Characterisation of Palmitoylation in Alpha$_{2A}$ Adrenoceptor and 5-HT$_{1A}$ Serotonin Receptor – G$_{o1\alpha}$ G Protein Fusion Proteins

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by

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I dedicate this thesis to my Mum, Dad and Wee Dodna. I did it! Yipee!
All my love
Lainey xx

Abbreviations

α	alpha
α_{2A}-AR	alpha$_{2A}$ adrenoceptor
α_{2A}-adrenoceptor-$G_{o1}\alpha C^{351}I$	alpha$_{2A}$ adrenoceptor fused to the pertussis toxin-resistant $Go_{1\alpha}Cys^{351}$Ile mutant
ADP	adenosine-5'-diphosphate
AGS	accelerator of G protein signalling
Ala	alanine
AMP	adenosine-5'-monophosphate
AMPA	(S)-2-amino-3-(3-hydroxy-5-methyl-4-isoxazolyl) propionic acid
Arg	arginine
Asn	asparagine
Asp	aspartate
ATP	adenosine-5'-triphosphate
β	beta
β-AR	beta adrenoceptor
$\beta\gamma$-dimer	complex formed between the beta and gamma subunits of heterotrimeric G proteins
BCA	bicinchoninic acid
B_{max}	total number of binding sites (maximum expression level)
BSA	bovine serum albumin
cAMP	adenosine-3',5'-monophosphate
CCR5	CC-chemokine receptor 5
cDNA	complementary deoxyribonucleic acid
cGMP PDEγ	cyclic guanosine-3',5'-monophosphate phosphodiesterase-γ
Ci	Curie
COS cells	*Cercopithecus aethiops* (monkey, African green) cells
CNS	central nervous system
cpm	counts per minute
CRF	corticotropin-releasing factor

C terminal/COOH	carboxyl terminal
Cys	cysteine
$C^{417}S$	5-HT_{1A}-receptor-$G_{o1}\alpha Cys^{351}$Ile fusion with mutated 1st GPCR palmitoylation site
$C^{420}S$	5-HT_{1A}-receptor-$G_{o1}\alpha Cys^{351}$Ile fusion with mutated 2nd GPCR palmitoylation site
$C^{442}A$	α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}$Ile fusion with mutated GPCR palmitoylation site
$C^{417}S$, $C^{420}S$	5-HT_{1A}-receptor-$G_{o1}\alpha Cys^{351}$Ile fusion with both the GPCR palmitoylation sites mutated
$C^{3}S$	α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}$Ile or 5-HT_{1A}-receptor-$G_{o1}\alpha Cys^{351}$Ile fusion with mutated G protein
$C^{417}S$, $C^{3}S$	5-HT_{1A}-receptor-$G_{o1}\alpha Cys^{351}$Ile fusion with both the 1st GPCR site and the G protein palmitoylation site mutated
$C^{420}S$, $C^{3}S$	5-HT_{1A}-receptor-$G_{o1}\alpha Cys^{351}$Ile fusion with both the 2nd GPCR site and the G protein palmitoylation site mutated
$C^{442}A$, $C^{3}S$	α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}$Ile fusion with both the GPCR and G protein palmitoylation sites mutated
$C^{417}S$, $C^{420}S$, $C^{3}S$	5-HT_{1A}-receptor-$G_{o1}\alpha Cys^{351}$Ile fusion with both GPCR palmitoylation sites and the G protein palmitoylation site mutated
Da	Dalton
DIG regions	detergent insoluble glycolipid-enriched regions
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
dNTPs	deoxynucleotide triphosphates
dpm	disintegrations per minute
DRY	Asp-Arg-Tyr
DTT	dithiothreitol
EA wax	enhanced autoradiography wax
EC loops	extracellular loops

EC_{50}	effective concentration 50% (concentration of agonist producing half maximal response)
ECL	enhanced chemiluminescence
EDTA	ethylene diamine tetra-acetic acid
eNOS	endothelial nitric oxide synthase
ER	endoplasmic reticulum
fmol	femtomole
γ	gamma
G protein	guanine nucleotide binding protein
GAP	GTPase activating protein
GAP-43	Growth-associated protein-43
GDP	guanosine-5'-diphosphate
$G\alpha$	G protein alpha subunit
$G\alpha\beta\gamma$	G protein heterotrimer
$G\alpha$-GDP	G protein alpha subunit with GDP bound
$G\alpha$-GTP	G protein alpha subunit with GTP bound
$G\beta\gamma$	G protein beta gamma subunit
$G_i\alpha$	alpha subunit of G protein originally characterised by its ability to inhibit adenylyl cyclase activity
$G_{o1}\alpha$	alpha subunit of G_o protein
$G_{o1}\alpha Cys^{351}Ile$	alpha subunit of G_o protein incorporating a cysteine to isoleucine mutation at amino acid residue 351
$G_s\alpha$	alpha subunit of G protein originally characterised by its ability to stimulate adenylyl cyclase activity
G_i class	class of G proteins responsible for inhibition of adenylyl cyclase
GFP	green fluorescent protein
GIP	glucose-dependent insulinotropic peptide
GLP-1	Glucagon-like peptide 1
Glu	glutamine
Gly	glycine
GnRH	Gonadotropin releasing hormone
GPCR	G protein coupled receptor

GRK	G protein-coupled receptor kinase
G_s class	class of G proteins responsible for stimulation of adenylyl cyclase
GRKs	G protein-coupled receptor kinases
GST	glutathione-S-transferase
GTP	guanosine-5'-triphosphate
GTPγS	guanosine 5'-O-(3-thiotriphosphate)
^3H	Tritium radionuclide
HA	haemagglutinin
HEK293T cells	human embryonic kidney 293 large T antigen cells
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid
HRP	horseradish peroxidase
8-OH-DPAT	8-hydroxy-2-(di-n-propylamino)tetralin
5-HT	5-hydroxytryptamine
5-HT_{1A}-receptor	5-hydroxytryptamine$_{1A}$ receptor
$5\text{-HT}_{1A}\text{-receptor-G}_{o1}\alpha C^{351}I$	5-HT_{1A}-receptor fused to the pertussis toxin-resistant $Go_{1\alpha}Cys^{351}Ile$ mutant
IC loops	intracellular loops
IC_{50}	concentration of inhibitor molecule required to produce half maximal response
IgG	Immunoglobulin-G
Ile (I)	isoleucine
IPTG	isopropylthio-beta-D-galactoside
kDa	kiloDalton
K_d	dissociation constant (concentration of ligand that will bind to half the receptors at equilibrium)
K_i	affinity of the receptors for a competing drug
K_m	affinity of an enzyme for substrate
L	litre
LB	L-broth
Leu	leucine
LH/hCG	luteinizing hormone/human chorionic gonadotropin
μg	micrograms

µl	microlitres
µM	micromolar
M	molar
MAPK	mitogen-activated protein kinase
Met	methionine
mg	milligrams
M-Glu-R	metabotropic glutamate receptor
min	minutes
min^{-1}	per minute
ml	millilitres
mm	millimetres
mM	millimolar
MOPS	4-morpholinepropanesulfonic acid
mRNA	messenger ribonucleic acid
NAD	nicotinamide adenine dinucleotide
NBCS	new born calf serum
ng	nanograms
nm	nanometres
nM	nanomolar
NMR	nuclear magnetic resonance
NO	nitric oxide
N-terminal/NH_2	amino terminal
OD	optical density
^{32}P	phosphorus-32 radionuclide
PACAP	Pituitary adenylate cyclase activating polypeptide
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PBS-T	phosphate buffered saline containing 0.2% Tween-20
PCR	polymerase chain reaction
PDE	phosphodiesterase
P_i	inorganic phosphate
PKA	protein kinase A
PKC	protein kinase C

PLC-β	phospholipase C-β
pM	picomolar
pmol	picomoles
Pro	proline
PSD-95	post synaptic density protein-95
PTH	parathyroid hormone
ptox	pertussis toxin
PVDF	polyvinylidenfluoride
RGS	regulator of G protein signalling
RS-79948-197	(8a R,12aS,13a-S)-5,8,8a,9,10,11,12,12a,13,13a-decahydro-3-methoxy-12-(ethylsulphonyl)-6H-isoquino[2,1-g] [1,6] naphthyridine
[^{35}S]	Sulphur-35 radionuclide
SDS	sodium dodecylsulphate
S.E.M	standard error of the mean
Ser	serine
S (nM)	substrate concentration (nM)
TAE	tris-acetate-EDTA
TE	tris-EDTA
TEM	tris-EDTA-magnesium
Thr	threonine
TM	transmembrane
TRH	thyrotropin releasing hormone
VIP	vasoactive intestinal polypeptide
Val	valine
V_{max}	maximum reaction rate
(v/v)	volume per volume
WAY100635	N-[2-[4-(2-methoxyphenyl)-1-piperazinyl[ethyl]-N-2-pyridinyl-cyclohexane-carboxamide
WT	wild type α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}$Ile or 5-HT_{1A}-receptor-$G_{o1}\alpha Cys^{351}$Ile fusion protein with all palmitoylation sites as normal
(w/v)	weight per volume

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Abstract

Palmitoylation variant GPCR-G protein fusion proteins were created between the porcine α_{2A}-adrenoceptor or the human 5-HT_{1A}-serotonin receptor and the pertussis toxin resistant, $Cys^{351}Ile$, form of the rat $G_{o1}\alpha$ protein. These palmitoylation-variant fusions were transiently expressed in HEK293T cells prior to analysis of the regulation of palmitoylation and the functional consequences of palmitoylation for both the GPCR and G protein parts of the fusions.

When the regulation of palmitoylation was studied for α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ fusion proteins, dynamic palmitoylation and depalmitoylation of both the Cys^{442} residue of the α_{2A}-adrenoceptor and the Cys^{3} residue of the $G_{o1}\alpha Cys^{351}Ile$ protein were found to occur. However, only the $G_{o1}\alpha Cys^{351}Ile$ protein part of the fusion was found to undergo adrenaline-stimulated regulation of palmitoylation and the effect of adrenaline required G protein activation. Adrenaline regulation proceeded in a concentration-dependent manner correlating with agonist occupancy of the α_{2A}-adrenoceptor. Such agonist effects were found to be, at least in part, due to agonist-stimulation of $G_{o1}\alpha Cys^{351}Ile$ protein depalmitoylation.

The requirements for palmitoylation of the α_{2A}-adrenoceptor and $G_{o1}\alpha Cys^{351}Ile$ protein elements of the α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ fusion proteins were subsequently assessed for various functional properties. Palmitoylation of neither the α_{2A}-adrenoceptor nor the $G_{o1}\alpha Cys^{351}Ile$ protein parts of the fusion determined fusion protein expression levels, affinity for the agonist adrenaline, affinity for the antagonist RS-79948-197, ability to bind or to hydrolyse GTP or their ability to influence the efficiency of RGS16 protein to accelerate the GTPase reaction.

In regulation of palmitoylation studies for 5-HT_{1A}-receptor-$G_{o1}\alpha Cys^{351}Ile$ fusion proteins, dynamic palmitoylation of the Cys^{3} residue of the $G_{o1}\alpha Cys^{351}Ile$ protein and the Cys^{417} residue of the 5-HT_{1A}-receptor was observed as well as a lack of incorporation of palmitate into Cys^{420} of the 5-HT_{1A}-receptor. Dynamic depalmitoylation was only observed for the Cys^{3} residue of the $G_{o1}\alpha Cys^{351}Ile$ protein,

not for the 5-HT_{1A}-receptor. In the latter case, palmitate once incorporated appeared to remain stably attached. Both the 5-HT_{1A}-receptor and the $G_{o1}\alpha Cys^{351}Ile$ protein parts of the fusion were found to undergo 8-OH-DPAT-stimulated regulation of palmitoylation. 8-OH-DPAT was able to regulate palmitoylation levels of both proteins in a concentration-dependent manner. For the regulation of $G_{o1}\alpha Cys^{351}Ile$ protein palmitoylation such agonist effects were found likely to be, at least in part, due to an agonist-stimulated rate of depalmitoylation. For the regulation of 5-HT_{1A}-receptor palmitoylation such agonist-stimulated increases in observed palmitoylation levels were only attributable to the addition of palmitate, given that no depalmitoylation of the 5-HT_{1A}-receptor could be detected.

The requirements for palmitoylation of the 5-HT_{1A}-receptor and $G_{o1}\alpha Cys^{351}Ile$ protein elements of the 5-HT_{1A}-receptor-$G_{o1}\alpha Cys^{351}Ile$ fusion proteins were also assessed for a selection of functional properties. Similar to the results obtained with $G_{o1}\alpha Cys^{351}Ile$ protein constrained to the α_{2A}-adrenoceptor, the palmitoylation of the $G_{o1}\alpha Cys^{351}Ile$ protein did not determine fusion protein expression levels, their affinity for the antagonist WAY100635, or their ability to bind GTP. Palmitoylation of 5-HT_{1A}-receptor did not alter fusion protein expression levels or their affinity for the antagonist WAY100635. However, in contrast, it did cause enhanced levels of GTP binding to the 5-HT_{1A}-receptor-$G_{o1}\alpha Cys^{351}Ile$ fusion proteins.

The results of this investigation suggest that there are different requirements for regulation of GPCR and G protein palmitoylation dependent on the GPCR-G protein fusion in question. These requirements may be responsible for the specific functional properties displayed by such fusions. The current study also demonstrates that GPCR-G protein fusion proteins can be successfully used as tools to study both the regulation of palmitoylation and the functional consequences of this modification.

Chapter 1

Introduction

1.1 Introduction

1.1.1 Cell Signalling

Cell signalling is the communication of individual cells with their environmental surroundings and other cells. This communication is a fundamental aspect of co-ordinating responses in multicellular organisms and it depends upon an efficient network for signal transduction across the lipid bilayer. Cells receive a multitude of signals at their extracellular surface via molecules such as hormones, neurotransmitters and the like. The majority of these signals, which do not enter the cell, must be transmitted to the interior of the cell through membrane-bound receptor systems. Some of these receptor systems function as ion channels, enzymes or sites for diffusion of lipid soluble ligands across the membrane into cells. However, the most abundant type of membrane-bound receptors are the G protein-coupled receptor (GPCR) family, which transduce signals to intracellular enzymes and ion channels via guanine nucleotide binding proteins (G proteins). This superfamily of GPCRs accounts for about 1000 of the ~30,000 genes encoding specific proteins in humans, making it one of the largest protein families in nature.

