-5-HT_{2A}R- and S453A-5-HT_{2A}R-expressing cells stimulated with 10 μM 5-HT for between 0-180 min. It can be seen that the S453A mutation does not have any discernable effect upon 5-HT_{2A}R-mediated PLC activity, with the maximum PLC responses of 5-HT_{2A}R and S453A-5-HT_{2A}R reaching 8.70 ± 0.22 and 7.69 ± 0.61 fold of basal respectively. Figure 4.9b illustrates PLC responses of H452Y-5-HT_{2A}R- and H452Y-S453A-5-HT_{2A}R-expressing cells stimulated with 10 μM 5-HT for between 0-180 min. In contrast, the S453A mutation does appear to confer a significant increase to the time-dependent PLC responses of the H452Y variant 5-HT_{2A}R between time-points 100-180 min, with the maximum PLC response of the H452Y-5-HT_{2A}R reaching 7.00 ± 0.21 fold of basal, but that of the H452Y-S453A5-HT_{2A}R reaching 10.97 ± 0.33 fold of basal. This difference was found to be statistically significant ($p<0.05$ by Wilcoxon test). The influence of the S453A mutation was further assessed on the concentration-dependence of PLC responses (Figure 4.10a & b). COS7 cells were transfected with wild-type -5-HT_{2A}R, S453A-5-HT_{2A}R, or H452Y-5-HT_{2A}R or H452Y-S453A-5-HT_{2A}R and stimulated with 1-

10,000 nM 5-HT for 180 min. Again, as illustrated in Figure 4.10a, the S453A mutation did not have any discernable effect upon 5-HT$_{2A}$R-mediated PLC activity with the maximum PLC response of the 5-HT$_{2A}$R and S453A-5-HT$_{2A}$R reaching 4.28 ± 1.87 and 4.33 ± 0.21 fold of basal, respectively. In contrast, in Figure 4.10b, PLC responses of the H452Y-S453A-5-HT$_{2A}$R reached a maximum of 8.11 ± 1.45 fold of basal and were found to be significantly increased at concentrations 100-10,000 nM over those of the H452Y-5-HT$_{2A}$R, which demonstrated maximum PLC responses of 4.71 ± 0.31 fold of basal. There was no marked difference between EC_{50} values, which all lay within the range 9.23 ± 1.67 nM and 24.34 ± 2.21 nM respectively ($p<0.05$ by Wilcoxon test).

We then asked whether the S453A mutation might disrupt the inhibitory effect of β-arrestin 2 on PLC responses that is apparent only in the setting of the H452Y variant 5-HT$_{2A}$R. Figure 4.11 illustrates the PLC responses of COS7 cells co-transfected with either the H452Y-5-HT$_{2A}$R or the H452Y-S453A-5-HT$_{2A}$R and pcDNA$_{3.1}$ or β-arrestin 2, and stimulated with increasing concentrations of 5-HT (1-10,000 nM). Again H452Y-5-HT$_{2A}$R-mediated PLC responses were enhanced in the case of the S453A mutation (as in Figure 4.10b) and β-arrestin 2 acted to decrease H452Y-5-HT$_{2A}$R-mediated PLC responses (as in Figure 4.5b). The addition of β-arrestin 2 also resulted in a significant decrease of S453A-H452Y-5-HT$_{2A}$R-mediated PLC responses at 5-HT concentrations 10-10,000 nM, resulting in an approximate 46% loss of 5-HT-induced PLC responses and a maximum response value of 3.75 ± 0.60 fold of basal compared to 7.8 ± 0.25 fold of basal in the absence of β-arrestin 2 ($p<0.05$ by Wilcoxon test).

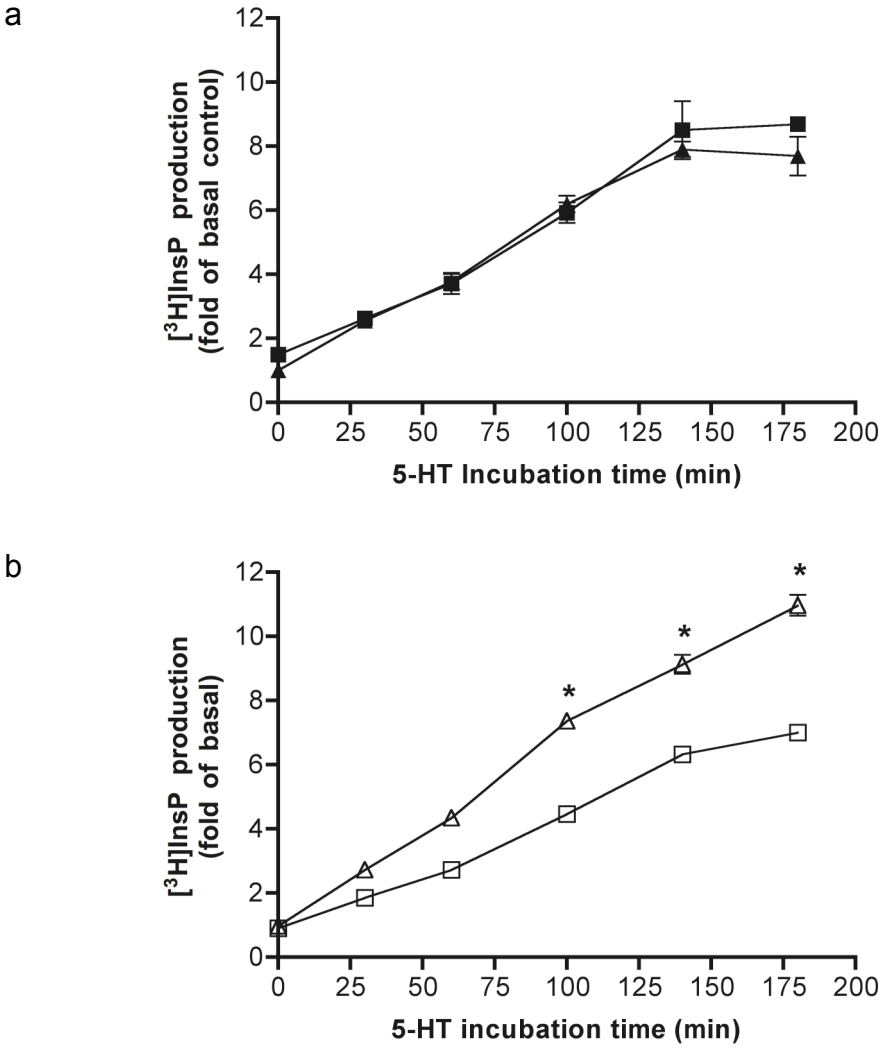


Figure 4.9 5-HT-induced PLC activation (time-course) in COS7 cells transfected with wild type, S453A, H452Y or S453A-H452Y variants of HA-5HT$_{2A}$R. Time-course of 5-HT-induced PLC activation in COS7 cells transfected with HA-tagged wild-type (■) or S453A (▲) variants of the receptor (a) and H452Y (□) or S453A-H452Y (△) variants (b). Cells were stimulated with 10 μM 5-HT for 0-180 min. PLC activity is measured as [^{3}H]InsP production. PLC responses of the S453A-H452Y variant 5-HT$_{2A}$R were found to be significantly greater than for H452Y variant controls at time-points 100-180 min. Significance denoted by * ($p<0.05$ by Wilcoxon test). The points plotted represent means ± SEM, n = 4.