1.1.2 History of GPCRs and G proteins

The existence of a hormone-linked G protein signalling system began to be realised in 1957 upon the elucidation of cAMP as a second messenger for adrenaline and glucagon and the first description of this messengers synthesis by a membrane-associated enzyme now known as adenylyl cyclase (Berthet *et al.*, 1957; Sutherland and Rall, 1958). At this time, and for around a decade afterwards, it was thought that hormone acted directly on this enzyme causing allosteric activation of an intrinsic catalytic subunit. It was then discovered that hormone receptors and adenylyl cyclase were actually separate entities (Birnbaumer and Rodbell, 1969; Birnbaumer *et al.*, 1969). Shortly after this, in 1971, Rodbell *et al.* made the key observation that GTP was vital for effective hormonal stimulation of adenylyl cyclase enzyme complex. The discovery that guanine-nucleotide-sensitive adenylyl cyclase actually consisted of two components, a GTP-

sensitive protein (G_s) and a catalytic moiety (Pfeufer and Helmreich, 1975; Ross and Gilman, 1977), paved the way for the purification and characterisation of the GTP-binding protein, G_s (Northup *et al.*, 1980).

Around the same time, the first GPCRs, the β_2-adrenergic and the rhodopsin receptors, were being purified and characterised (Shorr *et al.*, 1981 and Nathans and Hogness, 1983). Much of our understanding of G protein-linked receptors is based on information derived from studies on rhodopsin, the receptor responsible for mediation of vision in dim light. This GPCR constitutes more than 90% of membrane protein in bovine retina, making its purification and sequencing a relatively easy task. In 1986, Dixon *et al.* cloned the gene and cDNA for the mammalian β-adrenergic receptor and assessed its homology to rhodopsin. It was found to have a very similar structure, containing seven hydrophobic segments predicted to form transmembrane (TM) regions of the protein. This seven TM (7TM) motif had been identified previously in the bacterial light-sensitive protein, bacteriorhodopsin. This protein is a proton pump and it does not interact with G proteins, however, it helped in providing a good insight into the predicted structure of GPCRs because this analogous seven-times-transmembrane-spanning protein had already been subject to electron crystallography and 3-D reconstruction (Henderson and Unwin, 1975; Henderson *et al.*, 1990).

As work continued in the GPCR-G protein signalling area it became apparent that the binding of many ligands, of various structural classes, was sensitive to guanine nucleotides. As a result, throughout the 80s and 90s with the advent of cDNA cloning techniques, a variety of new G proteins and GPCRs were discovered. They were found to function in a wide spectrum of biological areas such as sensory transduction (e.g. olfaction), hormonal signalling and cell growth. It was also found that besides adenylyl cyclase, a number of amplifiers and effector systems like phospholipases and phosphodiesterases, as well as ion channels, are regulated by the G protein subunits (Birnbaumer *et al.*, 1990).

1.1.3 The Adrenergic Receptors: A Brief Introduction

Adrenaline is a catecholamine hormone synthesised in chromaffin cells of the adrenal gland. It is released into the plasma at times of stress or increased energy need and stimulates glycogenolysis in the liver and exerts potent actions on the cardiovascular system. Adrenaline is also present, to a limited extent, in neurons in the central nervous system (CNS). In the periphery, the adrenergic system has an essential role in the regulation of the cardiovascular system. Increased sympathetic discharge to the heart leads to an increased rate and force of contraction mediated through β_1-adrenoceptors. Circulating adrenaline also acts on cardiac tissue but, in addition, acts on α_1-adrenoceptors in arterial smooth muscle stimulating vasoconstriction and on β_2-adrenoceptors in vascular beds of skeletal muscle stimulating vasodilation leading to increased blood flow (Summers and McMartin, 1993).

A large number of clinically important drugs exert their actions through adrenoceptors. β_2-selective agonists, e.g. salbutamol, are used in the acute treatment of asthma while α–agonists prolong the action of local anaesthetics and act as nasal decongestants. Clonidine, an α_2-selective agonist, is used to treat hypertension through a central action. α_1-antagonists, e.g. prazosin, are also used to treat hypertension but have only limited theraputic application. β-antagonists, e.g. propanolol or atenolol, are the agents of choice in the treatment of hypertension and a number of other cardiovascular disorders, e.g. angina, certain cardiac dysrhythmias and cardiac infarction; they are also used in the treatment of anxiety and glaucoma (Insel, 1996).

Adrenoceptors can be divided into three main types based on sequence information, receptor pharmacology and signalling mechanisms (*http://www.gpcr.org*). These types are the α_1, α_2 and β-adrenoceptors. Further subdivisions exist within each class: α_{1A}, α_{1B}, α_{1D} (members of the α_1 type), $\alpha_{2A/2D}$, α_{2B}, α_{2C} (members of the α_2 type), β_1, β_2, β_3 and β_4 (members of the β type). A large number of agonists and antagonists are available which distinguish between the three main classes of adrenoceptor. However, only relatively small differences in affinity for agonists and antagonists exist within each class, especially within the α_1-adrenoceptor and α_2-adrenoceptor families.

The adrenoceptor used in this study is the α_{2A}-adrenoceptor. The intronless gene for this receptor is found on chromosome 10 in man and encodes a 450 amino acid protein of 49kDa. The predominant effector pathways for this receptor are the inhibition of adenylyl cyclase and L-type Ca^{2+} channels, and activation of K^+ channels through pertussis-toxin-sensitive G proteins of the G_i/G_o class. Several analyses have demonstrated wide distribution of mRNA for the α_{2A}-adrenoceptor, with high levels in rat CNS, e.g. brainstem, cerebral cortex, hippocampus, pituitary gland and cerebellum, and in peripheral tissues, e.g. kidney, aorta, skeletal muscle, spleen and lung (Lanier *et al.*, 1991; McCune *et al.*, 1993; Sternweis and Robishaw, 1984).

Adrenaline, being the natural ligand for adrenoceptors, is widely used in its study. It does not show any significant selectivity for particular types or sub-types of these receptors. UK14304 is an α_2-selective agonist which can be employed when it is not necessary to distinguish between α_2 subtypes (Pipili, 1986). Some agonists do exist which allow selectivity between the subtypes, e.g. the weak partial agonist, oxymetazoline, which is 50-100 times more selective for α_{2A} relative to α_{2B} and α_{2C}-adrenoceptors (Godfraind *et al.*, 1982).

For the purposes of this study the natural ligand adrenaline (**Figure 1.1** part **a**) was used to stimulate the receptor. The tritiated antagonist [^{3}H]-RS-79948-197 (**Figure 1.1** part **b**) was used in receptor binding studies to calculate expression level of the α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ constructs, as well as affinity of the radioligand for the receptor portion of the constructs (Wise *et al.*, 1997c). In receptor binding studies the α_2-selective antagonist idazoxan (**Figure 1.1** part **c**) was also used to allow calculation of non-specific binding (Langer and Hicks, 1984).

Figure 1.1

Structure of α_{2A}-adrenoceptor-interacting molecules used in this study

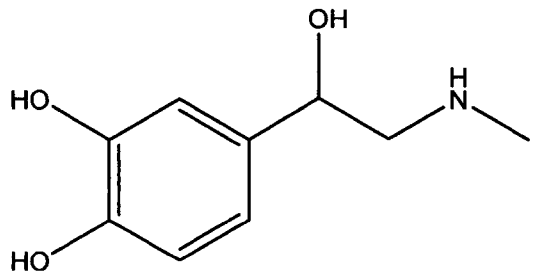
a) Adrenaline

b) RS-79948-197

c) Idazoxan

Figure 1.1

a)



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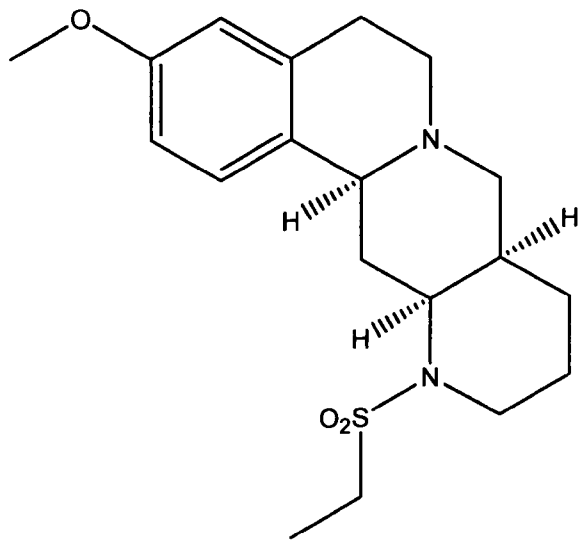

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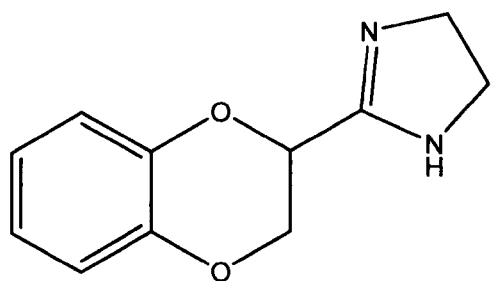


1.1.4 The Serotonin Receptors: A Brief Introduction

The name serotonin was originally given to an unknown vasoconstrictor substance found in the serum after blood has clotted. It was identified chemically as 5-hydroxytyptamine (5-HT) in 1948 (Rapport *et al.*, 1948a and 1948b). It is now known to be synthesised from the amino acid tryptophan, via 5-hydroxytryptophan, and it has a widespread distribution in plants and animals. It is an important neurotransmitter and local hormone in the CNS and intestine, and is implicated in a vast array of physiological and pathophysiological pathways (Houston and Vanhouette, 1986). In the periphery, 5-HT contracts a number of smooth muscles including the large blood vessels, the intestine and the uterus. It also induces endothelium-dependent vasodilation through release of nitric oxide from endothelial cells. 5-HT stimulates sensory nerve endings and is also a mediator of peristalsis. It is thought that in addition it may be involved in platelet aggregation, haemostasis, inflammatory mediation and microvascular control. In the CNS also, 5-HT is thought to be involved in a wide range of functions: including control of appetite, mood, anxiety, hallucinations, sleep, vomiting and pain perception. 5-HT receptor ligands have found clinical use in the treatment of depression, migraine and post-operative vomiting, and there is a strong potential for their use in other conditions (Lundberg, 1996).

5-HT is present in its highest concentrations in three regions in the body. About 90% of 5-HT is present in chromaffin cells in the wall of the intestine with a small amount in the nerve cells of the myenteric plexus. 5-HT is also found in blood, in high concentration in platelets: since it is important for platelet aggregation. In the CNS, 5-HT neurons originate primarily in the raphe nuclei of the brainstem and project to most areas of the brain.

5-HT receptors can be divided into seven main types based on sequence information, receptor pharmacology and signalling mechanisms (Hoyer *et al.*, 1994 and *http://www.gpcr.org*). These types are the $5\text{–}HT_1$, $5\text{–}HT_2$, $5\text{–}HT_3$, $5\text{–}HT_4$, $5\text{–}HT_5$, $5\text{–}HT_6$ and $5\text{–}HT_7$ receptors. Further subdivisions exist within the $5\text{–}HT_1$ ($5\text{–}HT_{1A}$, $5\text{–}HT_{1B}$, $5\text{–}HT_{1C}$, $5\text{–}HT_{1D}$, $5\text{–}HT_{1E}$, $5\text{–}HT_{1F}$), the $5\text{–}HT_2$ ($5\text{–}HT_{2A}$, $5\text{–}HT_{2B}$, $5\text{–}HT_{2C}$) and $5\text{–}HT_5$ ($5\text{–}HT_{5A}$, $5\text{–}HT_{5B}$) types.

The 5-HT receptor used in this study is the 5-HT_{1A} receptor. The intronless gene for this receptor is found on chromosome 5 in man and encodes a 421 amino acid protein of 46kDa (Kobilka *et al.*, 1987 and Chanda *et al.*, 1993). The predominant effector pathways for this receptor are the inhibition of adenylyl cyclase and activation of K^+ channels through pertussis-toxin-sensitive G proteins of the G_i/G_o class. The 5-HT_{1A} receptor is found presynaptically and postsynaptically in neurons in the CNS, e.g. dorsal raphe, hippocampus, medulla and cerebral cortex, and in the periphery, e.g. ileum. It is also abundant in foetal lymphatic tissue, particularly lymph nodes, spleen and thymus (Emerit *et al.*, 1991). Clinically, 5-HT_{1A} receptor ligands represent potential anxiolytic (Tunnicliff, 1991) and anti-hypertensive agents (Lundberg, 1996).

In terms of pharmacology, 5-HT (**Figure 1.2** part **a**), the natural ligand for serotonin receptors, induces activation of the 5-HT_{1A} receptor in nanomolar concentrations. However, it does not show any significant selectivity for particular types or sub-types of these receptors. Many potent agonists, selective for the 5-HT_{1A} receptor, are also available, such as 8-OH-DPAT (**Figure 1.2** part **b**), which has a selectivity of more than 100-fold for this subtype (Hjorth *et al.*, 1982). For the purposes of this study the 5-HT_{1A} selective agonist, 8-OH-DPAT, was used to stimulate the receptor. The tritiated antagonist [^{3}H]-WAY100635 (**Figure 1.2** part **c**) was used in receptor binding studies to calculate expression level of the 5-HT_{1A}-$G_{o1}\alpha$ constructs as well as affinity of the radioligand for the receptor portion of the constructs (Fletcher *et al.*, 1996). In receptor binding studies non-radiolabelled WAY100635 was used to allow calculation of non-specific binding. The 5-HT_{1A} selective inverse agonist spiperone (**Figure 1.2** part **d**) was also used in [^{35}S] GTPγS binding assays to reduce constitutive binding in the assay (Newman-Tancredi *et al.*, 1997).

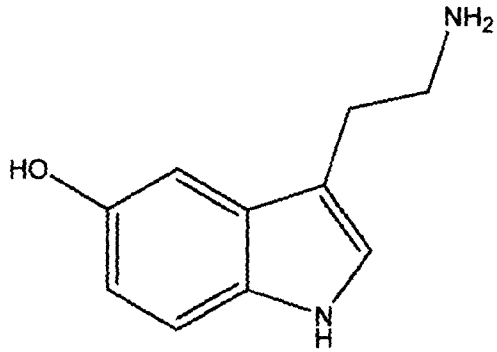
Figure 1.2

Structure of serotonin and the 5-HT_{1A} receptor-interacting molecules used in this study

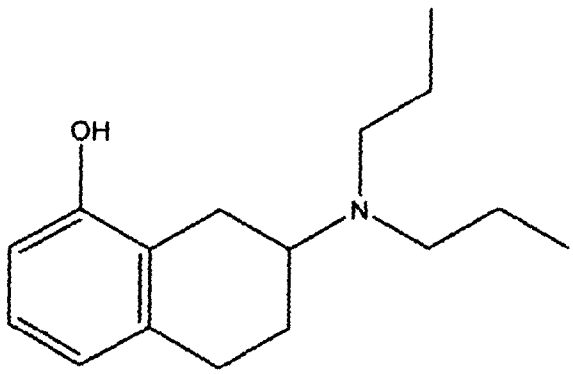
a) Serotonin
b) 8-OH-DPAT
c) WAY100635
d) Spiperone

Figure 1.2

a)



b)



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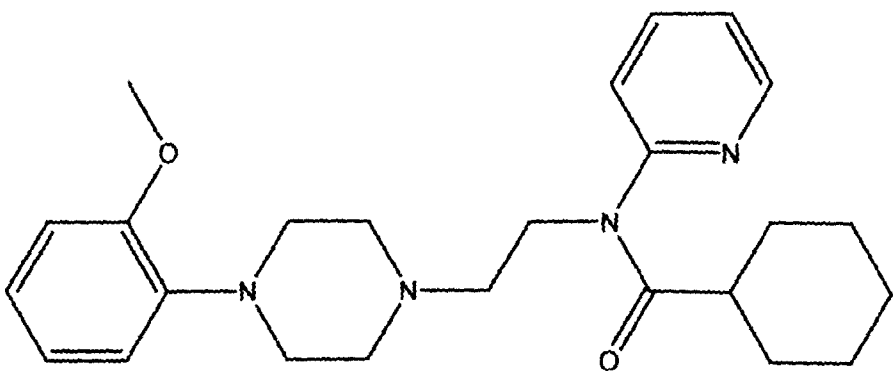

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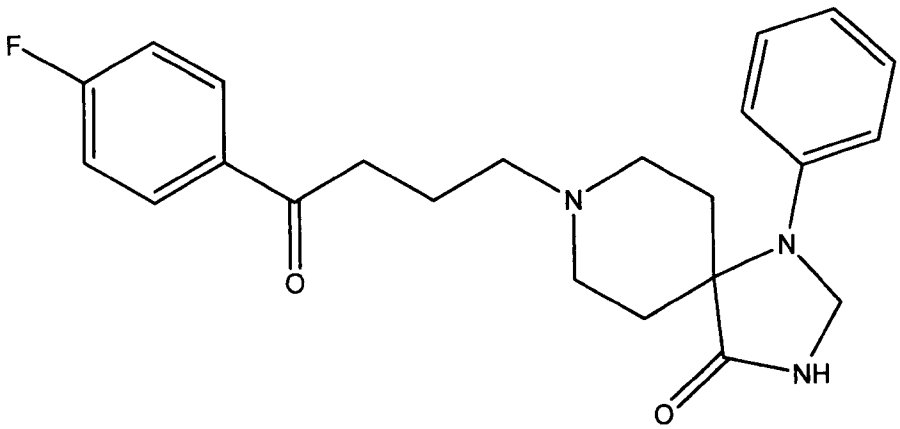


1.1.5 The $G_{o1}\alpha$ G protein: A Brief Introduction

G_o is a member of the G_i family of G proteins, responsible for the inhibition of adenylyl cyclase. However, this specific G protein's main function seems to be inhibition of neuronal calcium channel activity (Heschler *et al.*, 1987). G_o is expressed predominantly in CNS and heart, with high levels in various regions of the brain (Neer *et al.*, 1984; Sternweis and Robishaw, 1984); where it accounts for around 1% of the total membrane protein. High G_o levels are also found in neuronal growth cones where activation of this G protein may be promoted by interaction with another major growth cone protein, GAP-43 (Strittmatter *et al.*, 1990), and also amyloid precursor protein (Nishimoto *et al.*, 1993).

Two variant forms ($G_{o1}\alpha$ and $G_{o2}\alpha$) of the 40kDa G_o protein α-subunit, both with 354 residues, result from differential RNA splicing of a single $G_o\alpha$ gene, with the C-terminal 113 residues encoded by alternative use of duplicated exons 7 and 8 (Tsukamoto *et al.*, 1991; Kaziro *et al.*, 1991). $G_o\alpha$ splice variants are regulated selectively during neuronal development, with $G_{o1}\alpha$ predominating in differentiated neurons (Asano *et al.*, 1992). $G_o\alpha$ splice variants show selective interaction with different receptors and different β and γ-subunits (Kleuss *et al.*, 1991).

For the purposes of this study the $G_{o1}\alpha Cys^{351}Ile$ G protein was fused with either the α_{2A}-adrenergic or 5-HT_{1A} serotonin receptors. Use was made of antisera (Mullaney and Milligan, 1989) capable of recognising various regions of the $G_{o1}\alpha$ protein. These antisera were ON1 (against residues 1-16 of the G protein) and OC2 (against residues 345-354 of the G protein).

One further feature of the $G_o\alpha$ protein, advantageous in this study, is the site for pertussis toxin-mediated ADP-ribosylation at the amino acid Cys^{351} (Jones and Reed, 1987; Lochrie and Simon, 1988), four amino acids from the C-terminus of the protein. Pertussis toxin works by catalysing the transfer of an ADP-ribose group from NAD onto this cysteine residue. This bulky group renders the $G_o\alpha$ protein inactive, since it

prevents productive contacts between the GPCR and the G protein. This is because amino acid 351 is located within a key contact region for GPCRs. A number of studies have assessed the importance of this residue for signalling (Carr *et al.*, 1998; Jackson *et al.*, 1999; Bahia *et al.*, 1998). In the work by Bahia *et al.*, using α_{2A}-adrenoceptor-$G_i\alpha$ fusions, the toxin-sensitive residue was mutated to every other possible amino acid. They found that there was a central role for hydrophobicity in defining productive GPCR-G protein interactions, such that the wild-type Cys^{351} residue did not result in maximal stimulation by agonist, but that certain branched chain aliphatic or aromatic amino acids did. Nevertheless, although the mutation of this key residue may alter the pharmacology of the GPCR response, it is still an invaluable tool to confer toxin resistance to G proteins and therefore to ensure agonist function reflects only the G protein of interest in an experimental system. It has been previously shown (Burt *et al.*, 1998) that if toxin is not used to inactivate the endogenous G proteins, they too can be activated by the receptor part of the fusion protein. For this reason, all GPCR-G protein fusion constructs studied herein were $Cys^{351}Ile$ mutants and the human embryonic kidney cells (HEK293T) used to express them were pertussis toxin treated prior to experiments, to inactivate endogenous $G_i\alpha/G_o\alpha$ G protein.

1.2 G Protein Coupled Receptors

1.2.1 Introduction

Since the cloning of the first GPCRs, nearly 2000 new family members have been reported. This has resulted in the classification of the superfamily into over 100 subfamilies according to sequence homology, receptor function, and ligand structure. However, virtually all GPCRs can be grouped into one of three main classes, based on sequence similarity (**Table 1.1**): the rhodopsin-like family, the glucagon/VIP/calcitonin family and the metabotropic glutamate/chemosensor family (Gether, 2000).

Initially the classification of a GPCR as belonging to a particular subfamily was based on the high affinity binding of the natural ligand to that receptor. However, it was soon discovered that distinct members of these subfamilies existed which had differences in their structure, expression pattern and/or different reactions with synthetic agonists and

antagonists. In some cases the functional significance of receptor subtypes is rather obvious; giving the transmitter or hormone the opportunity to couple through different G proteins and thereby activate different effector systems. However in many cases the functional significance of receptor subtypes is found to be more subtle; for example where subtypes only display slight differences in desensitisation properties or in their extent of constitutive activity (Hoyer *et al.*, 1994).

Techniques for detecting protein-protein interactions such as yeast two-hybrid, phage display and fusion protein overlays have also revealed associations of heptahelical receptors with a variety of intracellular partners other than G proteins (Milligan and White, 2001). This is perhaps not surprising considering that with ~1500 heptahelical receptors there are only ~20 different heterotrimeric G proteins. Therefore, it is thought that each receptor may activate its own relatively specific set of intracellular signalling pathways by both G protein–dependent and G protein-independent mechanisms. However, the roles of GPCRs relevant for this work will only concern G protein-dependent mechanisms.

All GPCRs share a common structural homology characterised by amino acid sequences containing an extracellular N-terminal segment, seven transmembrane spanning domains linked by three extracellular and three intracellular loops, and an intracellular C-terminal segment. Between each of the main classes, this may be the only homology to exist, whereas within each class the homology also extends to some conserved amino acid residues and motifs (Gether, 2000).

GPCR signalling is initiated by the binding of a specific ligand to the extracellular side of the receptor. These ligands can be hormones, neurotransmitters, small peptides, proteins, lipids, ions, odorants, pheromones and photons (Hur and Kim, 2002). This activates the receptor, and causes it to undergo a conformational change allowing the GPCR, at its intracellular side, to interact with a specific G protein. The sheer diversity of ligands involved in GPCR-G protein signalling, as well as the degree of homology between GPCRs that bind extremely different ligands, is testament to the importance of this family and demonstrates that the structure of these receptors has been highly conserved through evolution.

Table 1.1

The main classes of G protein-coupled receptor

The table gives the main classes plus examples of each class. Note that both the α_{2A}-adrenoceptor and the 5-HT_{1A}-receptor used in this study bind monoamine ligands and therefore belong to the rhodopsin-like family of GPCRs.

Table 1.1

Rhodopsin-like family	Glucagon/VIP/ calcitonin family	Metabotropic Glutamate & Chemosensor family
Opsins Odorant Monoamine Lipid messengers Nucleotide Neuropeptides – most Peptide hormones – many Glycoprotein hormones Chemokines – some Protease receptors	Glucagon & GLP-1 Calcitonin & PTH Secretin & GIP PACAP & VIP GHRH & CRF	mGLU-R 1-7 calcium sensors

Figure 1.3

The main structural characteristics of the rhodopsin-like family of G protein-coupled receptors

The diagram shows the main structural features of the rhodopsin-like family of GPCRs. Residues located in the transmembrane (TM) helices are highlighted by enclosure in cylinders. G proteins are believed to interact mainly with residues located in the segments shaded dark grey. The key fingerprint residues (common to most rhodopsin-like GPCRs) are highlighted.

Shown are:

a) **N-terminal domain:** Site for glycosylation

b) **Conserved TM residues (Helix number is denoted by roman numerals and the relative residue number within that helix is indicated after the colon):**

Conserved polar residues (AsnI:18, AsnVII:16 and AspII:10).

Totally conserved residue ArgIII:26, part of DRY motif.

Conserved proline residues (ProV:16, ProVI:15 and ProVII:17).

c) **Extracellular (EC) loops:**

Two conserved cysteine residues (which form a disulphide bond in most family members)

Potential sites for N-linked glycosylation in the first and second EC loops.

d) **Intracellular (IC) loops:**

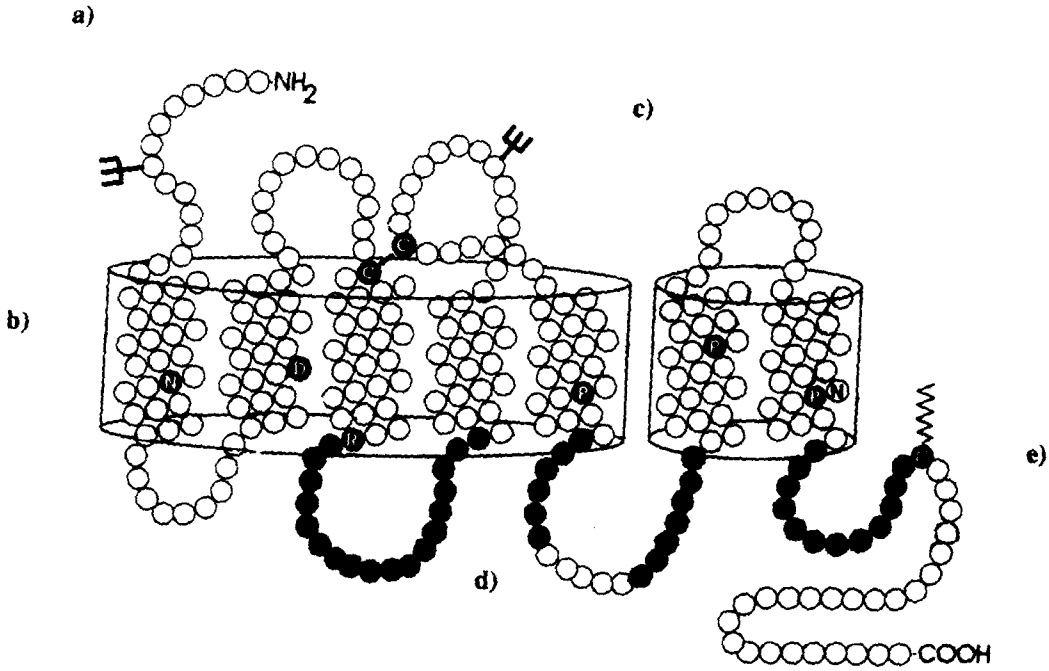
Second and third IC loops are often involved in G protein interaction. Third IC loop is frequently targeted for phosphorylation by various kinases.

e) **The C-terminal domain:**

Potential site for palmitoylation; creates a fourth IC loop. Residues here are also believed to be important for interaction with G proteins. This is a serine/threonine rich region (potential for phosphorylation).

Adapted from Figure 2.4, *Textbook of Receptor Pharmacology*. 1996. Foreman, J.C. and Johansen, T. (Editors). CRC Press, New York.

Figure 1.3



1.2.2 The Structure of GPCRs

Characterising the three-dimensional structure of GPCRs has proved problematic by traditional means such as x-ray crystallography or NMR. This is largely because they are complicated membrane proteins, which presents two main problems. They are difficult to produce in sufficiently large quantities and when they can be produced, they seldom form useful crystals. However, over the years a number of attempts have been made to elucidate the crystal structure of rhodopsin, as a model for other GPCRs. In the beginning, very low-resolution cryoelectron micrograph studies of two-dimensional crystals crudely indicated tilts and orientations of rhodopsin's transmembrane helices (Schertler *et al.*, 1993). To assign the densities to specific helices and to locate specific residues in each helix, modellers depended entirely on inference from the effects of many GPCR mutations and from comparing primary structures of hundreds of other GPCRs (Baldwin *et al.*, 1997). It was not until the first atomic-resolution experimental three-dimensional structure of rhodopsin was published (Palczewski et al., 2000) that it was realised that Baldwin *et al.*'s model of the seven-helix bundle superimposed reasonably well on the actual crystal structure.