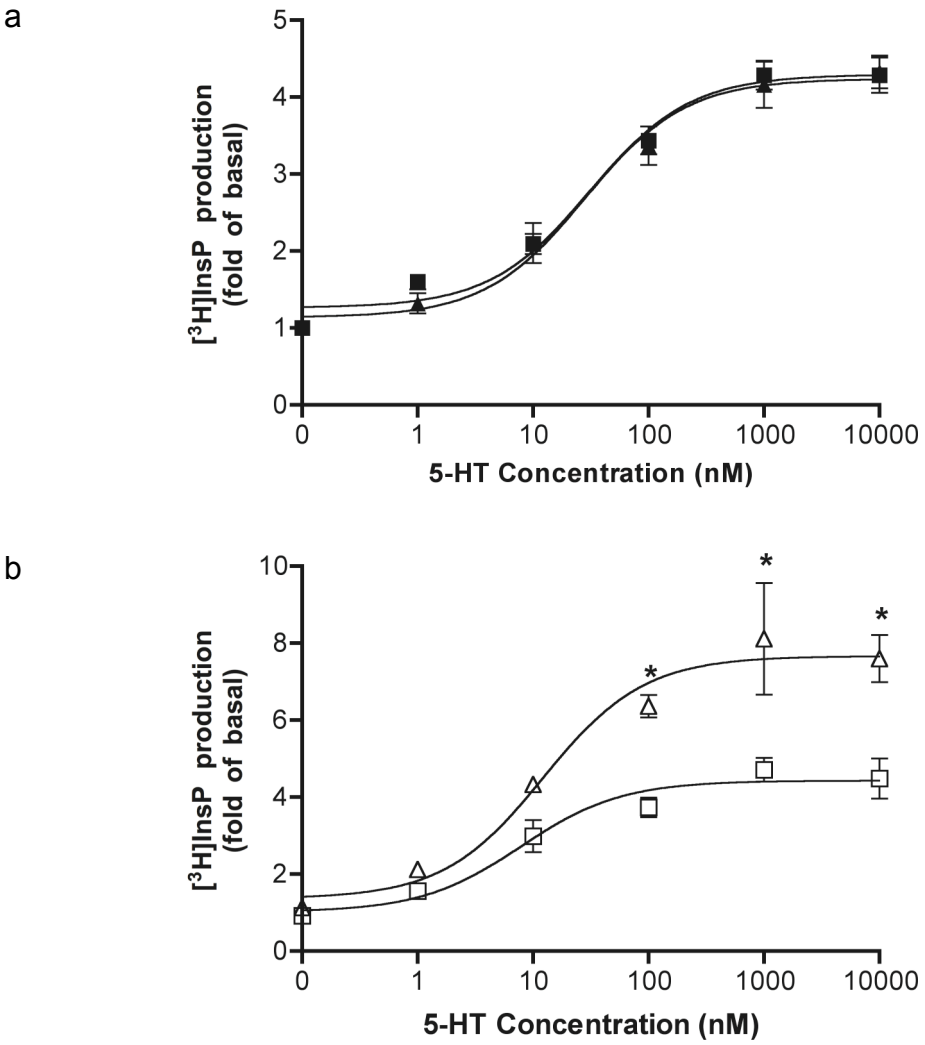


Figure 4.10 5-HT-induced PLC activation (concentration-dependence) in COS7 cells transfected with wild-type, S453A, H452Y or S453A-H452Y variants of HA-5HT$_{2A}$R. Concentration-dependence of 5-HT-induced PLC activation in COS7 cells transfected with HA-tagged wild-type (■) or S453A (▲) variants of the 5-HT$_{2A}$R (a) and H452Y (□) or S453A-H452Y (△) variants (b). Cells were stimulated with 1-10,000 nM 5-HT for 180 min. PLC activity is measured as [^{3}H]InsP production. PLC responses of the S453A-H452Y variant of the 5-HT$_{2A}$R were found to be significantly greater than for H452Y variant controls at concentrations 100-10,000 nM. Significance denoted by * ($p<0.05$ by Wilcoxon test). The points plotted represent means ± SEM, n = 3.

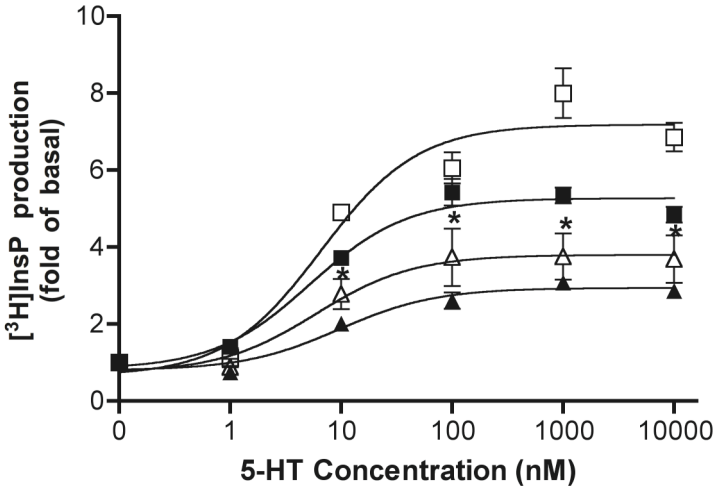


Figure 4.11 Concentration-dependence of 5-HT-induced PLC activation in COS7 cells co-transfected with H452Y or S453A-H452Y variants of HA-5HT$_{2A}$R and pcDNA$_{3.1}$ or β-arrestin 2. PLC responses of COS7 cells co-transfected with HA-tagged H452Y-5-HT$_{2A}$R and pcDNA$_{3.1}$ (■), HA-H452Y-5-HT$_{2A}$R and β-arrestin 2 (▲), HA-H452Y-S453A-5-HT$_{2A}$R and pcDNA$_{3.1}$ (□) or HA-H452Y-S453A-5-HT$_{2A}$R and β-arrestin 2 (△). Cells were stimulated with 1-10,000 nM 5-HT for 180 min. PLC activity is measured as [^{3}H]InsP production. PLC responses in cells transfected with the HA-H452Y-S453A-5-HT$_{2A}$R variant and β-arrestin 2 were found to be significantly less than those in cells co-transfected with the HA-H452Y-S453A-5-HT$_{2A}$R and pcDNA$_{3.1}$ at concentrations 10-10,000 nM. Significance denoted by * ($p<0.05$ by Wilcoxon test). The points plotted represent means ± SEM, n = 3.

To determine that the differences in PLC responses observed were not simply a consequence of differences in expression of 5-HT$_{2A}$R variants, COS7 cells were transfected with either the wild-type, H452Y, S453A, or H452Y-S453A receptor variant and assayed for levels of receptor binding. In a pilot study, specific 5-HT$_{2A}$R ligand-binding was determined by the incubation of membranes from appropriately transfected COS7 cells with the selective 5-HT$_{2A}$R ligand [^{3}H]ketanserin, in the presence/absence of excess mianserin, to define non-specific binding. Table 4.1 shows total and non-specific binding was at approximately similar levels for each of the four receptor variants suggesting that they were likely to be expressed at similar numbers of receptors.

	[^{3}H]ketanserin binding (dpm/assay)			
	Wild-type 5-HT$_{2A}$R	**H452Y 5-HT$_{2A}$R**	**S453A 5-HT$_{2A}$R**	**H452Y/S453A 5-HT$_{2A}$R**
Total	437 ± 51	515 ± 42	432 ± 36	399 ± 44
Non-specific	198 ± 21	174 ± 20	215 ± 17	178 ± 23

Table 4.1 Ligand binding to wild type, H452Y S453A and H452Y-S453A variant constructs of the 5-HT$_{2A}$R. Levels of expression of wild-type, H452Y, S453A and H452Y-S453A variant 5-HT$_{2A}$R were assessed in a pilot study by binding of the selective 5-HT$_{2A}$R ligand [^{3}H]ketanserin (0.8 nM) in the absence/presence of excess 10 μM mianserin, to define non-specific binding. Values are means ± SEM, n = 5. There were no discernable differences between the receptor constructs in either total or non-specific binding.

4.2.4 5-HT_{2A}R activation of ERK MAP kinase, potentially downstream of arrestins

Arrestins are well established to act as scaffolds for the components of signalling complexes, thereby facilitating the activation of various signalling molecules, including ERK 1/2. In a pilot study, we therefore assessed the time-course of 5-HT-stimulated ERK activation in COS7 cells transfected with either wild-type 5-HT_{2A}R, or H452Y-5-HT_{2A}R. Cells were stimulated with 10 μM 5-HT for 0-120 min before lysis in Laemmli buffer, separation of proteins by SDS-PAGE and Western blot. ERK phosphorylation was subsequently assessed with the use of polyclonal rabbit anti-phospho ERK 1/2 antibody (Cell Signalling Technologies) and HRP-linked anti-rabbit antibody (Cell Signalling Technologies) or polyclonal rabbit anti-pan ERK 1/2 antibody (Cell Signalling Technology) and HRP-linked anti rabbit antibody (Cell Signalling Technologies). In these preliminary findings wild-type-5-HT_{2A}R-mediated ERK activation was found to peak at approximately 30-60 min (Figure 4.12a). However, H452Y-5-HT_{2A}R-mediated ERK activation appeared to be more robust and remained elevated from 10 min up to 120 min following 5-HT addition (Figure 14.12b). Further work would be required to fully substantiate these preliminary findings.