GPCRs have many structural features that contribute to function. From their characteristic serpentine structure, to the many motifs, domains and modifications (such as glycosylation, phosphorylation, and palmitoylation), the GPCRs are complicated structures. A summary of these features, for the rhodopsin-like family of GPCRs (which includes the α_{2A}-adrenoceptor and 5-HT_{1A}-serotonin receptors), is given in **Figure 1.3** as well as in the following paragraphs.

The N-Terminal Domain

The N-terminal segment of GPCRs varies in length from 7-595 amino acids (Ji *et al.*, 1998). Most exhibit at least one consensus sequence (Asn-X-Ser/Thr, where X is any amino acid except proline or aspartate) for N-linked glycosylation. In many experiments the molecular mass of GPCRs is found to be greater than predicted from amino acid structures, suggesting glycosylation. Direct evidence for glycosylation has been obtained for a number of GPCRs, where endoglycosidase treatment results in a decrease in molecular mass (Rands *et al.*, 1990). Glycosylation is proposed to be functionally important for correct folding and cell surface expression of GPCRs (George *et al.*,

1986). For some receptors there may also be predicted glycosylation sites in the first and second extracellular loops. Note that in the other two families of GPCR (the glucagon/VIP/calcitonin family and the metabotropic glutamate/chemosensor family), the relatively larger N-terminus is also thought to be involved in ligand binding (Strader *et al.*, 1995; Takahashi *et al.*, 1993) and receptor activation (Takahashi *et al.*, 1993; metabotropic glutamate receptor).

Transmembrane Domains

Sequence analysis and hydrophobicity plots suggest that all GPCRs contain seven domains of 20-25 predominantly hydrophobic amino acids. By extrapolation from the structure of rhodopsin these stretches are predicted to form α-helical membrane spanning domains of unequal length, which can extend beyond the lipid bilayer (Unger *et al.*, 1997). It is thought the domains are arranged in a barrel shape (Baldwin *et al.*, 1997), orientated roughly perpendicular to the plane of the membrane in an anti-clockwise arrangement, imposing specificity on a ligand's entry into and binding to the TM core. Therefore, important residues in the transmembrane domains are involved in forming this dynamic structure, which allow conformational changes upon agonist binding. It was shown by Sealfon *et al.* (1997) that the core primarily contains the extremely hydrophobic TMs II, III, V, and VI with the more hydrophilic TMs I, IV, and VII having greater exposure to the bilayer. Most of the well-conserved residues are involved in forming the structure of the core. Proline residues (e.g. ProV:16, ProVI:15 and ProVII:17; conserved in most rhodopsin-like GPCRs) are thought to be important because their structure can cause a kink in the helix backbone by 26°, and the angle of the helices influences how they interact with each other and with ligand molecules (Ji *et al.*, 1998). A hydrogen bond network (formed by AsnI:18, AsnVII:16 and AspII:10) and salt bridges between residues of the same TM region as well as other TM regions, are critical for maintaining a tightly packed core (Pebay-Peyroula *et al.*, 1997).

The core structure forms the ligand-binding pocket for small molecular weight ligands such as adrenaline; where the carboxylate group on the conserved aspartate in TM III acts as a counter–ion for the catecholamine nitrogen, and two conserved serines in TM V interact with the meta- and para-hydroxyls on the catechol ring (Strader *et al.*, 1989). Note that serotonin is believed to bind in a similar fashion to adrenaline, via

corresponding residues in its receptor. Mutations at some specific residues ($Asp^{82}Asn$, $Asp^{116}Asn$, $Ser^{199}Ala$ and $Thr^{200}Ala$) in TM regions of the 5-HT_{1A} serotonin receptor, have been shown to decrease the affinity for the natural ligand, 5-HT (Ho *et al.*, 1992). Many antagonists for monoamine receptors also bind to the same site as the corresponding agonists (classic competitive antagonists which are structurally very similar to the agonist molecules). However, several antagonists have been shown to have interaction points not shared with agonists or to bind completely different sites (Kobilka, 1992). For larger stimuli, such as glycoprotein hormones, the extracellular region of the receptor is also involved in the binding of ligands (Reichart *et al.*, 1991).

At the boundary between TM III and IC loop 2, there is a DRY motif (aspartate-arginine-tyrosine), which is highly conserved in all rhodopsin-like GPCRs but not in the other families. This motif is assumed to be crucial for the signal transduction process. It contains an arginine residue (ArgIII:26 in **Figure 1.3**) which is believed to be constrained in a hydrophilic pocket formed by conserved polar residues in TM I, II and VII. It is suggested that upon receptor activation, protonation of this Arg causes it to shift out of the pocket and leads to exposure of previously hidden sequences in IC loops 2 and 3 on the cytoplasmic face (Scheer *et al.*, 1996; Wess, 1997).

Intracellular Loops

The intracellular loops vary in length between 10 and 40 amino acids in length, with the notable exception of the third intracellular loop, which can be more than 150 residues long. Obviously, the whole intracellular region affects the three-dimensional structure of the cytoplasmic side of GPCRs and it is this overall structure, which is important for G protein coupling. However, as outlined in **Figure 1.3**, the highly conserved second (Wess, 1998), the third intracellular loop (Cotecchia *et al.*, 1992), and the C-terminal tail (O'Dowd *et al.*, 1988) have been reported to be particularly important for this function. Furthermore, it has now been suggested that some of these regions may be responsible for particular aspects of receptor-G protein coupling. Regions of IC loop 2 as well as about 8 amino acids at the amino terminal portion of IC loop 3 may be responsible for determining the selective binding of particular types of G proteins (Wess, 1998). It is also believed that a region of about 12 positively charged amino acids residues, at the carboxyl terminal portion of IC loop 3 may be involved in the

induction of the high affinity conformation of the receptors. Mutations in this region (just below TM VI) can lead to constitutive activity of the GPCR (Cotecchia *et al.*, 1992). For the 5-HT_{1A} serotonin receptor, synthetic peptides of the entire IC loop 2 (Asp^{133}-Arg^{153}) and of regions of IC loop 3 (residues Ala^{331}-Leu^{347}, but not Ala^{336}-Val^{344}) were shown to be important for G protein interaction (Verrault *et al.*, 1994).

The intracellular region of GPCRs also has other important functions, since the third intracellular loop is also a known target for phosphorylation by G protein receptor kinases (GRKs) and second messenger activated kinases (Benovic *et al.*, 1985). As a result, this region is thought to be involved in phosphorylation and desensitisation events. In the 5-HT_{1A} serotonin receptor, there are a number of PKC phosphorylation sites in IC loop 2 (Thr^{149}) and IC loop 3 (Thr^{229}, Ser^{253}, Thr^{343}) which have been discovered to be important for signalling specificity and desensitisation (Lembo *et al.*, 1995 and 1997). The roles of phosphorylation and desensitisation are discussed in more detail in section 1.2.3.

Extracellular Loops

The majority of rhodopsin-like GPCRs contain two conserved cysteine residues in extracellular loops 1 and 2. In bovine rhodopsin and some other GPCRs these amino acids are known to be linked by a disulphide bond. This linkage is thought to constrain the loops and receptor, maintaining the tertiary structure for ligand binding (Karnik and Khorana, 1990; Ji and Ji, 1995). Substitution of these cysteine residues in the first and second extracellular loops of the β_2-AR receptor (Cys^{106} and Cys^{184}) induced destabilisation of the tertiary structure and alterations in ligand binding properties (Dohlman *et al.*, 1990).

The C Terminal Domain

All GPCRs except the mammalian GnRH receptor (which completely lacks an intracellular C-terminal domain (Sealfon *et al.*, 1997)) have an intracellular C-terminal tail. This varies considerably in length from ~12-359 amino acids. This region of the GPCR is usually rich in serine and threonine residues, present in consensus sequences for phosphorylation by GRKs and second messenger kinases. Like the intracellular loops, the C-terminal domain is thought to be important for receptor desensitisation

(Freedman and Lefkowitz, 1996). Most GPCRs also contain cysteine residues 15-20 residues C-terminal to TM VII, which have been shown to bind palmitate via a thioester linkage. It is believed that the palmitic acid residues become intercalated into the membrane bilayer, thereby creating a fourth IC loop (Ganter *et al.*, 1992) and possibly affecting G protein interaction (Milligan *et al.*, 1995). The process of palmitoylation appears to be dynamically regulated by receptor occupancy (Qanbar and Bouvier, 2003) however, the function of this modification is not yet fully understood and may vary dependent upon the individual receptor. There are some suggestions that the processes of palmitoylation and phosphorylation may be connected, in that the conformational constraints induced by palmitoylation may alter the accessibility of adjacent phosphorylation sites (Moffett *et al.*, 1996). The roles of palmitoylation will be fully discussed in section 1.5.

1.2.3 Further Important Features of GPCRs

GPCR Phosphorylation and Desensitisation

The functional consequences of phosphorylation vary considerably among receptors and can also be dependent upon which type of G protein is involved in the coupling process. The main recognised consequence of phosphorylation is desensitisation or down-regulation of the receptor (Hausdorff *et al.*, 1990). Desensitisation of GPCRs is the decrease in responsiveness to agonist after repeated or continuous stimulation (Ferguson, 2001). This is obviously an important mechanism to prevent acute or chronic receptor stimulation. The level of desensitisation can vary from complete termination of receptor signalling to the reduction in agonist potency and maximal responsiveness.

The process of desensitisation has many steps, which may not be the same for every receptor. In general the receptor must be uncoupled from the G protein, causing attenuation of signalling before sequestration into an intracellular compartment (internalisation) and then down-regulation if the stimulation is present chronically. These desensitisation processes occur over a range of time periods (phosphorylation in seconds, endocytosis within minutes and down-regulation within hours). As mentioned previously, the mammalian GnRH receptor lacks a C-terminal tail. This GPCR is therefore resistant to agonist-dependent phosphorylation and consequent desensitisation (Willars *et al.*, 1999). Deletions or mutations in cytoplasmic phosphorylation sites in

GPCRs (including the α_{2A}-adrenoceptor) have also been shown to reduce desensitisation, due to loss of phosphorylation (Liggett *et al.*, 1992).

Two types of desensitisation exist, homologous (receptor-specific) desensitisation and heterologous (cross-) desensitisation. Homologous desensitisation results in only the activated GPCR being desensitised. In this case the kinases involved are linked specifically to a particular receptor (e.g. the β-adrenergic receptor kinase, GRK2). In heterologous desensitisation, activation of one GPCR leads to the desensitisation of other GPCRs. This occurs via relatively promiscuous kinases such as protein kinase A and protein kinase C (Chuang *et al.*, 1996). The methods of protein kinase–mediated desensitisation have not been studied nearly as much as those of GRK-mediated desensitisation, perhaps since the latter is specific for GPCRs. Therefore it is unclear how similar desensitisation is in each case. It has been suggested that GRK phosphorylation is not absolutely necessary for desensitisation (Dickers *et al.*, 1999 and Bouvier *et al.*, 1998) but that it might enhance interaction with adaptor proteins involved in promoting the process, such as arrestins (Bouvier and Rousseau, 1998 and Zhang *et al.*, 1996). In support of this, Lohse *et al.* (1992) have shown that arrestins preferentially bind to agonist activated and GRK-phosphorylated GPCRs as opposed to second messenger protein kinase-phosphorylated or non-phosphorylated receptors.

In general, the processes of phosphorylation and desensitisation proceed with the following sequence of events (Ferguson and Caron, 1998). After phosphorylation of GPCRs by either specific G protein receptor kinases (GRKs) or second messenger kinases the GPCR-G protein interactions are reduced. For GRK-mediated phosphorylation this uncoupling is markedly promoted by the translocation and binding of adaptor proteins, called β-arrestins, to the receptor. This enhanced GPCR-G protein uncoupling results because of steric hindrance, since the regions of the receptors that arrestins bind to, generally IC loop 3 and the membrane-proximal portion of the C-terminal tail, are also the regions for G protein interaction (Law *et al.*, 2000). After uncoupling, the β-arrestin-bound receptors are targeted for internalisation into clathrin-coated vesicles (CCVs) (Zhang *et al.*, 1996 and Goodman *et al.*, 1996) and the system can then undergo resensitisation or down-regulation (Koenig and Edwardson, 1997).

GPCR resensitisation and down-regulation

GPCR resensitisation is achieved by the dephosphorylation of internalised receptors, by a receptor phosphatase, and subsequent recycling of receptors back to the cell surface (Pitcher *et al.*, 1995). During prolonged agonist exposure, sequestered receptors are instead retained intracellularly and are then targeted for down-regulation in lysosomes. Down-regulation involves a loss in receptor number due to reduction of mRNA and protein synthesis as well as degradation of the existing receptor protein (Bohm *et al.*, 1997). After down-regulation has occurred, resensitisation must proceed to allow recovery of the signalling system. In this case, resensitisation involves mobilisation of an intracellular pool of naïve receptors and/or *de novo* receptor synthesis (Shapiro and Coughlin, 1998).

Alternative pathways for GPCR internalisation

It is now recognised that not all GPCRs internalise via a β-arrestin and clathrin dependent route (Zhang *et al.*, 1996; Vogler *et al.*, 1999). Internalisation of GPCRs in non-coated vesicles has also been reported as an alternative pathway for endocytosis; this process can be dynamin-dependent (via caveolae) (Raposo *et al.*, 1989) or dynamin-independent (exact mechanisms as yet unknown) (Claing *et al.*, 2000). Dynamin-dependent internalisation is believed to proceed via small microdomains of plasma membrane rich in cholesterol, glycosphingolipids and caveolin proteins, known as caveolae. Potocytosis is the method by which caveolae can concentrate or move molecules into the cell (Simionescu, 1983). Molecules internalised by this method can then travel to the cytoplasm, the endoplasmic reticulum, the opposite cell surface or caveolae-derived tubular/vesicular compartment (Anderson, 1998). A wide variety of cell signalling molecules, including GPCRs, G proteins and effectors, have been shown to associate with caveolae (Anderson, 1998). It is proposed that the function of caveolae is to sequester/cluster molecules and act as a scaffold for the formation of signalling complexes. This localised concentration and specificity of signalling components would presumably lead to highly efficient signal transduction. Caveolae are highly enriched in detergent-insoluble glycolipid-enriched (DIG) regions of the plasma membrane known as rafts (Sargiacomo *et al.*, 1993). Electron microscopy of A431 cells showed that β_2-ARs could internalise via microdomains with the caveolae marker protein, caveolin-1 (Raposo *et al.*, 1989).