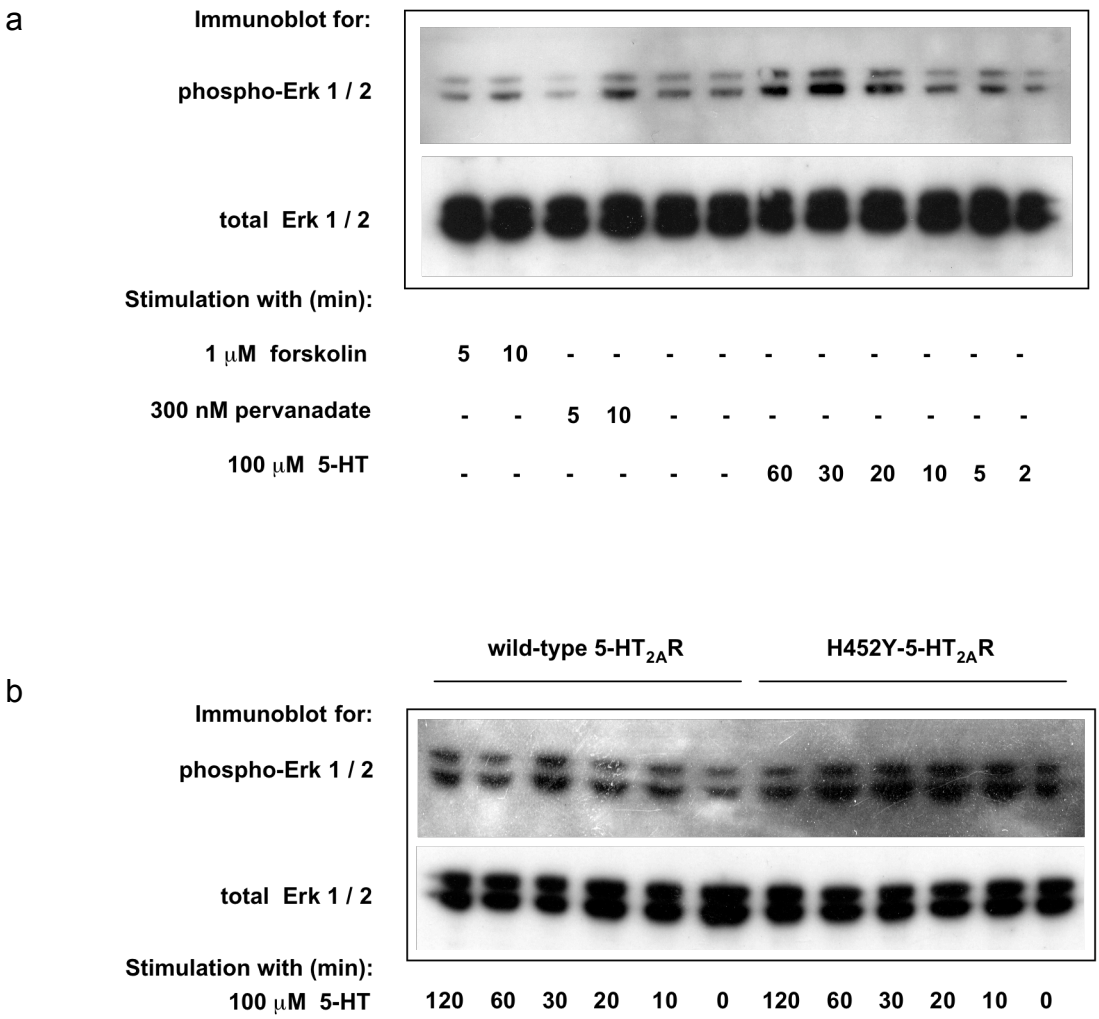


Figure 4.12 Time-course of 5-HT-induced ERK1/2 phosphorylation by the wild-type 5-HT$_{2A}$R and the H452Y-5-HT$_{2A}$R. COS7 cells were transfected with the wild-type 5-HT$_{2A}$R (a) and either wild-type or H452Y-5-HT$_{2A}$R (b) and stimulated with 10 μM 5-HT for periods of time between 0-120 min. 1 μM forskolin and 300 nM pervanadate were used as positive controls. Proteins were separated by SDS-PAGE and Western blot and ERK activation assessed with the use of phospho-ERK and pan-ERK antibodies.

4.3 Discussion

The first indication that there may be some change in the regulation of PLC signalling in the H452Y variant of the 5-HT_{2A}R came from experiments on Ca^{2+} mobilisation in platelets from heterozygous patients in which 5-HT-induced Ca^{2+} responses were attenuated or curtailed (308). These findings were followed up by Hazelwood et al, who determined the H452Y-5-HT_{2A}R variant of the receptor to exhibit a significantly reduced ability to activate PLC in NIH3T3 cells (309). To further investigate the possible underlying basis for this we initially examined the time-course of [^{3}H]InsP accumulation in COS7 cells expressing either wild-type-5-HT_{2A}R or H452Y-5-HT_{2A}R and stimulated with 5-HT. Both receptor types were found to demonstrate a delayed time-dependent attenuation of the rate of [^{3}H]InsP accumulation at 5-HT incubation times of >140 min. This appears to be generally in line with time-dependent desensitisation of PLC activity demonstrated by other class A receptors, such as the angiotensin II receptor and the FP prostaglandin $F_{2\alpha}$ receptor, have been shown to desensitise between ~ 90 and >120 min ref (462,463). However, our current studies showed there to be no discernable differences in the time-course of wild-type- or H452Y-5-HT_{2A}R-mediated PLC activation. Thus we repeated the assay in C6 glioma cells which endogenously express the 5-HT_{2A}R, and have previously been demonstrated to rapidly desensitise following agonist-pre-incubation (128). Endogenous 5-HT_{2A}R-mediated PLC responses were again found to desensitise between ~ 140-180 min and again there were no discernable differences in the time-courses of PLC responses mediated by wild-type- or H452Y-5-HT_{2A}R additionally expressed in these cells (although desensitisation of these responses was less marked).

To more directly assess any early phase of 5-HT-induced desensitisation of phospho-inositide hydrolysis that may not have been clearly identifiable in the time-course assay, we examined wild-type- and H452Y-5-HT_{2A}R-mediated phospho-inositide hydrolysis in COS7 cells that had been pre-incubated with 5-HT for periods of up to 30 min. Again, in contrast to findings in NIH3T3 cells in which the H452Y-5-HT_{2A}R demonstrated a significantly altered desensitisation profile to the wild-type-5-HT_{2A}R (309), we found that in COS7 cells the H452Y substitution did not appear to confer any significant alteration to receptor desensitisation of PLC responses. Hazelwood et al described the wild-type-5-HT_{2A}R to exhibit a biphasic mode of desensitisation, demonstrating a loss of ~ 40% phospho-inositide hydrolysis with 10 min 5-HT pre-treatment whereas the H452Y-5-HT_{2A}R desensitised in a monophasic fashion, demonstrating a significantly altered loss of only ~ 25% phosphoinostide hydrolysis with 10 min 5-HT pre-treatment but apparently being already in a partially desensitised state (309). Our results in COS7 cells, however, did not indicate any discernable loss of responses following pre-incubation with 5-HT for between 0-30 min. 5-HT_{2A}R desensitisation has been reported in various cell models, including in PS200 hamster fibroblast cells, in which desensitisation was found to occur relatively slowly, with an initial loss of ~ 25% [H^3]InsP formation by 12 minutes, and an ~ 50% loss of response by 1 hour (454) and in HEK293 cells stimulated with quipazine, in which phospho-inositide hydrolysis was determined to decrease by ~ 20% at 10 min, ~ 40% at 1 h and ~ 60 % at 240 min of agonist pre-treatment (130). Studies examining the endogenous expression of β-arrestins in a variety of cell-types determined COS7 cells to express very low levels of endogenous β-arrestin, the

lowest of all cell-types studied, and HEK293 cells the highest (although Gray et al report arrestin-independent 5-HT$_{2A}$R desensitisation in HEK293 cells) (130)), which could be a factor in the differing 5-HT$_{2A}$R desensitisation profiles (464).

Due to the very low endogenous levels of β-arrestin expression in COS7 cells we investigated the possible role of β-arrestin 1/β-arrestin 2 by co-transfecting these with wild-type or H452Y-variant 5-HT$_{2A}$R in order to assess their influence on PLC responses. Although β-arrestin 1 had no effect upon wild-type- or H452Y-5-HT$_{2A}$R-mediated [^{3}H]InsP production, β-arrestin 2 induced a significant decrease in both time-dependent and dose-dependent H452Y-5-HT$_{2A}$R-mediated PLC responses. It was confirmed by Western blot that both arrestin isoforms were being adequately expressed. In addition to arrestins preferentially binding particular GPCRs over others (465), many GPCRs have been shown to selectively bind specific arrestin isoforms, and the arrestin isoform-specificity is thought to be largely dependent on C-terminal residues (437,438). Our studies do not demonstrate any isoform-dependent β-arrestin effect on wild-type-5-HT$_{2A}$R-mediated [^{3}H]InsP production, but do suggest that the H452Y substitution confers selectivity for the β-arrestin 2 isoform. To investigate this effect further we assessed the physical interaction of the β-arrestin 2 isoform with the receptor. Although previous studies have demonstrated an isoform-non-selective binding of arrestins to the 5-HT$_{2A}$Ri3 they did not investigate binding to the 5-HT$_{2A}$Rct (186). When using GST-fusion proteins of the 5-HT$_{2A}$Ri3 and 5-HT$_{2A}$Rct domains, we found that β-arrestin 2 bound with clearly much greater affinity to the 5-HT$_{2A}$Rct. Furthermore, GST-fusion proteins of wild-type- and H452Y-5-HT$_{2A}$Rct were used to compare levels of captured β-arrestin 2

and identified interaction with the H452Y-5-HT$_{2A}$Rct to be distinctly greater than that with wild-type-5-HT$_{2A}$Rct. Attempts to investigate these interactions in a cellular setting by co-immunoprecipitation and Western blot were not successful due to the proximity of non-specific IgG bands (~ 50 kDa) to β-arrestin 2 bands (~ 52 kDa), thereby obscuring any β-arrestin 2 protein which may have been immunoprecipitated.

Thus these data suggest that the presence of the H452Y substitution results in both increased β-arrestin 2 binding by the 5-HT$_{2A}$Rct, and the conferment of β-arrestin 2-dependent "desensitisation" to 5-HT$_{2A}$R-mediated PLC responses. The H452Y polymorphic residue is adjacent to a Ser residue (S453), a potential site for Ser/Thr kinases such as GRKs. Putative phosphorylation sites required for desensitisation in the wild-type-5-HT$_{2A}$R were previously determined by sequential mutagenesis of all 5-HT$_{2A}$R intracellular Ser/Thr residues, with the finding that only Ser 188 and Ser 421 were thought to be involved, in HEK293 cells at least (455). However, the consensus targeting sites for GRKs 1, 2 and 3 are thought to often involve serine/threonine residues in acidic environments (466), and residues 454 and 455 of the 5-HT$_{2A}$R (adjacent to S453) are both glutamate. We hypothesised that the presence of a tyrosine at position 452 in place of the usual histidine makes the adjacent serine more susceptible to phosphorylation by Ser/Thr kinases. Single point mutagenesis was used to generate S453A 5-HT$_{2A}$R constructs and assess any evidence for a role of this residue in desensitisation/attenuation of PLC signalling by β-arrestin 2. The new mutation was not found to affect wild-type-5-HT$_{2A}$R-mediated PLC responses, although it appeared to significantly increase H452Y-5-HT$_{2A}$R-

mediated PLC activity. Whether this might be due to attenuation of an event contributing to endogenous desensitisation of PLC signalling by this receptor variant is a possibility, but could not be explicitly proved in the present study. Radio-ligand binding showed that the affinity and number of binding sites were very similar between the wild-type and H452Y-5-HT$_{2A}$R (Chapter 3) and that addition of the S453A mutation to these did not appear to modify the levels of expression of these constructs. However, the addition of β-arrestin 2 resulted in a significant decrease in H452Y-S453A-5-HT$_{2A}$R-mediated PLC activity below control H452Y-S453A-5-HT$_{2A}$R responses, suggesting that the increase in H452Y-S453A-5-HT$_{2A}$R-mediated PLC responses in the absence of β-arrestin 2 is not subsequent to the loss of a Ser/Thr kinase site which may be a GRK target and a resulting resistance to β-arrestin 2. These results suggest that increased susceptibility of the H452Y variant receptor to β-arrestin 2 is potentially a direct result of the H452Y mutation, as opposed to its influence on the putative Ser/Thr kinase target site at residue 453.