Specificity of GPCR Signalling

GPCRs can couple to more than one Gα subunit and can hence activate multiple effectors. In order for specificity of GPCR signalling to be maintained there is separation of the various receptors and Gα subunits involved in the signalling process (e.g. compartmentalisation via differential tissue expression, or by association with signalling complexes; which control the available molecules at any given time).

Signal Integration

With so many different signalling molecules in one location, caveolae are a logical place to look for signal integration. These organised structures may be able to account for the rapid and specific coupling of signalling molecules to more than one effector system, concentrating the required molecules within close proximity for interaction (Anderson, 1998).

Ligand-Receptor Binding and Receptor Activation

Ligand binding to GPCRs encompasses two main properties, affinity and efficacy. The affinity of a ligand for its GPCR is how tightly it associates with the receptor. The efficacy of a ligand at a GPCR is defined as the degree of activation produced upon binding. A GPCR agonist is defined as an agent, endogenous or synthetic, whose binding to the receptor results in activation of the G protein and the effector system. Three types of agonists exist; full (100% efficacy), partial (exerts limited effector responses) and inverse (that decrease the basal level of receptor modulation of effectors). An antagonist is an agent whose binding does not result in activation (zero efficacy).

The general mechanism of receptor activation can be summarised as follows (from the allosteric ternary complex model of Lefkowitz *et al.*, 1993). The GPCR can be thought of as a dynamic membrane protein, existing naturally in equilibrium between active and inactive conformations (Gether and Kobilka, 1998). Agonist molecules stimulate the receptor by stabilising an active conformation. They will therefore have significantly lower affinity for the G protein-uncoupled form of the receptor than the G protein-coupled form. Antagonist molecules bind the receptor G protein-independently and stabilise the inactive conformation of the receptor. Therefore, agonists and antagonists

bind preferentially to distinct conformational populations of their common receptor target (Kobilka, 1992).

A certain proportion of GPCRs can bind G protein and initiate signalling without any agonist present, a feature known as constitutive activity. In some cases, constitutive activity arises due to mutations in the GPCR, stabilising the active form of the receptor (Cotecchia *et al.*, 1992). Constitutively active receptors have been shown to account for some diseased states (e.g. mutations in the luteinising hormone receptor resulting in precocious puberty; Kawate *et al.*, 1995). Inverse agonists can be used to reduce constitutive activity in GPCRs, by inhibiting the unstimulated functioning of the receptor. An example of one such inverse agonist is spiperone, which can reduce the constitutive activity exhibited by some 5-HT receptor subtypes (Newman-Tancredi *et al.*, 1997).

1.3 G proteins

1.3.1 Introduction

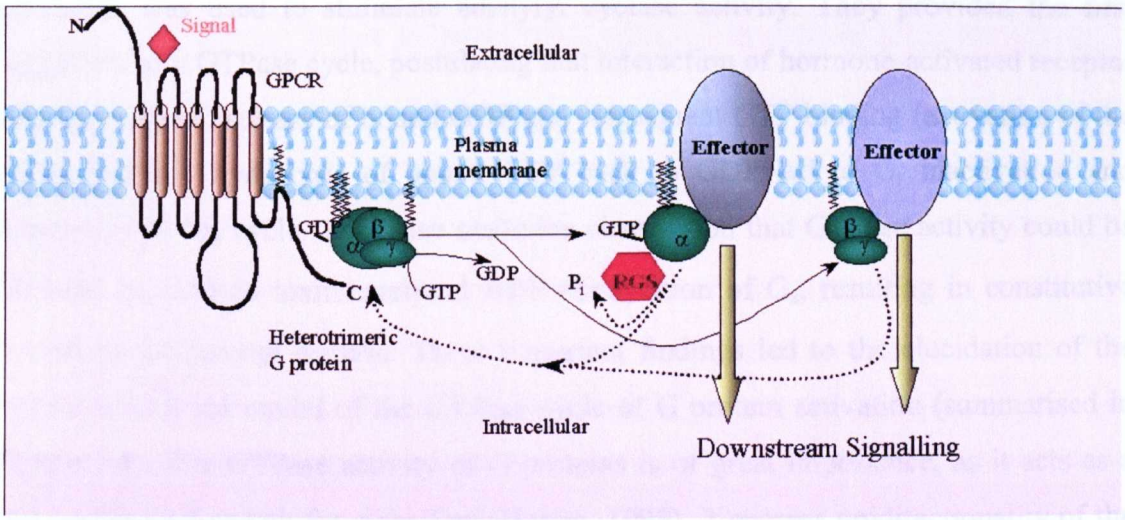
After the purification and characterisation of the first GTP-binding protein, G_s, in 1980 (Northup *et al.*) and with the advent of cDNA cloning techniques, more than 20 other GTP-binding proteins were discovered, including G_i, G_o and transducin (Spiegel, 1987), the $G_{q/11}$ family (Strathmann and Simon, 1990) and the $G_{12/13}$ family (Hooley *et al.*, 1996). Further research showed that Gα proteins (39-46kDa) formed complexes with two other proteins, the β-subunit (35-36 kDa) and the γ-subunit (6-10 kDa). It was found that β and γ-subunits tightly associate and function as a βγ-dimer. Initially it was thought that the role of the βγ-subunit was just to keep the α-subunits in check and to help with membrane anchorage. However, βγ-dimers have since been shown to be involved in the activation of signalling pathways, independently of the Gα subunit (Clapham and Neer, 1997).

Figure 1.4

General features of G protein signalling and the GTPase cycle

Diagram outlining the general features of G protein signalling and the GTPase cycle. The agonist (which can be a neurotransmitter, olfactory molecule, or glycoprotein hormone) binds to and stimulates the seven-times transmembrane spanning GPCR to undergo conformational changes triggering GPCR-G protein interactions. These interactions reduce the affinity of the heterotrimeric form of the G protein for bound GDP, promoting the exchange of GTP in place of bound GDP at a site within the α subunit. Once GTP is bound, both the receptor and the $\beta\gamma$ dimer no longer have high affinity for the α subunit, causing dissociation of the complex. Both the α and the $\beta\gamma$ units are then free to interact with and modulate effector systems, leading to metabolic or ionic changes within the cell. The cycle is terminated upon the hydrolysis of $G\alpha$ bound-GTP to GDP by the intrinsic GTPase activity of the α subunit; a process accelerated by RGS proteins. The high-affinity binding of $\beta\gamma$ is thus restored, promoting re-association of the heterotrimeric G protein with the receptor for another round of signalling.

Figure 1.4


N
Signal
GPCR
Extracellular
Plasma membrane
Effector
Effector
GDP
β
γ
GTP
α
GDP
GTP
P_i
RGS
C
Heterotrimeric G protein
Intracellular
Downstream Signalling

1.3.2 Overview of GPCR-G Protein Signalling

In work done in 1978 by Cassel and Selinger, GTPase activity of G_s was reported when adrenaline was used to stimulate adenylyl cyclase activity. They provided the first suggestion of a GTPase cycle, postulating that interaction of hormone-activated receptor with G_s triggered release of bound GDP and subsequent GTP binding (activation of the cycle) and that hydrolysis of bound GTP back to GDP led to G_s inactivation and completion of the cycle. They also made the observation that GTPase activity could be inhibited by cholera toxin-catalysed ADP-ribosylation of G_s, resulting in constitutive activation of adenylyl cyclase. These important findings led to the elucidation of the currently accepted model of the GTPase cycle of G protein activation (summarised in **Figure 1.4**). The GTPase activity of G proteins is of great importance, as it acts as a rate-limiting off-switch for signalling (Hamm, 1998). It ensures unidirectionality of the signalling system and also allows signal amplification before deactivation of the system. GTPase activity is found to vary enormously between G proteins (Vaughan, 1998), leading to very different rates of GTP hydrolysis.

1.3.3 Alpha Subunits

On the basis of amino acid homology of the α-subunits, G proteins can be classified into 4 major classes, namely, G_s, $G_{i/o}$, $G_{q/11}$, and $G_{12/13}$ (Simon *et al.*, 1991). In general, the G_s family is responsible for the activation of adenylyl cyclase, whilst the G_i family is responsible for the inhibition of adenylyl cyclase. Additional members of the G_i family, with some alternative functions, have also been characterised. These additional members are the G_t proteins, which stimulate cGMP phosphodiesterase, the G_o proteins, which are involved in Ca^{2+} ion channel closure (Hsu *et al.*, 1990) and the G_{gust} and G_z proteins. G_{gust} is expressed in the taste buds and is thought to couple to cGMP phosphodiesterase. G_z is expressed in neuronal cells where it inhibits adenylyl cyclase (Taussig and Gilman, 1985). The $G_{q/11}$ family predominantly couples to phosphoinositide turnover (Strathmann and Simon, 1990). The last G protein family, the $G_{12/13}$ family, is ubiquitously expressed and has been shown to be involved in both the regulation of Na^+/H^+ ion exchange in cells (Hooley *et al.*, 1996) and the maintenance of

the cell cytoskeleton through the activation of the small GTPase Rho (Klages *et al.*, 1999).

Another important feature of G protein α-subunits is their susceptibility to be ADP-ribosylated by toxins. It has already been explained in section 1.1.5 how $G_{o1}\alpha$, a member of the G_i class of G proteins, can act as a substrate for pertussis toxin-catalysed ADP-ribosylation. This toxin (from *Bordetella pertussis*) can also mediate ADP-ribosylation of the other members of the G_i class, apart from G_z. Cholera toxin (from *Vibrio cholerae*) is a toxin, which catalyses the ADP-ribosylation of the G_s family of G proteins, G_{gust} and G_t. Both of these toxins are A-B toxins (containing a similar A-B subunit structure). The B subunits bind to a cell surface receptor, which permits the internalisation of the whole toxin by the cell. Once inside the cell the disulphide bridge holding the subunits together is broken to release the A subunit. It is this A subunit which then catalyses ADP-ribosylation of the target protein.

1.3.4 Beta and Gamma Subunits

Several crucial roles have been assigned to the βγ-dimer including promoted association of GDP-bound α-subunits with ligand-activated receptors. This function presents the heterotrimeric G protein, ready for receptor-dependent G protein activation (Birnbaumer *et al.*, 1990). The βγ-subunits show preferential binding of Gα-GDP over Gα-GTP and it has been shown experimentally that they stabilise the GDP-bound form of Gα; since GDP dissociates from Gαβγ more slowly than from Gα alone (Higashijima *et al.*, 1987). Taken together these facts can explain why, in terms of affinity, α and βγ-subunits dissociate from activated receptors.

Gβγ-subunits have also been implicated in the mediation of signal transduction by interaction with various effector molecules. Some of the effectors regulated by the βγ-subunit are; adenylyl cyclase (Tang and Gilman, 1991), phospholipase C β (PLC β) (Camps *et al.*, 1992), inwardly rectifying G protein-gated K^+ channels (Logothetis *et al.*, 1987), voltage-sensitive calcium channels, phosphoinositol-3-kinase (PI3K) and molecules in the mitogen activated protein kinase (MAPK) pathway (Crespo *et al.*,

1994). Just as is the case for Gα-subunits, multiple β and γ-subunits exist. To date, 6 β-subunits and 12 γ-subunits have been cloned and identified. This multiplicity of subunits means there are many possible combinations (not all exist). It is thought that each of these combinations has a unique role. Specificity of different β and γ-subunits have been investigated in some systems (Garcia *et al.*, 1998), but many more combinations exist which have still to be investigated.

1.3.5 Structural features of G proteins

General structural features

X-ray crystallography of a number of α-subunits, including $G_t\alpha$ (Noel *et al.*, 1993) and $G_{i1}\alpha$ (Coleman *et al.*, 1994), show that in general, the structure of the G protein superfamily is highly conserved; reflecting the common mechanism of action. All Gα subunits contain two domains. One domain is organised around a core structure, involved in the binding and hydrolysis of GTP. This domain is structurally homologous to the GTPases of monomeric G proteins and elongation factors. The other domain is a unique α-helical domain which masks the guanine-nucleotide binding site from the surrounding solvent by burying bound GTP or GDP deep in the protein core (Bourne *et al.*, 1991). An equilibrium is thought to exist between a "closed" conformation (containing the buried nucleotide) and an "open" conformation (leading to GDP dissociation and subsequent GTP binding); with activated receptor presumably stabilising the "open" conformation.

The GTP-binding domain of the core consists of 5 α-helices surrounded by 6 β-strands, and also contains a binding site for Mg^{2+} ions (found to be essential for catalysis (Sprang, 1997)). The β-subunit of heterotrimeric G proteins comprise of an N-terminal helix followed by a 7 membered β-propeller structure (containing seven WD-40 repeats (Sondek *et al.*, 1996)). The γ-subunit contains two helices but no inherent tertiary structure. βγ interactions are maintained via an N-terminal coiled-coil and the β-propeller (Sondek *et al.*, 1996). The interaction of Gα with Gβγ involves binding of the Gα N-terminal helical domain to the propeller structure of the β-subunit. Upon receptor

activation, conformational changes in Gα cause a reduction in helical content, which leads to dissociation of the Gβγ dimer away from Gα (Lambright *et al.*, 1996).

Insight into the structural mechanism of GTP hydrolysis was derived from the structure of Gα subunits activated by AlF_4^- (Coleman *et al.*, 1994). This complex occupies the γ-phosphoryl binding site and, together with GDP, mimics the action of GTP. These studies reveal a planar transition state for GTP hydrolysis containing a conserved Arg residue (Arg^{178} in $G_{i1}\alpha$) interacting with fluoride atoms, and also a conserved Glu residue (Glu^{204} in $G_{i1}\alpha$) interacting with a fluoride atom and the axial water molecule required for hydrolysis. Substantial rearrangement of three regions of the α-subunit occurs upon GTP hydrolysis. These are designated switch I, switch II and switch III (Lambright *et al.*, 1994). When GTP is bound, basic residues in Switch II form ionic interactions with residues in Switch III. Upon GTP hydrolysis, these linkages are broken as Switch II and III collapse. Switches II and III are proposed effector-binding regions in activated $G_s\alpha$ (Sprang, 1997).

The two terminii of Gα proteins are thought to be important for different functions. The N-terminus has been implicated in membrane anchorage, since this is the site for palmitoylation and/or myristoylation (see following paragraphs and section 1.5), and tryptic removal of this region has been shown to result in cytosolic, soluble Gα subunits (Eide *et al.*, 1987). The other important role of the N-terminus is thought to be interaction with the βγ–subunit. Support for this hypothesis comes from the results of several studies, which observe that βγ binding is lost upon mutation of (Denker *et al.*, 1992), removal of (Navon and Fung, 1987), or direction of monoclonal antibodies to (Mazzoni and Hamm, 1989) the N-terminal 20-21 residues of the G protein.

The C-terminus of the G protein is the region thought to be important for receptor and effector interaction; perhaps why this region of Gα proteins is found to contain most of the class specific sequence variation and why this region has been successfully used to produce G protein-specific polyclonal antipeptide antisera (Mullaney and Millgan, 1989). There are many examples of studies providing proof of the importance of the C-terminus in receptor and effector coupling. The uncoupling of $G_{i/o}\alpha$ interaction with the

receptor was shown to result from ADP-ribosylation of Cys^{351}, close to the C-terminus (West *et al.*, 1985). In addition, it has been shown that antibodies directed against the extreme C-terminus of the α-subunits are capable of inhibiting receptor-mediated activation of G proteins (Simonds *et al.*, 1989). A proline to arginine mutation, at the sixth amino acid from the C-terminus of $G_s\alpha$, has been shown to abolish adenylyl cyclase activation upon receptor stimulation (Sullivan *et al.*, 1987); supporting the role of the C-terminus in receptor-coupling. The development of chimeric G proteins also provided insight into the role of the C-terminus. Creation of a chimera where the C-terminus of $G_q\alpha$ was replaced with the corresponding residues of $G_i\alpha$ resulted in stimulation of phospholipase C by receptors otherwise coupled exclusively to G_i (Conklin *et al.*, 1993), indicating the role of this region in specific receptor interactions.

Lipid modifications of G proteins

Every G protein contains some form of lipid modification. These can be of three types: isoprenylation, myristoylation and palmitoylation. All G protein βγ-dimers become isoprenylated on the cysteine residue of a CAAX motif (Cys residue followed by any two aliphatic residues then any other residue), located at the C-terminal end of the γ-subunit. After attachment of the isoprenyl group (through a stable thioether linkage), a protease then cleaves the AAX residues and the Cys residue becomes carboxymethylated (Higgins and Casey, 1994). Different Gγ subunits undergo modification with different isoprenyl groups: either farnesyl (a 15-carbon moiety) or geranylgeranyl (a 20-carbon moeity) (Wedegaertner *et al.*, 1995). Removal of the isoprenylated Cys residue results in Gβγ not being properly targeted to the plasma membrane and being found in the cytosol (Spiegel *et al.*, 1991).

Lipid modifications are also present on G protein α-subunits. $G_i\alpha$ subunits undergo co-translational addition of a 14-carbon myristate group onto an N-terminal glycine residue. This glycine is at codon 2 of the $G_i\alpha$ subunit and is part of a general MGXXXS consensus sequence (Met, then Gly, then any three residues, followed by Ser). After cleavage of the N-terminal Met residue, attachment of myristate (via an amide bond) is catalysed by N-myristol CoA transferase. Mutation of this glycine residue has been shown to result in unmyristoylated Gα subunits, located predominantly in the cytosol.

However, this lipid modification does not account in full for G protein membrane location, since $G_s\alpha$ does not undergo myristoylation but is still membrane associated (Jones *et al.*, 1990). Therefore, for some α-subunits other lipid modifications and/or protein domains are thought to mediate membrane anchorage. Another Gα modification reported to promote membrane association is palmitoylation. In general terms palmitoylation involves the attachment of palmitate, a 16-carbon fatty acid, to a substrate. In terms of Gα subunit palmitoylation, the fatty acid is sometimes attached post-translationally to an N-terminal cysteine residue, through a labile thioester linkage. However, the attachment of palmitate to other residues, not via a thioester bond, has also been observed. Due to the nature of the thioester bond this modification is reversible, suggesting that changes in palmitoylation status can lead to variations in Gα membrane affinity and modulation of signalling (Parenti *et al.*, 1993). Increased turnover of palmitate on Gα subunits has been observed upon activation for $G_s\alpha$ (Wedegaertner and Bourne, 1994) and $G_i\alpha$ (Chen and Manning, 2000). Non-palmitoylated mutants of $G_s\alpha$ have been reported to exhibit a markedly decreased capacity to associate with the membrane (Wedegaertner *et al.*, 1993). In terms of the relative contributions of myristoylation and palmitoylation to membrane association of Gα subunits, it has been found that myristoylation alone is insuffient for membrane localisation (Peitzsch and McLaughlin, 1993) but that it is a prerequisite for palmitoylation for most G proteins (Mumby *et al.*, 1994; Galbiati *et al.*, 1994). The kinetic bilayer-trapping hypothesis (Shahinian and Silvius, 1995) suggests that proteins with a single lipophilic group (such as myristoylation) can interact transiently with intracellular membranes, facilitating rapid palmitoylation by a plasma-membrane-bound palmitoyl transferase. The protein is then proposed to remain stably attached, due to the strong hydrophobicity and slow kinetic off rate of the dual fatty acid anchor. The roles of palmitoylation will be discussed in more detail in section 1.5.

1.3.6 Other molecules acting at sites within the GTPase cycle

Although it has been known for some time that the G protein α-subunits possess an intrinsic GTPase activity it was found that many G protein-mediated physiological responses turned off much more rapidly than *in vitro* for pure Gα subunits. This

discrepancy was explained by the discovery of proteins called GTPase activating proteins (GAPs) that accelerate the GTPase activity of the α subunits. Two classes of GAPs for trimeric G proteins have been reported. One class includes G protein effectors such as PLCβ1 (Mukhopadhyay and Ross, 1999; Montell, 2000) and cGMP PDE γ-subunit (Slepak *et al.*, 1995), which stimulate GTP hydrolysis by α_q and α_t respectively. The second class is a family of regulators of G protein signalling (RGS proteins), which also stimulate the rate of α-subunit GTP hydrolysis. To date more than 20 RGS proteins have been described, all possessing a conserved domain of about 115 amino acids known as the RGS box (Ross and Wilkie, 2000). By studying the crystal structure of RGS4 bound to $G_{i1}\alpha$-GDP-AlF^{4-} it was found that the active core of the RGS box forms a four-helix bundle. This bundle stabilises the transition state for hydrolysis of Gα-bound GTP (resulting in an increase in k_{cat} for GTP hydrolysis) by interacting with the three flexible switch regions of Gα, regions whose conformation is dependent on the identity of the bound guanine nucleotide (Tesmer *et al.*, 1997). The family of RGS proteins have been shown, using the yeast 2 hybrid system, *in vitro* binding, and co-immunoprecipitation assays, to have a range of selectivities for the various Gα subunits. At present, these *in vitro* studies with mammalian RGS proteins seem to indicate most, if not all, act selectively as GAPs for Gα proteins in the α_i family (α_i, α_o, α_z and α_t) and/or $G_{q/11}$ but do not appear to interact with, or affect, α_s or $\alpha_{12/13}$ classes (Ross and Wilkie, 2000). It is also now known that certain adaptor proteins can interact with regulators of G protein signalling and modulate their activity (e.g. 14-3-3 proteins have been shown to bind to RGS7 and inhibit its GAP function (Benzing *et al.*, 2000)).

It has now been suggested that as well as RGS proteins, which accelerate the GTPase activity of G proteins (terminating GPCR-G protein signalling), AGS proteins (accelerators of G protein signalling) also exist. Although each AGS protein activates G-protein signaling, they do so by different mechanisms within the context of the G-protein activation/deactivation cycle. The role of these proteins will, however, not be discussed further for the purposes of this study, for a recent review the reader is referred to Cismowski *et al.* (2001).

1.4 GPCR-G Protein Fusion Proteins

The construction and analysis of GPCR-G protein fusion proteins has been the work of various groups (Bertin *et al.*, 1994; Wise and Milligan, 1997; Seifert *et al.*, 1998; Guo *et al.*, 2001). In these fusion proteins the N terminus of the desired Gα subunit is directly attached to the C-terminus of a GPCR. These constructs have proved useful tools to study the enzymatic capacity of G proteins, to measure ligand efficacy, and to evaluate receptor-coupling specificities of related G proteins (reviewed by Seifert *at al.*, 1999; Milligan, 2000). The very nature of the fusion is advantageous for these purposes, as it defines the stoichiometry of expression of the two entities as 1:1 and also ensures their co-localisation following expression. In most of these studies, toxin-resistant G proteins (the reasons for which have been previously discussed in section 1.1.5) have been used.

In the present study, the N-terminus of palmitoylation variant $G_{o1}\alpha$ proteins are directly linked to the C-terminus of palmitoylation-variant versions of either the α_{2A}-adrenoceptor or the 5-HT_{1A}-serotonin receptor. The structure of these fusions is outlined in **Figure 1.5** and **Appendices 8.1-8.4**.

Some may consider fusion protein systems not to be a worthwhile area of research, believing the artificially constrained nature means they cannot be physiologically relevant. This is true to a certain degree since events downstream of GPCR-G protein interaction, such as internalisation and desensitisation may be different for fusions as opposed to the native proteins (Loisel *et al.*, 1999). Therefore, results obtained from using this system must be interpreted with an air of caution. However, for certain areas of study, without fusion-protein investigations, advancement in our understanding would not have been possible.

Figure 1.5

The structure of GPCR-G protein fusion proteins.

The diagram shows the general structure of GPCR-G protein fusions in terms of:

a) **linear arrangement in a fusion protein:**

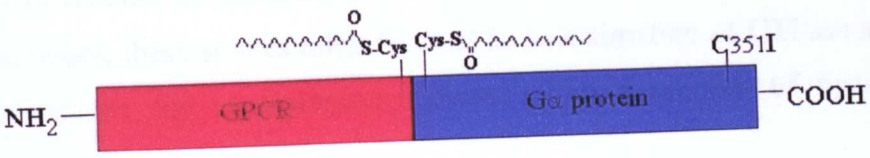
This diagram depicts the general linear structure of both types of fusion protein used in this study. It shows the $Cys^{351}Ile$ mutation, present in all these constructs, and indicates the presence of palmitate in each part of the fusion protein. Note that in the case of $5HT_{1A}$-receptor-$G_{o1}\alpha Cys^{351}Ile$ fusions there would be two palmitoylstion sites in the GPCR portion.

b) **2-dimensional representation of a fusion protein expressed in a cell:**

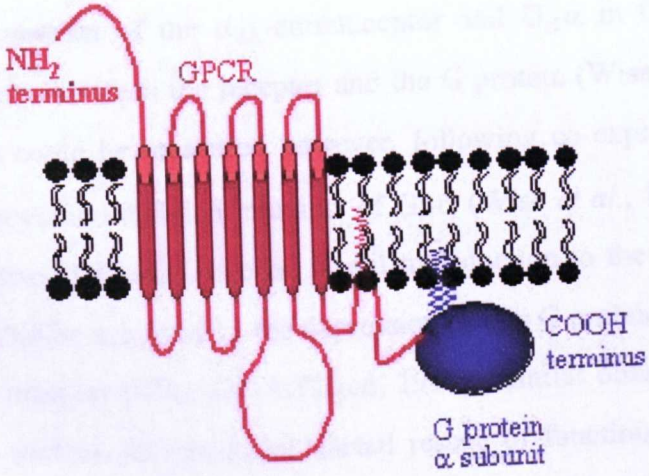
The 2-D diagram depicts the general arrangement of the GPCR and Gα protein parts of the fusion protein when expressed in a cell. It shows the insertion of palmitate, attached to each protein, into the lipid bilayer. The diagram also shows the insertion of myristate, attached to the $G_{o1}\alpha$ protein, into the bilayer.

Figure 1.5

a)


O
S-Cys
Cys-S
O
C351I
NH2
GPCR
Gα protein
COOH

b)


NH2
terminus
GPCR
COOH
terminus
G protein
α subunit

The usefulness of fusion-proteins is perhaps best illustrated in the study of GTPase activity of various G proteins. Before the use of GPCR-G protein fusions there was a lack of information on the absolute levels of GPCRs and their cognate G proteins. Their localisation in relation to one another in cells and at the plasma membrane was also unclear. Therefore, these studies often resulted in an estimation of GTPase activity too low to account for the physiologically observed rapid kinetics of ligand-induced activation.

In the present study, involving the use of a palmitoylation deficient G protein, targeting of the G protein to the plasma membrane for efficient coupling and signalling could have been a problem if a non-fusion approach was taken (by inference from a previous study using acylation-deficient mutants of the $G_{i1}\alpha$). It has previously been shown that independent co-expression of the α_{2A}-adrenoceptor and $G_{i1}\alpha$ in COS cells allowed functional interactions between the receptor and the G protein (Wise *et al.*, 1997a). No functional contacts could be measured however, following co-expression of the α_{2A}-adrenoceptor and acylation-deficient mutants of $G_{i1}\alpha$ (Wise *et al.*, 1997b). It was also shown that the rescue of agonist-induced signal transduction to the acylation-deficient mutants of $G_{i1}\alpha$ could be achieved by the expression of this G protein as part of a fusion construct with the receptor (Wise and Milligan, 1997). Similar observations have been made for the $G_s\alpha$ protein. In this study, partial rescue of functional interactions of a non-palmitoylated mutant of $G_s\alpha$ was observed by fusion of the $G_s\alpha$ subunit to the β-adrenergic receptor (Ugur *et al.*, 2003).

1.5 Palmitoylation

1.5.1 Introduction

The first report of covalent attachment of lipids to proteins was made in 1951 (Folch and Lees). It was not, however, until about 30 years later that the first descriptions of the three main classes of lipid modification were given. In 1979 the characterisation of palmitoylation was reported (Schmidt and Schlesinger, 1979), followed in 1982 by the

description of myristoylation (Carr *et al.*, 1992) and in 1989 by the characterisation of prenylation (Casey *et al.*, 1989). These three lipid modifications have been shown to be present on many types of proteins and are thought to play a role in a variety of functions (Resh, 1999). However, the focus of the present study will concern only the role of the first of these lipid modifications, palmitate. This lipid modification will be studied in terms of its regulation and the role that it plays in signalling of GPCRs and G proteins.