β-arrestins are well established to act as scaffolds for MAP kinase signalling complexes, thereby facilitating agonist-induced activation of MAP kinases. Thus in pilot studies we assessed time-course of 5-HT-induced ERK 1/2 phosphorylation in both wild-type- and H452Y-5-HT$_{2A}$R-expressing COS7 cells. The preliminary findings appeared to indicate that H452Y-5-HT$_{2A}$R-mediated ERK 1/2 phosphorylation did appear to be both stronger and to occur earlier. This could possibly be as a result of increased β-arrestin 2 interactions with the H452Y variant receptor, hence increased scaffolding of MAP kinase signalling components. These observations would be consistent with the idea that the H452Y substitution leads not

only to enhanced binding of β-arrestin 2 at the receptor, and desensitisation-like attenuation of PLC signalling, but may also facilitate arrestin-dependent signalling. Extensive further study with a range of techniques; including siRNA knockdown of endogenous arrestin isoforms, would be needed to establish and reveal the extent of such a pathway.

CHAPTER 5.0

5.0 Influence of Ca^{2+}-binding proteins on 5-HT_{2A}R-mediated signalling

5.1 INTRODUCTION

An independent proteomic study performed in the lab resulted in the identification of several previously non-recognised 5-HT_{2A}Rct binding partners which have been further investigated here. In that study, GST-fusion protein constructs of the 5-HT_{2A}Rct were incubated with rat brain cytosol, and specifically bound proteins were separated by SDS-PAGE and identified using Matrix-Assisted Laser Desorption/Ionization-Time-Of-Flight (MALDI-TOF) mass spectrometry. Two such detected proteins were the Ca^{2+}-binding proteins annexin A2 and S100B.

5.1.1 Annexin family

Ca^{2+} binding proteins are classified on the basis of the structure of their Ca^{2+} binding-domains, into EF-hand proteins, C2-domain proteins and annexin-related, or type-II Ca^{2+} binding-site proteins (467). The annexin superfamily is sub-categorized into family A (vertebrate annexins), family B (invertebrate annexins), family C (fungi and unicellular eukaryote annexins), family D (plant annexins) and family E (protist annexins) (467,468). The vertebrate annexin family comprises 12 annexin proteins, annexins A1-A11 and A13 (469). All vertebrate annexin proteins are composed of a conserved C-terminal protease-resistant core, and a divergent amino-terminal region. The N-terminus varies widely in both size and amino acid composition and is thought to influence function, thereby conferring specificity within the annexin family (470). The Ca^{2+}/phospholipid binding C-terminal core domain is composed of four 70-75 residue homologous repeats (except in annexin A6, which contains eight)

(469,471-473). X-ray crystallographic data suggests that in the case of annexin A2, each of the four repeating regions comprises five α-helices that are arranged into similar structures (472). Additionally, hydrophobic interactions occur between residues of the repeating units and the core domain forms a tightly packed, curved disc, to produce what is known as the 'annexin fold' (472). The concave side of the annexin core, which is orientated away from the membrane, forms interactions with the N-terminal domain and cytosolic signalling molecules (471,474). The convex side of the disc, which faces the membrane, contains binding sites for actin, phosphoinositide and Ca^{2+} (471,474). The binding of Ca^{2+} by annexin, creates a bridge between the negatively charged carbonyl/carboxyl groups of annexin and phosphoryl groups of membrane phospholipids, thereby facilitating the reversible association of the annexin core domain at the negatively charged phospholipid membrane surface (475). This Ca^{2+}-induced bridging mechanism may facilitate further direct annexin-phospholipid interactions that contribute to membrane binding (476).

5.1.2 Annexin A2

Annexin A2 exists both as a soluble monomeric form (annexin A2 ~ 36kDa), and as a heterotetramer (annexin A2t ~ 90 kDa), that is composed of two annexin A2 molecules and a dimer of the annexin binding partner and regulatory subunit S100A10 (alternatively known as p11) (477). p11, an 11kDa EF-hand protein, forms a Ca^{2+}-independent interaction with the N-terminal region of annexin A2, to form this heterotetramer consisting of a central p11 dimer, linked to two annexin protomers (478). Formation of annexin A2t enables an increased association of the

annexin A2 amino-terminus with the membrane, allowing annexin A2 to aggregate membranes at micromolar Ca^{2+} concentrations (467,479). Annexin monomers and complexes are involved in a diverse array of physiological processes, many of which are attributed to their ability to bind phospholipids in a Ca^{2+}-dependent manner. Annexins typically associate with negatively-charged phospholipids, but annexin A2 is known to specifically interact with the plasma membrane component phosphatidylinositol 4,5-bisphosphate (PtdIns 4,5-P_2) (480,481). The affinity of annexin A2 association with the membrane is also reguated by cholesterol and pH; annexin A2 can undergo cholesterol-mediated association with endosomal membranes in a Ca^{2+}-independent manner (482), and the annexin A2 monomer has been demonstrated to form Ca^{2+}-independent plasma membrane interactions at mild acidic pH (pH levels 6.6-6.8), whilst at the same pH, annexin A2t appears to undergo conformational changes comparable to those induced by Ca^{2+} (483). Furthermore, annexin A2 undergoes modification by both Tyr and Ser/Thr kinases. Nicotine stimulation has been demonstrated to promote PKC-mediated annexin A2 phosphorylation in chromaffin cells(484), and annexin A2 has additionally been shown to undergo insulin-triggered tyrosine phosphorylation subsequent to insulin receptor activation(485). The latter phosphorylation event has recently been demonstrated to be important for promoting Rho/Rho kinase-dependent and actin-mediated changes in cell morphology (486). Phosphorylation of annexin A2 regulates its function, reducing its affinity for phospholipids and interfering with the ability of annexin A2t to aggregate chromaffin secretory granules at micromolar Ca^{2+} concentrations (487,488).

5.1.3 Annexin A2 in vesicular trafficking

Annexin A2 is also capable of binding to F-actin and there is strong evidence to support the involvement of annexin proteins in intracellular trafficking (489,490). Annexin A2t is reportedly required for Ca^{2+}-dependent exocytosis and influences the aggregation and fusion of lipid vesicles in chromaffin cells (484,491); it is also reported to induce the formation of plasma membrane cholesterol-rich microdomains (lipid rafts), essential for exocytosis (492,493). The localisation of annexin A2t within plasma membrane lipid rafts that also contain transmembrane receptors such as CD44, Src-related kinases and caveolins indicates that annexin A2 may also play an important role in the recruitment and arrangement of signal transduction components (493,494). Annexin A2t also appears to localize between intracellular secretory granules and the plasma membrane in chromaffin cells, and has been demonstrated to undergo conformational changes to form cross-links between the vesicles and the plasma membrane, following cellular stimulation (495,496). Annexin A2 is additionally found to be associated with endosomes within Baby Hamster Kidney (BHK) cells (497). Furthermore, annexin A2t is implicated in the recycling of receptor complexes such as the transferrin receptor and the epidermal growth factor receptor (EGFR) (498,499), and is demonstrated to be important for the uptake of the transferrin receptor via clathrin-coated pits (498).

5.1.4 Trafficking of ion channels

Additionally, annexin A2 is known to be involved in modulation of ionotropic signalling pathways; it is demonstrated to regulate the functional expression of transient receptor potential calcium channels TRPV5 and TRPV6 in kidney and

intestinal epithelial cells (500,501), the cystic fibrosis transmembrane conductance regulator (CFTR) chloride channel in epithelial cells(502), and volume-activated chloride currents in vascular endothelial cells (503). Furthermore, the annexin A2 binding partner p11 is also implicated in the targeting of several ion channels to the plasma membrane, including the Na_v 1.8 ion channel (504), acid-sensing ion channel (ASIC1a channel) (505), as well as TRPV5 and TRPV6, and was recently identified to act as a mediator in the upregulation of 5-HT_{1B} receptor movement to the plasma membrane surface (506).

5.1.5 Annexin A2 activity at the nucleus

Monomeric annexin A2 is also capable of localising at, and entering the nucleus. It is suggested that monomeric annexin A2 may be involved here together with protein kinases, in regulating DNA replication and cellular proliferation (494). Reports have suggested that it forms part of a primer recognition complex that increases DNA polymerase-α catalytic activity on DNA substrates (507), is able to bind mRNA thereby promoting its association with the cytoskeleton and perinuclear localisation (508), and has been identified to associate with PKCε undergoing phorbol ester-induced translocation to the nucleus in NIH3T3 fibroblast cells (509).