1.5.2 Role of Palmitoylation

Initially, the 16-carbon fatty acid nature of the palmitate moiety, lead to the suggestion that it might enhance various hydrophobic interactions (Wedegaertner *et al.*, 1995). These were thought to be interactions such as allowing the protein to interact with the lipid membrane or another protein (via contact between hydrophobic regions). Whilst there is evidence for the role of palmitoylation in both of these cases (see Dunphy and Linder, 1998, for a review), it is now assumed that hydrophobic interactions are not the only functional consequence of attachment of palmitate. This view arose upon appreciation of the nature of the thioester bond through which palmitate is attached to some proteins. The lability of the thioester linkage means that palmitate attached via this bond can be readily removed (Magee *et al.*, 1987) and thus the reaction has the potential to be regulated. In addition, there has been an increasing body of evidence to support a role for palmitoylation in targeting of proteins to specialised regions of the cell, known as rafts (regions of the cell thought to be important for signal complex formation) (Anderson, 1998).

Palmitoylation status has been implicated to affect a vast array of signalling interactions and processes. Some examples are GPCR-G protein interactions, RGS-G protein interactions, GPCR phosphorylation, GPCR desensitisation and down-regulation, as well as caveolar targeting and membrane localisation of many proteins. The reversibility of thioester-attached palmitate is thought to be critically important for these interactions, because regulation would offer co-ordinated control of the membrane binding and/or protein-protein interactions of these proteins (Milligan *et al.*, 1995; Wedegaertner *et al.*, 1995).

Many suggestions for the role for palmitoylation in GPCRs and G proteins have derived initially from the study of other cellular proteins capable of undergoing palmitoylation. This lipid modification has been shown to be particularly prevalent for proteins involved in processes such as cell adhesion, cell growth and signal transduction, providing further support that palmitoylation may play a more important role than just hydrophobic interactions. Some examples of other cell proteins known to be palmitoylated include GAP-43 (Arni *et al.*, 1998), endothethial nitric oxide synthase (eNOS) (Garcia-Cardena *et al.*, 1996), several tyrosine kinases (Shenoy-Scaria *et al.*, 1994), post-synaptic density protein (PSD-95) (El-Husseini *et al.*, 2002), RGS proteins (Tu *et al.*, 1999), and GRKs (Premont *et al.*, 1996). To summarise, the role of palmitoylation for many (e.g.GAP-43, TKs, eNOS) seems to be in targeting to caveolae, whereas, the role for others (GRKs and RGS proteins) appears to be in promoting hydrophobic interactions (e.g. protein-membrane or protein-protein interactions). However, the role of palmitoylation for one of these proteins, PSD-95, seems to be in functional regulation. The AMPA-type glutamate receptors are involved in the control of synaptic strength and PSD-95 is a protein involved in the regulation of this process. Palmitate turnover on PSD-95 is regulated by the activity of the glutamate receptor and blocking palmitoylation is found to disperse synaptic clusters of PSD-95, causing a selective loss of synaptic AMPA receptors. It has also been shown that rapid glutamate-mediated AMPA receptor internalisation requires depalmitoylation of PSD-95 (El-Husseini *et al.*, 2002).

1.5.3 Palmitoylation and caveolae

Caveolae are subdomains of glycolipid rafts. Structurally, they are small invaginations of the plasma membrane and they can be found in various cells. The major components of caveolae are cholesterol, sphingolipids and structural proteins named caveolins. Three caveolin isoforms are known: caveolin-1, caveolin-2 and caveolin-3. Caveolin has been shown to co-purify with many lipid modified signalling molecules, including GPCRs, G proteins, H-Ras, eNOS, and Src family tyrosine kinases (Song *et al.*, 1996). It has been suggested that protein-protein interactions with caveolin protein may drive fatty acylated proteins into caveolae. The C-terminal domain of caveolin-1 undergoes palmitoylation on three residues, Cys^{133}, Cys^{143}, and Cys^{156} (Dietzen *et al.*, 1995).

Palmitoylation of caveolin is not required for its localisation to caveolae but appears to facilitate its interaction with $G_i\alpha$ (Galbiati *et al.*, 1999) and is required for cholesterol binding, chaperone complex formation and rapid transport of cholesterol to caveolae (Uittenbogaard and Smart, 2000).

A role for palmitoylation in directing signalling proteins to caveolae is suggested by mutagenesis (Robinson and Michel, 1995), fractionation and immunofluorescence experiments (Garci-Cardena *et al.*, 1996). This has been shown for a number of $G\alpha$ subunits, including $G_{i1}\alpha$ (Galbiati *et al.*, 1999; Moffett *et al.*, 2000) and $G_{o1}\alpha$ (Guzzi *et al.*, 2001). In further support of this hypothesis, myristoylation (Gly^2Ala) and palmitoylation (Cys^3Ser) mutants of $G_{i1}\alpha$ were poorly targeted to caveolae-enriched membrane fractions (Song *et al.*, 1997). Mutational or pharmacological activation of $G_s\alpha$ has also been shown to prevent its cofractionation with caveolin (Li *et al.*, 1995). These authors proposed a mechanism whereby palmitoylation is necessary for initial targeting to caveolae and subsequent interaction with the other molecules required for signal transduction. Upon agonist activation, the ensuing depalmitoylation of the G protein observed by many groups (Iiri *et al.*, 1996) is thought to allow the deacylated protein to leave the caveolar membrane (Li *et al.*, 1995) and localise to either the cytoplasm (Wedegaertner *et al.*, 1996) or non-caveolar membrane (Huang *et al.*, 1999). GPCRs have also been reported to associate with caveolae (Moffett *et al.*, 2000). Some GPCRs have been shown to localise to caveolae in response to receptor agonist but not to antagonists (Feron *et al.*, 1997; Raposo and Benedetti, 1994), suggesting that clustering or receptor activation by ligand binding is sufficient to promote regulated translocation into caveolae membranes.

1.5.4 Palmitoylation and βγ Subunits

It has been observed in a number of studies that the βγ-subunits of G proteins are important for various aspects of GPCR-G protein signalling. It is thought that a further aspect of the regulation by βγ-subunits involves the palmitoylation of G protein α-subunits. It has previously been shown that reciprocal regulation of $G_s\alpha$ can occur by palmitate and the βγ-dimer (Iiri *et al.*, 1996). These authors suggested that

palmitoylation of Gα as well as binding of βγ to α-subunits promotes $G_s\alpha$ membrane attachment. Subsequent hormonal activation of $G_s\alpha$ is then proposed to promote dissociation of $G_s\alpha$-GTP away from the βγ-dimer, accelerating depalmitoylation of the α subunit and ultimately triggering release of some of the $G_s\alpha$ to the cytosol. However, more recent studies have provided evidence that the βγ-subunit is not the only moiety involved in regulation of palmitoylation on Gα subunits. In these studies a role for N-myristoylation, as a pre-requisite for palmitoylation, was highlighted (Wang *et al.*, 1999). This work used mutants of $G_o\alpha$ unable to bind βγ but able to be N-myristoylated. It was found that these G proteins could still undergo palmitoylation. However, if N-myristoylation was removed from these proteins the βγ-subunit then became necessary for subsequent palmitoylation to occur. In light of this work, these authors suggested an (at least partly) overlapping role for N-myristoylation and βγ-subunits exists in the regulation of palmitoylation of Gα subunits. This is thought to be the reason that Gβγ has been observed to be so important for membrane targeting and palmitoylation of Gα-subunits such as $G_s\alpha$ and $G_q\alpha$ which do not undergo myristoylation. When the βγ binding regions in these α-subunits were mutated the Gα no longer localised to plasma membranes and also exhibited reduced palmitoylation (Evanko *et al.*, 2000). It has since been shown that the effects of these mutations can be overcome upon overexpression of specific Gβγ subunits, indicating the importance of the dimer in proper membrane localisation and subsequent palmitoylation (Evanko *et al.*, 2001).

1.5.5 Cycles of Palmitoylation

In 1985, it was realised that the thioester bond, through which palmitate was attached to some proteins, was one of its most important and distinctive features. The chemical reactivity of the thioester linkage was already known to make it sensitive to various treatments, such as mild alkali (Schmidt *et al.*, 1979), nucleophilic agents (Schlesinger *et al.*, 1980) and thiol reagents (O'Brien *et al.*, 1987). The knowledge of this chemical reactivity led to the now widely accepted proposal that protein palmitoylation, via a thioester bond, is a reversible modification with the potential to undergo dynamic regulation (Mumby, 1997). To date, turnover of covalently attached palmitate has been reported for several proteins such as PSD-95 (see section 1.5.2), GPCRs (Mouillac *et*

al., 1992) and G proteins (Wedegaertner and Bourne, 1994). In addition it is now known that this process can be affected by external stimuli (James and Olsen, 1989; Chen and Manning, 2000).

Several studies have shown that agonist stimulation can selectively modulate the palmitoylation state of GPCRs (Ng *et al.*, 1994; Mouillac *et al.*, 1992) by promoting an increase in the turnover rate of receptor-bound palmitate (Loisel *et al.*, 1996). Similar effects have been observed for palmitate attached to $G_s\alpha$ following G protein activation by direct stimulation (Mumby *et al.*, 1994; Degtyarev *et al.*, 1993) as well as by cholera toxin treatment (Degtyarev *et al.*, 1993). These results indicated that palmitoylation-depalmitoylation cycles might occur at the cell surface where the receptor is accessible to agonists. It was suggested that the role of such a cycle might be to co-ordinate regulation of signalling, by determining either the location of proteins or their propensity to interact with other molecules (Mumby, 1997). Some investigators have attempted quantitative analyses of the stoichiometry of G protein palmitoylation. For $G_s\alpha$, it was found that the overall percentage of the protein palmitoylated before and after receptor activation did not change (Jones *et al.*, 1997). This makes the important point that it may be alteration in turnover rate of palmitate on the protein, not necessarily alterations in absolute levels of palmitoylation which may prove to be important.

It is now generally accepted that palmitoylation-depalmitoylation cycles do occur, presumably facilitated by enzymes which catalyse palmitate addition (palmitoyl transferase) and removal (palmitoyl thioesterase) (**Figure 1.6**). However, the exact molecular mechanisms of palmitate regulation are still partially shrouded in mystery since, despite rigorous efforts, there has been no definitive characterisation of an appropriate palmitoyltransferase enzyme (Qanbar and Bouvier, 2003), and in addition, the two proposed classes of palmitoylthioesterases, APT-1 and PPT-1, still require further characterisation (Linder and Deschenes, 2003).

Figure 1.6

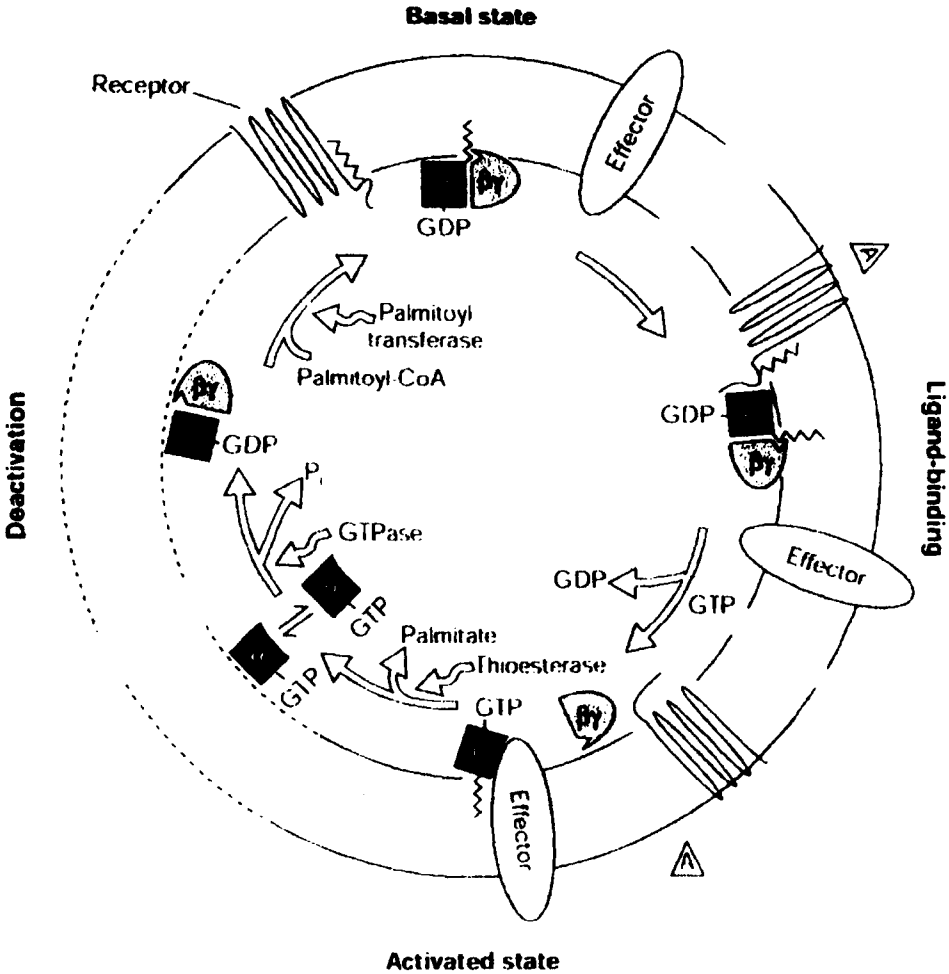
Cycles of Palmitoylation

The diagram depicts the proposed regulation of palmitoylation status at various stages of the G protein activation cycle. It also depicts the role played by the enzymes thought to be involved in catalysis of these changes (palmitoyltransferase and palmitoylthioesterase) and palmitoyl CoA, the proposed palmitate donor for the acylation reactions. Thioesterification by palmitate is represented by the jagged line appended to either the GPCR or the Gα subunit. Gaps within the membrane serve to separate the different steps of the cycle. A triangle surrounding the letter A represents ligand.

In the basal state (top) and during ligand-binding (right), the receptor and G protein are palmitoylated. In the activated state (bottom), receptor and Gα-GTP (dissociated from Gβγ) become depalmitoylated either non-enzymatically or by a thioesterase. Acylated or deacylated Gα can modulate effector, although in this example only palmitoylated Gα is shown to interact with effector. Depalmitoylated Gα may partition out of the plasma membrane subdomain that is enriched in receptor and effector (the light-density caveolar-like subdomain, represented by the two solid parallel lines) into another subdomain (represented by the two dotted parallel lines) and/or into the cytoplasm (partitioning into the cytoplasm is represented by the reversible arrow symbol). Hydrolysis of GTP by the intrinsic GTPase activity of Gα triggers the conformational change in this subunit and Gα-GDP rebinds to membrane-associated Gβγ. The heterotrimer is the preferred reactant in either the enzyme-catalysed palmitoylation or the autopalmitoylation of Gα. The repalmitoylated protein may partition back to the receptor- and effector- rich subdomain of the plasma membrane.