5.1.6 S100B

S100B is a member of the S100/calmodulin/troponin C Ca^{2+}-binding protein superfamily. Members of this family are EF-hand proteins, thus they contain a helix-loop-helix domain, consisting of two α-helices, linked by a short loop region that typically binds Ca^{2+} ions. It has a moleular mass of 21kDa, and exists in various

forms; as a monomer, as a homodimer linked via disulphide bonds, and as a heterodimer attached to the S100A1 protein (510-512). S100B is distributed throughout the central and peripheral nervous systems and is primarily produced by astrocytes (513). It is implicated in a variety of cellular functions, such as cellular communication, growth, signal transduction and energy metabolism (514), but is primarily thought to act as a Ca^{2+} sensor. Following the binding of Ca^{2+} by the four binding sites of the EF hand domain, S100B undergoes a conformational change and exposes key residues involved in interactions with various target proteins (515). This enables the protein to convey signals to intracellular proteins such as the tumour suppressor protein p53 (516), in a Ca^{2+}-dependent manner, (515,517). Furthermore, increased levels of S100B have been associated with neurodegenerative disorders such as Alzheimer's Disease (518) and neuropsychological disorders such as schizophrenia (519).

5.2 Results

5.2.1 Physical interactions of Ca^{2+}-binding proteins with the 5-HT_{2A}R

In order to further assess interactions of the 5-HT_{2A}R with annexin A2, COS7 cells were co-transfected with HA-5-HT_{2A}R and annexin A2, and the receptor was immunoprecipitated in a nominally Ca^{2+}-free environment with anti-HA antibody, or non-immune IgG as a control. Annexin A2 was identified by Western blot analysis with anti-annexin A2 rabbit polyclonal (H-50; Santa Cruz) and polyclonal anti-rabbit HRP-conjugated antibody (Chemicon). It is evident from Figure 5.1 that annexin A2 demonstrates a specific association with the HA tagged-5-HT_{2A}R; annexin A2 pull-down is illustrated in the bottom panel. In lane 1, annexin A2 immunoreactivity is clearly associated with anti-HA antibody-directed immunoprecipitation of the receptor, whereas in lane 2, barely detectable quantities of annexin A2 were co-immunoprecipitated with the control non-immune IgG.

To broadly isolate the region of the receptor that annexin A2 preferentially interacts with (Figure 5.2), rat brain cytosol was incubated with GST-fusion proteins of the 5-HT_{2A}Rct or i3 immobilised on glutathione Sepharose-4B matrix. Following exposure to brain proteins, the samples were washed and bound proteins were eluted from the matrix columns. Annexin A2 was detected and identified by Western blot and antibody analysis. Annexin A2 was found to interact strongly with the C-terminal construct of the receptor, whereas binding to the i3 construct appeared minimal. In both cases the extent of annexin A2 binding was increased in the presence of added 1 mM Ca^{2+}. This would be consistent with the idea that conformational changes within

annexin A2 upon Ca^{2+}-binding may facilitate its binding to the 5-HT_{2A}R, which appears to involve predominantly the c-terminal domain of the receptor.

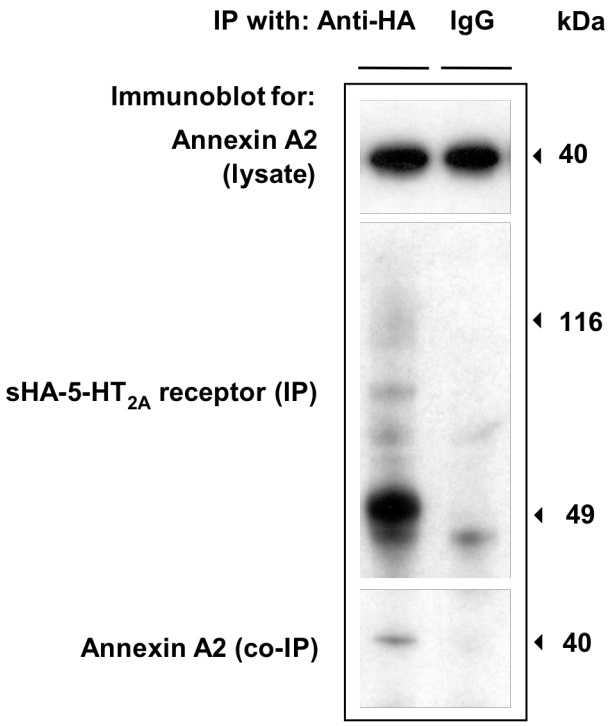


Figure 5.1 Co-immunoprecipitation of annexin A2 with HA-tagged 5-HT_{2A}R. Solubilised extracts from COS7 cells co-transfected with annexin A2 and sHA-5-HT_{2A}R (signal sequence and HA-tagged 5-HT_{2A}R) were co-immunoprecipitated with anti-HA antibody or non-immune IgG control and proteins were visualised by Western blot and enhanced chemiluminescence. Annexin A2 was found to associate specifically with the receptor.

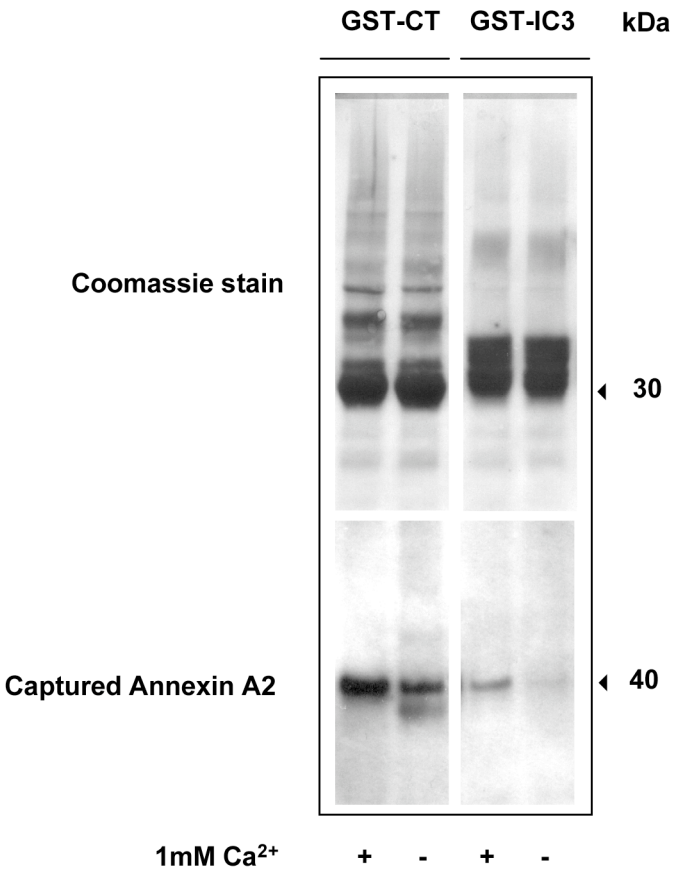


Figure 5.2 Capture of annexin A2 from rat brain cytosol by GST-fusion proteins of the 5-HT_{2A}Rct and i3, in the presence and absence of Ca^{2+}. Annexin A2 captured by GST-fusion proteins of 5-HT_{2A}R domains of interest was revealed by Western blot and antibody analysis. Annexin A2 was found to associate selectively with the c-terminus of the receptor in a Ca^{2+}-facilitated manner (construct ~ 40kDa), to a greater extent than the i3 domain (construct ~36 kDa). This figure was provided by Derek Robertson.

5.2.2 Functional influences of Ca^{2+}-binding proteins on 5-HT_{2A}R signalling

To establish potential functional consequences of 5-HT_{2A}R interactions with annexin A2 (and also S100B), the effects of annexin A2 and S100B upon the conventional signalling pathway of the 5-HT_{2A} receptor, Gq/11-mediated activation of PLC, were investigated in COS7 cells. Figure 5.3a illustrates the effects of annexin A2 and S100B expression upon the time-course of 5-HT_{2A}R-mediated PLC responses to 5-HT. Annexin A2 was found to evoke an increase in 1 μM 5-HT-induced PLC activation which was statistically significant over a time-period of 100-180 min, to approximately 6-fold of basal at 180 min. This represents an increase of approximately 33% in maximal PLC responses at 180 min compared to control responses, which were approximately 4-fold over basal. Conversely, expression of S100B did not cause any discernable changes in PLC responses, with the maximal response to 5-HT at 180 min being within 5% of the control response. The influence of annexin A2 upon PLC responses was further investigated in the presence of the annexin A2 regulatory subunit p11 (Figure 5.3b). Again, expression of annexin A2 induced a significant increase in 5-HT-induced PLC responses compared to control (at 100-180 min). However, co-expression of p11 with annexin A2 did not induce any further increase in PLC activity; in fact, PLC responses appeared slightly (but not significantly) reduced, with maximal PLC responses at 180 min reaching approximately 5.75-fold of basal responses, compared to approximately 6.4-fold of basal PLC activity seen with annexin A2 plus empty vector. Furthermore, transfection of COS7 cells with p11 plus empty vector did not induce any discernable increase in PLC responses over control, with maximal PLC responses at 180 min of approximately 4.85- and 4.72-fold of basal, respectively.

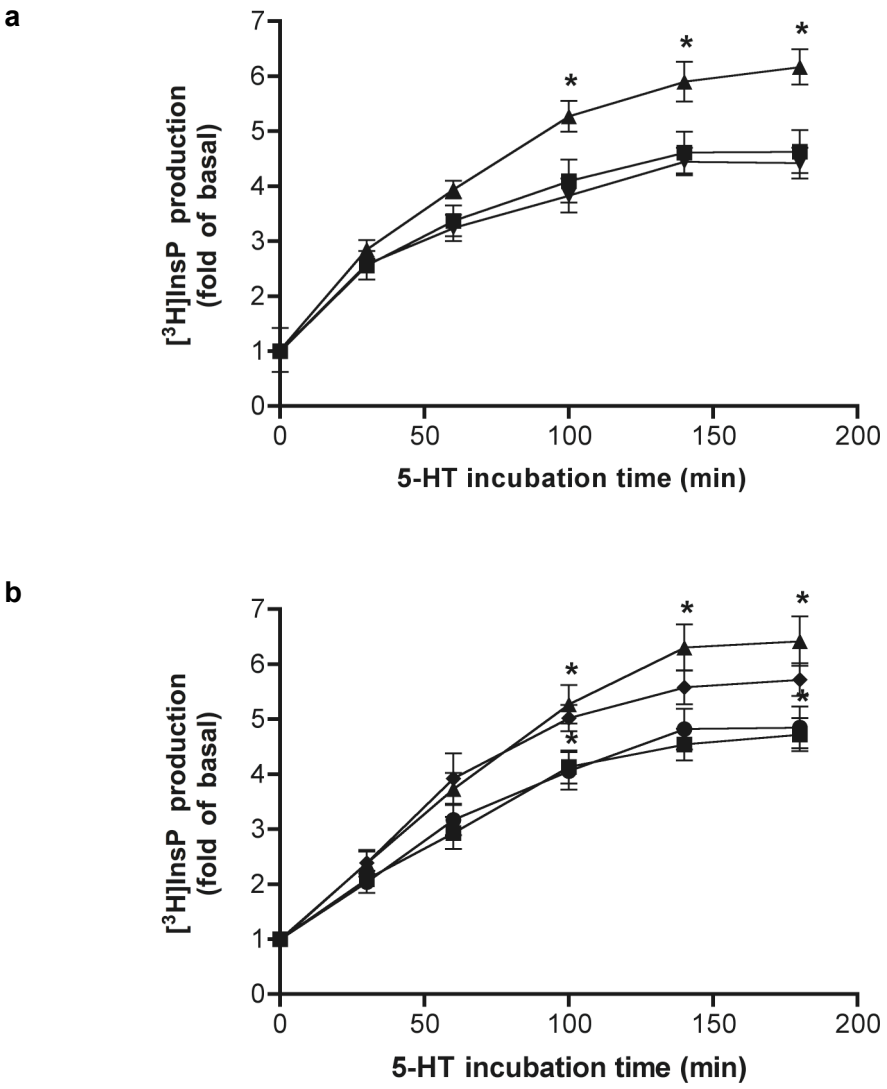


Figure 5.3 Effects of annexin A2, S100B and p11 on the time course of 5-HT PLC activation in COS7 cells transfected with 5-HT_{2A}R. PLC activation responses elicited by 1 μM 5-HT were measured by [^{3}H] labelled inositol phosphate production. **a.** Cells were additionally transfected with empty $pcDNA_{3.1}$ vector (■), as control, annexin A2 (▲), or S100B (▼). Annexin A2, but not S100B caused a significant upregulation of the 5-HT_{2A}R time-dependent signalling response. **b.** Cells were transfected with empty $pcDNA_{3.1}$ vector (■), as control, annexin A2 and $pcDNA_{3.1}$ (▲), annexin A2 and p11 (◆), or p11 and $pcDNA_{3.1}$ (●). p11 failed to elevate PLC responses above control and co-transfection of S100A10 with annexin A2 did not induce any further increase in PLC responses than that evident with annexin A2. All values are the mean ± SEM for n = 4 measurements. (∗ $p < 0.05$, Wilcoxon test).

Further experiments were also carried out in order to evaluate any effects of the Ca^{2+}-binding proteins on the concentration-dependence of 5-HT_{2A}R-mediated PLC activation. COS7 cells overexpressing control $pcDNA_3$ vector, annexin A2 (Figure 5.4a), or S100B (Figure 5.4b) were stimulated with increasing concentrations of 5-HT, from 1 nM - 10,000 nM for 30 min, and PLC responses measured (Figure 5.4a). Annexin A2-expressing COS7 cells demonstrated an increase in the amplitude of the 5-HT concentration-response curve. Levels of PLC activity at 5-HT concentrations 1 nM – 10,000 nM in annexin A2 cells were found to be significantly increased above control ($p<0.001$ by Wilcoxon test in each case). The EC_{50} for 5-HT in 5-HT_{2A}R plus $pcDNA_3$ cells was 4.41 nM ± 1.36 nM, which was slightly, but not significantly, decreased to 2.02 nM ± 0.92 nM with the addition of annexin A2, in the 5-HT_{2A}R plus annexin A2-transfected cells. However, the Emax of 5-HT_{2A}R plus annexin A2 cells was found to be significantly higher, at 3.49 ± 0.18 (fold of basal) than that demonstrated by control cells, at 2.37 ± 0.11 ($p<0.001$ by Wilcoxon test). In contrast, Figure 5.4b shows that S100B-expressing cells did not appear to demonstrate any upregulation of PLC responses. There were no significant changes to either EC_{50} values, or E_{max}, which were 4.73 nM ± 1.40 nM and 6.90 nM ± 1.70 nM, or 2.93 ± 0.15 and 3.04 ± 0.23 fold of basal, respectively.

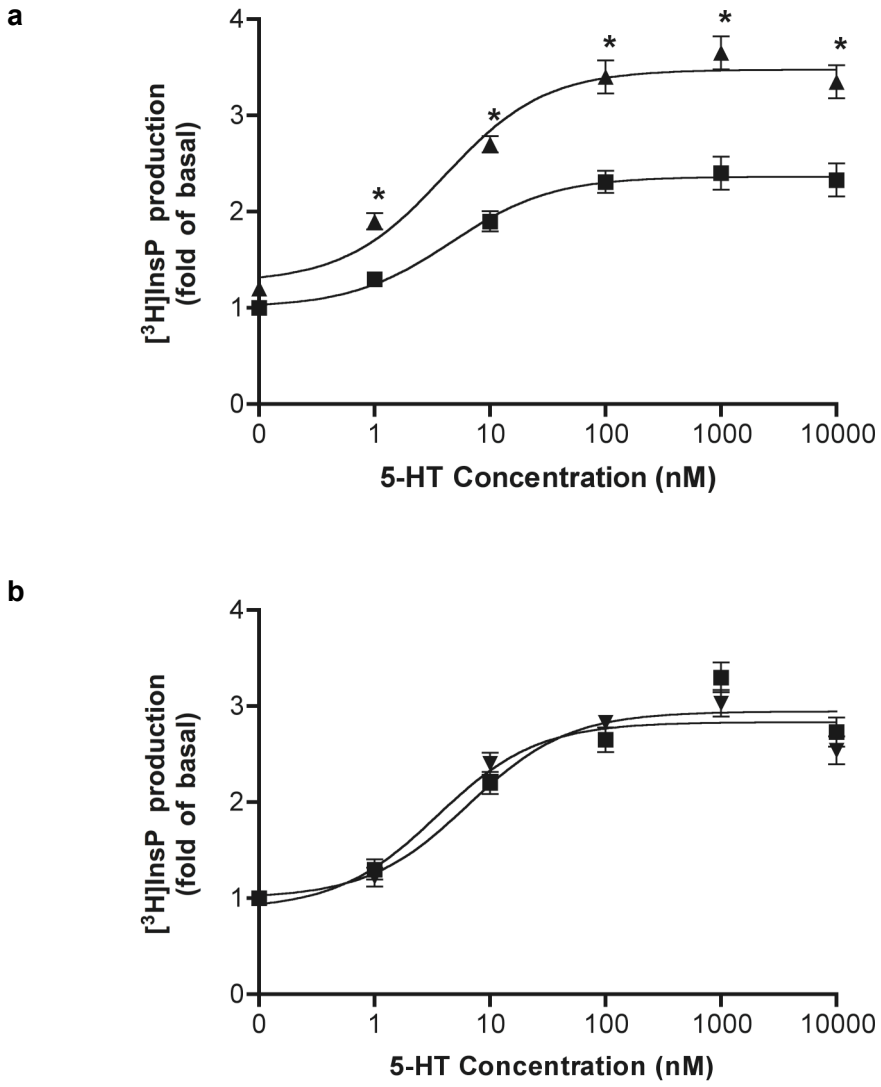


Figure 5.4 Effect of annexin A2 and S100B on the concentration-dependence of 5-HT-induced PLC activation in COS7 cells transfected with the 5-HT_{2A}R. Responses were measured by [^{3}H] labeled inositol phosphate production over 30 min. The cells were transfected with the 5-HT_{2A}R and either empty vector as control (■), annexin A2 (▲) (Figure 5.4a), or S100B (▼) (figure 5.4b). Annexin A2 caused a significant upregulation of the 5-HT_{2A}R signalling response with asterisks denoting significance (∗ $p < 0.001$, Wilcoxon test). S100B had no discernable effect on the concentration-response curve. All values are the mean ± SEM for n = 3 measurements.

5.2.3 Interaction between annexin A2 and arrestin - influences on 5-HT_{2A}R signalling

In Chapter 4 it was shown that β-arrestin 2 expression consistently attenuated 5-HT-induced PLC activation mediated by the H452Y-5-HT_{2A}R, although such an effect was not detectable with the wild-type-5-HT_{2A}R. The enabled influence of β-arrestin 2 in the H452Y variant would be consistent with the idea that β-arrestin 2 may interact with the receptor domain. As the c-terminal domain is also the site of interaction of annexin A2, experiments were carried out to investigate whether there was any functional interaction between the influence of annexin A2 and β-arrestin 2 on PLC responses of the H452Y-5-HT_{2A}R. COS7 cells were triple-transfected with a complement of H452Y-5-HT_{2A}R and either double quantities of $pcDNA_{3.1}$, $pcDNA_{3.1}$ plus annexin A2, $pcDNA_{3.1}$ plus β-arrestin 2, or annexin A2 plus β-arrestin 2, and assessed for PLC responses upon stimulation with 3 μM 5-HT for 180 min (Figure 5.5). The H452Y-5-HT_{2A}R-mediated PLC activity of annexin A2 plus $pcDNA_{3.1}$-expressing cells was found to be significantly greater than control cells, with mean [^{3}H]InsP production (dpm/assay) in response to 5-HT reaching 1322 ± 58, as opposed to 1012 ± 66 for control cells. Expression of β-arrestin 2 resulted in a significant reduction of 5-HT-induced PLC responses to 806 ± 72 (dpm/assay). However, cells expressing receptor, β-arrestin 2 and annexin A2 were still found to yield significantly elevated 5-HT-induced PLC responses with [^{3}H]InsP production activity reaching 1214 ± 54 (dpm/assay) ($p < 0.05$, in each case). The apparent ability of annexin A2 to abrogate the inhibitory influence of β-arrestin 2 on signalling by the H452Y-5-HT_{2A}R would be consistent with the idea that annexin A2 binding to the receptor c-terminal domain may prevent the docking of β-arrestin 2. Interestingly

this would appear to represent a secondary level modulatory influence on signalling that further distinguishes the H452Y variant from the wild-type receptor, but time was not available to extend the investigation.

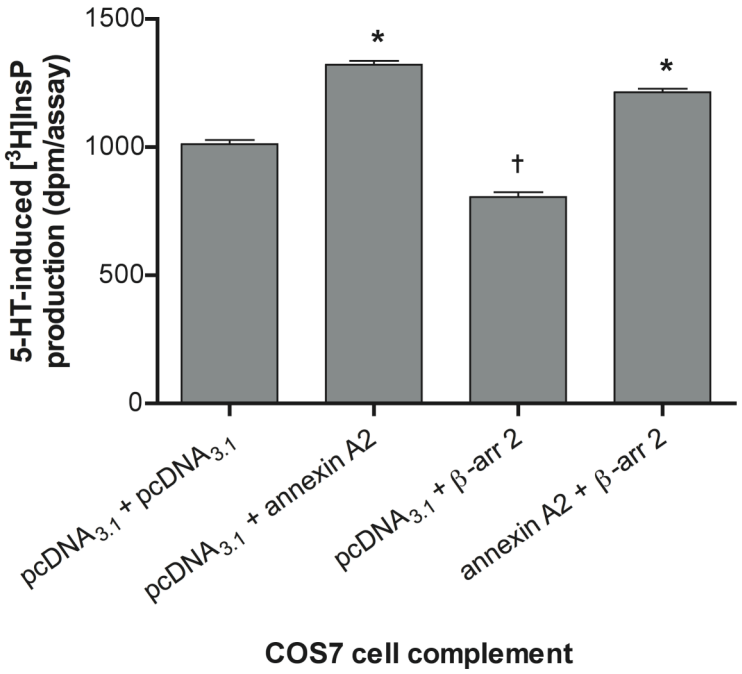


Figure 5.5 5-HT-stimulated PLC responses of COS7 cells co-transfected with the H452Y-5-HT_{2A}R and complements of annexin A2, β-arrestin 2 and $pcDNA_{3.1}$. Bar graph illustrating H452Y-5-HT_{2A}R-mediated PLC responses of COS7 cells triple-transfected with H452Y-5-HT_{2A}R and double quantities of $pcDNA_{3.1}$, or $pcDNA_{3.1}$ plus annexin A2, $pcDNA_{3.1}$ plus β-arrestin 2, or annexin A2 plus β-arrestin 2, and stimulated with 3 μM 5-HT for 180 min. Annexin A2 plus $pcDNA_{3.1}$ cells demonstrated significantly greater PLC responses than control cells, denoted by (* $p < 0.05$, unpaired t-test) and β-arrestin 2 plus $pcDNA_{3.1}$ cells demonstrated significantly decreased PLC responses compared to control cells, denoted by († $p < 0.05$, unpaired t-test). However, annexin A2 and β-arrestin 2 PLC responses were still significantly greater than control, denoted by (* $p < 0.05$, unpaired t-test). All values are the mean ± SEM for n = 4 measurements.

5.3 Discussion

Previous work in the laboratory suggested that annexin A2 physically associates with the c-terminal region of the 5-HT_{2A}R. In the current series of experiments, co-immunoprecipitation studies clearly demonstrated an association with the receptor and this was confirmed by results from GST-fusion protein experiments that suggest that the interaction site (which may or may not involve other proteins) was in the c-terminal domain of the receptor. Another Ca^{2+}-binding protein, calmodulin, has been identified to bind both the 5-HT_{2A}Ri2 and the 5-HT_{2A}Rct between residues 378 to 395, which are positioned close to tm7 and the NPxxY domain (520). It is possible that annexin A2 may bind to a similar region of the receptor ct. The exact annexin A2 binding site could be further investigated with the expression of the 5-HT_{2A}R serially truncated or mutated at the suspected annexin interaction site, and observing the effect on the physical associations and signalling responses of the receptor.

When experiments were carried out to explore any functional impact of this interaction, annexin A2 was demonstrated to act as a novel modulator of 5-HT_{2A}R-mediated PLC activation. However, neither S100B, a further calcium-binding protein found to interact with the 5-HT_{2A}ct, or the annexin A2 binding subunit, p11, induced a similar modulation of 5-HT-induced PLC activation. Evidence for an extensive role of annexin A2 and p11 within cellular trafficking (480), provides support for a putative similar role in the transport of the 5-HT_{2A}R, which could explain the increase in 5-HT-induced signalling that was seen. It is thus possible that annexin A2 could facilitate the expression of the receptor at the plasma membrane, potentially either by anchoring the receptor at the appropriate plasma membrane microdomain or

alternatively, by acting as a chaperone and increasing the delivery of the receptor from the endoplasmic reticulum to the plasma membrane. A subsequent increase in plasma membrane 5-HT_{2A}R expression would thus result in increased 5-HT_{2A}R-mediated signalling, such as an enhancement of PLC activation. However, unlike the present findings, previous studies have determined annexin A2 involvement in receptor membrane targeting and trafficking to be p11-dependent, or to involve the heterotetrameric form of annexin A2 bound to p11 (484,493). The apparent p11-independent annexin A2-mediated increase in PLC signalling could be explained by a reliance upon endogenous levels of p11. If basal p11 levels were sufficient for hetero-tetramer formation, the further addition of p11 may not induce any further facilitation of signalling.

It is also possible that annexin A2 might promote allosteric modulation of the 5-HT_{2A}R, thereby inducing a more active receptor conformation. For instance, the direct interaction of annexin A2 with the receptor could induce conformational changes that improve receptor and Gq/11 coupling, thereby increasing the efficiency of functional receptors at the membrane. A further possibility is that annexin A2 could function as a scaffold for the necessary protagonists of 5-HT_{2A}R-mediated PLC activation: annexin A2t has previously been suggested to act as a scaffold for the CD44 receptor and various signal transduction components when localised within lipid rafts (493). Additionally, annexin A6, which is not a PKCα phosphorylation target (467), has been found to associate with PKCα following PKCα activation and subsequent translocation to lipid raft microdomains (521,522), forming a basis for the hypothesis that annexin A6 may function as a scaffold for PKC (467).

Furthermore, PSD-95, a PDZ-domain binding protein that interacts with the 5-HT_{2A}Rct, has been shown to increase 5-HT_{2A}R-mediated PLC signalling, reportedly by acting as a scaffold to organise the necessary components of the PLC signalling pathway, thereby increasing PLC activity (191). Thus it could be conjectured that by acting in a similar manner, annexin A2/A2t could cause an increase in 5-HT_{2A}R-mediated PLC signal transduction.

As previously mentioned, calmodulin is known to associate with residues 378-395 of the 5-HT_{2A}Rct. Within this region is both a putative PKC target motif (^{384}NKTYR^{388}) (520), and the (^{376}NPxxY^{380}) motif, which as well as being involved in PLD signalling is known to act as an internalisation sequence for many class A GPCRs (47,374,523). It is theoretically possible that association of annexin A2 with the former motif could interfere with PKC association with the receptor, thereby inhibiting PKC-mediated 5-HT_{2A}R phosphorylation and subsequent desensitisation. Such a mechanism could allow for the increased expression of active receptors at the plasma membrane surface. However, annexin A2 appears to increase 5-HT-induced PLC responses at low agonist concentrations, at which point it may be less likely that desensitisation would be playing a major role. The binding of annexin A2 to the 5-HT_{2A}Rct could also theoretically act to hinder NPxxY-dependent internalisation. Although the mechanism by which various GPCRs are dependent upon this motif for efficient internalisation is not yet fully understood, interruption of the sequence by point mutagenesis has been demonstrated to inhibit internalisation (47,523). Thus, shielding of these residues could potentially result in an increase of receptor population at the cell surface. As previously mentioned, the NPxxY motif has

previously been demonstrated to be involved in ARF-mediated activation of PLD (274), thus it would also be of interest to investigate any possible effects of annexin A2 on receptor mediated-PLD responses.

Recent studies demonstrated that p11 acts to chaperone the translocation of the 5-HT_{1B} receptor to the plasma membrane (506). It was also noted that levels of p11 in rodent brain are increased following administration of antidepressants, and decreased in both animal models of depression and in brain tissues taken from patients suffering from depression (506). Furthermore, p11 knockout mice were found to exhibit depression-like behaviour, and following the induction of p11 overexpression in mice, specific behaviours were noted that mimicked those seen following treatment with antidepressants (506). The p11 partner annexin A2 is known to exist within lipid raft fractions of both the hippocampus and the cerebral cortex of rat brain and is putatively involved in learning processes (524). Additionally, as previously discussed, 5-HT_{2A}R activity is also strongly implicated in a wide range of neuropsychiatric disorders. Thus, it is possible that the increase in 5-HT_{2A}R-mediated PLC signalling seen with the addition of annexin A2 could have biological significance to psychotropic roles within the CNS. Annexin A2 is additionally expressed in high levels within lung, intestine, kidney, muscle, epithelial and fibroblast cells (525), and the 5-HT_{2A}R is known to be expressed in various peripheral regions, including platelets where it induces aggregation and smooth muscle where it promotes contraction (168), thus annexin A2-5-HT_{2A}R interactions could also be of importance within peripheral systems.

In experiments to investigate the functional interaction of two putative c-terminal domain interacting proteins we found that annexin A2 can abrogate the inhibitory effect of β-arrestin 2 on PLC signalling at the H452Y variant receptor (where the influence of β-arrestin 2 is more marked compared to the wild-type receptor). This suggests that annexin A2 docking to the receptor may intefere with arrestin-mediated processes such as desensitisation and internalisation (at the H452Y variant at least) and may be consistent with an interaction, at least in part, with a more distal region of the c-terminal domain. These observations suggest that association of the Ca^{2+}-binding protein annexin A2 with the 5-HT_{2A}R may have functional relevance not only to its signalling abilities but also to its secondary level modulation by other proteins such as arrestins. Furthermore it appears that the H452Y variant form of the 5-HT_{2A}R may differ in its susceptibility to some of these influences compared to the wild-type receptor.

CHAPTER 6.0

6.0 Final Discussion

The 5-HT_{2A}R is widely implicated to be involved in a range of psychiatric disorders, including bipolar disorder (306), obsessive compulsive disorder (526), alcoholism (304), personality disorders (527) and schizophrenia (172). Psychiatric disorders such as schizophrenia can not only be severely disabling for the individual, but also profoundly affect associated family and friends, and present a significant impact upon society and the economy. Treatment for schizophrenia is often open-ended: the majority of sufferers continue to be affected throughout their lives, and face the likelihood of chronic unwanted side-effects associated with ongoing pharmacological treatment. A multi-factorial disease-state such as schizophrenia is so complex in terms of its etiology that it is very difficult to target specifically and effectively. It is therefore very important to study the pharmacological and biochemical mechanisms of any associated signalling pathways, in an attempt to develop a more sophisticated model of this disease process and its treatment. Thus this thesis has focussed on signalling by the 5-HT_{2A}R and the impact of an H452Y receptor polymorphism, exploring downstream PLD activity, desensitisation of PLC responses and the effects of Ca^{2+}-binding proteins.

5-HT_{2A}R-mediated PLD activation has not been widely studied in comparison to the more conventional signalling pathways, thus we were able to identify some fundamental determinants of 5-HT_{2A}R-mediated PLD activity in COS7 cells. We employed co-immunoprecipitation and functional PLD assay to identify PLD1 as the active isoform, and determined the activation of PLD1 by the 5-HT_{2A}R to be

primarily ARF-mediated. Due to the potentially cell-specific nature of 5-HT_{2A}R-mediated PLD activation, it would be of interest to perform parallel studies in alternative cell-types to elucidate whether this is a typical pattern of PLD activity downstream of the 5-HT_{2A}R. We further determined the probable locus of PLD1 binding to be between residues 438 and 471 within the distal region of the receptor C-terminus, distinct to the ARF1 binding site at the beginning of the C-terminus. The agonist-stimulated activation of the 5-HT_{2A}R is known to be swiftly followed by the recruitment of ARF1 to the 5-HT_{2A}Rct (274) and, as presented in this thesis by the time-course of 5-HT-stimulated co-immunoprecipitation of PLD1 with the PrC-5-HT_{2A}R, we found that PLD1 was likely to be associated with the receptor prior to agonist-stimulation. Thus a likely model of ARF-mediated PLD activation would be the direct association of ARF1 with PLD1 following the recruitment of ARF1 to the receptor. This could be facilitated by conformational changes of the receptor bringing ARF1 and PLD1 into contact, the dissociation of activated ARF1 from the receptor and its subsequent association with PLD1 or, alternatively, despite binding at a site in the distal regions of the receptor, PLD1 could overlap the ARF1 binding region, thus ARF1 and PLD1 could interact upon the recruitment of ARF1 to the receptor. An appropriate extension of these studies would be to analyse the time-course of agonist-induced ARF1 and PLD1 association at the receptor with the use of fluorescence techniques such as fluorescence resonance energy transfer (FRET).

PLD activation was then investigated downstream of a physiologically relevant H452Y polymorphic variant of the receptor, and compared to wild-type 5-HT_{2A}R-mediated PLD activation. The H452Y polymorphic variant was found to evoke a

significantly decreased facilitation of PLD1 responses and association with the 5-HT_{2A}Rct. As previously discussed, the H452Y polymorphism is associated with a resistance to the atypical antipsychotic clozapine (301), as well as with bipolar disorder (306), ADHD (305) and poor memory (307). Clozapine acts as an anatognist of 5-HT_{2A}R signalling, thus it would be of particular interest to explore the effects of clozapine upon 5-HT-induced PLD1 activation downstream of both receptor variants. A pertinent extension of these studies would involve the pharmacological assessment of a further range of atypical antipsychotics, such as risperidone, olanzapine and ziprasidone, upon 5-HT_{2A}R-mediated PLD1 activation and any effects of the H452Y polymorphism.

The influence of the H452Y polymorphism was further explored with regard to 5-HT_{2A}R-mediated PLC activity. There did not be appear to be any discernable alteration of 5-HT-induced PLC responses as a result of the introduction of the H452Y polymorphism to the 5-HT_{2A}R. However, in the presence of β-arrestin 2, H452Y-5-HT_{2A}R-mediated PLC responses were significantly reduced below that of the wild-type receptor. Furthermore, GST-fusion protein studies revealed a substantial increase in β-arrestin 2 binding by the H452Y variant receptor when compared to wild-type. β-arrestin isoforms are now well-accepted to independently facilitate G protein-independent signalling, by acting as scaffolds of multi-signalling protein complexes (135). One well-studied consequence of β-arrestin-dependent signalling is the phosphorylation and subsequent activation of Map kinases, such as ERK1/2 (137). We explored the hypothesis that the increased binding of β-arrestin 2 seen with the H452Y-5-HT_{2A}R might also result in changes to potential 5-HT_{2A}R-

mediated G protein-independent ERK1/2 activation. Preliminary studies suggest that 5-HT-induced ERK1/2 phosphorylation is increased as a consequence of the H452Y polymorphism. The recent discovery that 'biased ligands' can act as differential activators of β-arrestin or G protein signalling (528,529), to selectively favour the activation of one pathway over the other, has generated further possibilities for the pharmacological targeting of certain GPCRs. Thus, it would be of much interest to further investigate H452Y- and wild-type 5-HT_{2A}R-mediated ERK1/2 phosphorylation in the presence of a greater array of ligands including, as discussed above, clozapine and a further range of atypical antipsychotics. To further substantiate the impact of β-arrestin 2 on both H452Y-5-HT_{2A}R-mediated PLC responses and ERK1/2 phosphorylation, it would be appropriate to knock down any endogenous arrestin using siRNA and evaluate any difference in PLC and ERK1/2 responses. A relatively new technique that could be utilized to quantify the agonist-induced recruitment of β-arrestin to both receptor variants involves using receptor and β-arrestin isoforms complementarily tagged with R-luc and YFP, which allows a BRET signal to be calculated following the recruitment of β-arrestin to within a 100Å distance of the receptor.

Finally, this thesis illustrates the ability of the Ca^{2+}-binding protein, annexin, to increase the facilitation of 5-HT_{2A}R-mediated PLC responses. To investigate further, it would be appropriate to explore the effects of annexin on the trafficking of the receptor to the cell surface. Various techniques could be employed to study this, including the quantification of membrane-expressed receptor by radioligand-binding studies, and protein localisation studies using confocal/fluorescent microscopy.

In conclusion, this thesis presents new insights into the signalling mechanisms of the 5-HT_{2A}R and its H452Y receptor variant, that are of potential value to the further understanding and development of treatments for psychiatric disorders such as schizophrenia.

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7.0 Bibliography

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