Mumby,S.M. 1997. *Current Opinion in Cell Biology.* **9(2),** 148-154.

Figure 1.6


Basal state
Receptor
Effector
GDP
Palmitoyl transferase
Palmitoyl-CoA
GDP
Deactivation
Ligand-binding
GTPase
GDP
GTP
Effector
Palmitate
Thioesterase
GTP
GTP
Effector
Activated state

The lack of clarity over the mechanisms of palmitate addition has proved the biggest problem. Currently there are two distinct views of how protein palmitoylation may occur. One view is continued support for the existence of a palmitoyltransferase, whilst the other challenges the existence of this enzyme, believing that palmitoylation occurs spontaneously in cells (autopalmitoylation). At present, it is unclear which of these two situations is most likely, or indeed, whether both mechanisms may play some role.

Regardless of which scenario is unveiled, the idea of a regulated cycle of palmitoylation is still feasible. Theoretically, it would suffice to regulate one step of the reversible reaction in order to achieve cycling of the overall process. The evidence supporting each of these suggested mechanisms of palmitate regulation has been recently reviewed (Qanbar and Bouvier, 2003; Linder and Deschenes, 2003).

The search for a palmitoyltransferase enzyme has resulted in numerous reports of palmitoyltransferase activity, carried out by many proteins in many different regions of the cell (reviewed in Qanbar and Bouvier, 2003). Perhaps in light of the diverse array of proteins to which this fatty acid is attached, this is not surprising. It may be somewhat naïve to expect one ubiquitously expressed enzyme could be responsible for all palmitate additions. Searching for this enzyme has been made difficult by two main problems. The first problem is the inherent instability of palmitoyltransferase activity. Some studies have reported the need for detergent to solubilise the activity (Berthiaume and Resh, 1995) whilst other studies report loss of activity during purification attempts (Dunphy *et al.*, 2000). The second problem in searching for a specific palmitoyltransferase enzyme comes from the distracting observation of palmitoyltransferase activity by many enzymes that have a primary role in lipid metabolism. To date, the most promising demonstrations of palmitoyltransferase activity are by proteins such as the yeast protein complex Erf2p-Erp4p (Bartels *et al.*, 1999), required for the ER to plasma membrane localisation of yeast Ras proteins. However, further work is obviously required to find proteins capable of performing this role at or near the plasma membrane.

The phenomenon of autopalmitoylation has been observed for a number of types of protein such as rhodopsin (O'Brien *et al.*, 1987), Gα subunits (Duncan and Gilman,

1996) and RGS4 and RGS10 (Tu *et al.*, 1999). This autopalmitoylation does not appear to occur randomly on any cysteine residue, with some reports suggesting a degree of sequence specificity for the process (Belanger *et al.*, 2001). In some cases, the sites for autopalmitoylation are the same as those demonstrated *in vivo* (Ross and Braun, 1988) but this is not always the case (Scholich *et al.*, 2000). The physiological relevance of autopalmitoylation is also cause for controversy, since kinetic calculations have predicted that autopalmitoylation would probably be too slow under physiological conditions (Leventis *et al.*, 1997).

In contrast to the debated existence of palmitoyltransferases there seems to be no question of the existence of palmitoylthioesterases. The first reports of this activity were made in 1986 (Berger and Schmidt, 1986), before subsequent purification of the first palmitoylthioesterase, PPT-1 (Camp and Hofmann, 1993) and a related protein, PPT-2 (Soyombo and Hofmann, 1997). It was later discovered that these two proteins were actually lysosomal enzymes, with primary function in the degradation of acylated peptides not the turnover of palmitate on proteins. Another enzyme, APT-1, was then discovered which was a thioesterase for palmitoylated proteins such as $G_s\alpha$ (Duncan and Gilman, 1998) and eNOS (Yeh *et al.*, 1999). The subsequent resolution of the crystal structure of APT-1 (Devedjiev *et al.*, 2000), as well as data from other functional studies, led to the realisation that structurally it belonged to the α/β hydrolase family (which includes lipases, esterases, dehalogenases and thioesterases). When coupled to the knowledge that the most recently purified palmitoylthioesterases were also lipid-modifying enzymes (Liu *et al.*, 1996; Ueno, 2000), a hypothesis for the existence of dual activity for these enzymes was proposed. However, this hypothesis still requires further research.

1.5.6 Experimental Methods for the Study of Palmitoylation

Palmitoylation of proteins is notoriously tricky to study and this has been responsible for the comparatively slow progress in this area of GPCR-G protein signalling. A variety of technical limitations make the study of this lipid modification difficult. Firstly, the instability of this modification, arising from the thioester bond, renders it susceptible to cleavage in the presence of various chemicals, e.g. the reducing agent

dithiothreitol (DTT) (O'Brien *et al.*, 1987). Another problem arising from the thioester bond is the inherent reversibility of the modification. Whilst this is presumed to be an invaluable feature for proteins *in vivo*, it can make things difficult when studying the stoichiometry of palmitate on proteins. The standard approach for studying palmitoylation is by incubation of cells with high levels of radiolabelled palmitate, and monitoring subsequent incorporation/removal of the radiolabelled lipid from proteins (Mouillac *et al.*, 1992). However, there are two main limitations to this metabolic labelling approach. Firstly, palmitic acid is a major fatty acid of cellular lipids, therefore the majority (>99.5%) of the radioactivity is incorporated into lipids, leaving only a tiny amount remaining for labelling of proteins (i.e. isotopic dilution) (Grange *et al.*, 1995). As a result, the protein of interest must be fairly abundant in the cell type analysed to detect palmitoylation successfully. The second limitation is the nature of the radiolabelled palmitic acid. At the moment, the best available form for this metabolic labelling is [9, 10 (n)-^{3}H] palmitic acid. Therefore, the poor penetrance of [^{3}H] as an emitter means that it often takes weeks to months for exposure of SDS-PAGE fluorograms or western blots, hampering the rate of the investigative progress.

The early studies of palmitoylation (McIlhinney *et al.*, 1985; Magee *et al.*, 1987) established the nature of this lipid modification as dynamic and post-translational. This was achieved by two experimental approaches. The first approach was the metabolic labelling of cells with radioactive palmitate in the presence of cycloheximide, an inhibitor of protein synthesis (since palmitate labelling in the absence of protein synthesis is indicative of a post-translational, dynamic process). The second approach was to compare the rate of turnover for the palmitate moiety with the rate of turnover for the protein itself (where a faster turnover rate for palmitate is indicative of a dynamic process). Since then, most studies have focused on either the functional consequences of palmitate exclusion, or the regulation of palmitate turnover for the various GPCRs and G proteins.

A very important point has arisen with respect to the methods by which the functional consequences of palmitoylation are assessed. To date, many studies have involved the mutational exclusion of palmitate. However, it has to be taken into consideration that loss of the cysteine residues, rather than lack of palmitate, could contribute to the

observed effects. There are some other methods to remove palmitate from proteins *in vitro*, such as hydroxylamine treatment or deacylating enzymes, which have been successfully used by a number of groups (Morrison *et al.*, 1991; Camp *et al.*, 1994; Hepler *et al.*, 1996). However, use of these alternative approaches are not amenable to every type of study, leaving site-directed mutagenesis of cysteine as one of the only other options. Concern has arisen because the cysteine sulphydryl group itself may be required for some aspects of protein structure or function. Palmitoylation of cysteine temporarily blocks these free sulphydryl groups, but depalmitoylation makes them re-available for interaction. Mutational exclusion of cysteine would consequently abolish all roles played by this residue. Therefore, appropriate caution must be taken before interpreting results from mutational studies (Hepler *et al.*, 1996). The importance of this caution has been well illustrated by the study of $G_q\alpha$ protein. Mutational exclusion of the Cys^9 and Cys^{10} residues gave rise to different experimental results than those for the wild type protein depalmitoylated with a thioesterase *in vitro* (Hepler *et al.*, 1996).

To study the regulation of palmitate turnover in GPCRs and G proteins a type of metabolic labelling assay (an *in vivo* palmitoylation assay) is carried out. The relatively low *in vivo* expression of molecules such as GPCRs would make it very difficult to assess palmitoylation in this context. This is why a system is necessary, where the protein can be overexpressed to reach much more easily detectable levels by use of a heterologous expression system. As already explained, a huge amount of the incorporated palmitate is present in cellular lipids and a substantial fraction also as palmitoyl-coenzyme A (the acyl donor for palmitoylation; formed from palmitic acid, after penetration into cells and transformation by ATP-driven esterification, with extra-mitochondrial CoA-SH, to yield palmitoyl-CoA). In light of this it is therefore necessary to purify the target protein in isolation from the various other palmitoylated proteins present in cell lysates. In order to do this an immunoprecipitation step with a protein-specific antibody is incorporated into the palmitoylation assay (Stevens *et al.*, 2001).

Two main types of palmitoylation assay have been routinely carried out; pulse-labelling and pulse-chase. The distinctions between the two assays and the processes they represent must be understood before results from each can be properly interpreted.

Pulse-labelling involves the incubation of cells with radiolabelled palmitate and measuring the incorporation of the radiolabel over a certain period of time. This can be done in the presence or absence of agonist. In this assay the observed incorporation of palmitate reflects a combination of the effects of both the palmitoylation and depalmitoylation reactions. In contrast, the other type of palmitoylation assay, a pulse-chase assay, allows an estimation of kinetics for the rate of depalmitoylation irrespective of the rate of repalmitoylation. In this assay, after an initial pulse period to achieve steady-state labelling of palmitate attached to the protein of interest, depalmitoylation can be observed by incubating the cells with non-radioactive palmitate, and monitoring the decrease in attached ^{3}H palmitate with increased chase time. The time taken for half the palmitoylated proteins to undergo fatty acid cleavage ($t_{1/2}$ depalmitoylation) can then be estimated from these experiments (Magee *et al.*, 1987).

1.5.7 GPCR palmitoylation

Palmitoylation of GPCRs was first demonstrated on two conserved cysteine residues (Cys^{322} and Cys^{323}) of the visual receptor rhodopsin (O'Brien and Zatz, 1984; Ovchinnikov *et al.*, 1988). Subsequent to this, by primary sequence comparison, similarly conserved cysteine residues were revealed to be present in the C-terminal tail of a number of GPCRs. This has ultimately led to the physical demonstration of palmitoylation on many GPCRs, including the α_{2A}-adrenoceptor (Kennedy and Limbird, 1994) and the 5-HT_{1A}-serotonin receptor (Ng *et al.*, 1993; Papoucheva *et al.*, 2004) used in this study. The reason for palmitoylation of integral membrane proteins, such as GPCRs, is considered somewhat enigmatic, given that the modification would presumably be unnecessary for stable membrane attachment or intracellular targeting (Veit *et al.*, 1991). It has therefore been suggested that palmitoylation may play an important role in the functioning of GPCRs.

To date, a number of very different responses have been observed upon mutation of GPCR palmitoylation sites (the details of which, for a selection of GPCRs, are outlined in **Table 1.2**), raising the possibility that a shared functional role may not exist.

Table 1.2

The effects of mutating various GPCR palmitoylation sites

Outlined in the table are a selection of GPCRs. The observed effects of mutating palmitoylation sites in these receptors is noted.

Table 1.2

GPCR	Fuctional Effects of Mutating Palmitoylation Sites
Rhodopsin receptor	No effect on ligand binding or Gα activation Cys^{323} mutant showed reduced light-dependent phoshorylation by rhodopsin kinase (Karnik *et al.*, 1993)
β_2-adrenoceptor	Decreased adenylyl cyclase stimulation Increased basal phosphorylation of receptor Decreased agonist-mediated phosphorylation of receptor (O'Dowd *et al.*, 1989; Moffett *et al.*, 1996, 2001)
α_{2A}-adrenoceptor	No effect on coupling to G_i/G_o proteins No effect on receptor phosphorylation No agonist-promoted down-regulation (Kennedy and Limbird, 1993, 1994; Eason and Liggett, 1992, 1993; Eason *et al.*, 1994)
m_2 muscarinic receptor	Still activates G protein but not to same extent as wild type. (Hayashi and Haga, 1997)
TRH receptor	Membrane expression level, high affinity agonist binding, Gs coupling, homologous desensitisation and agonist-promoted internalisation not affected by mutation. Decreased rate of intracellular trafficking (delayed but not trapped). (Tanaka *et al.*,1998). Decreased agonist-promoted internalisation (Groarke *et al.*, 2001)
LH/hCG receptor	Normal trafficking Normal cAMP production Increased agonist-promoted internalisation and down-regulation (Kawate *et al.*, 1997; Kawate and Menon, 1994; Munshi *et al.*, 2001) Decreased cell surface expression (Zhu *et al.*, 1995)
5-HT_{4A} receptor	Triple mutants: Still interacts with Gs Still stimulate Adenylyl Cyclase activity Still activate cyclic nucleotide-sensitive cation channels Double mutants (Cys^{328}Ser, Cys^{329}Ser): Increased capacity to be constitutively active (Ponimaskin *et al.*, 2002)
Vasopressin V_{1a} receptor	Decreased basal and agonist-stimulated phosphorylation Increased sequestration rate No effect on ligand binding No apparent effect on intracellular signalling (Hawtin *et al.*, 2001)

Vasopressin V_2 receptor	Decreased expression at cell surface with double mutant Still get normal Adenylyl Cyclase stimulation Still get normal ligand binding (Schulein *et al.*,1996; Sadeghi *et al.*, 1997) Reduced rate of agonist-promoted internalization/sequestration (Schulein *et al.*,1996; Charest and Bouvier, 2003) Significantly decreased agonist-promoted ERK1/2 activation (Charest and Bouvier, 2003)
Dopamine D_1 receptor	Still get normal Adenylyl Cyclase stimulation Still get high affinity agonist binding (Jin *et al.*, 1997)
A_1 adenosine receptor	Little difference in receptor-effector coupling, agonist-promoted internalisation and down-regulation Enhanced proteolysis of mutants (Gao *et al.*, 1999)
A_3 adenosine receptor	Appearance of agonist-independent basal phosphorylation of mutants (Palmer and Stiles, 2000)
Histamine H_2 receptor	Enhanced down-regulation of mutants
5-HT_{1A} receptor	Decreased G_i coupling and inhibition of Adenylyl Cyclase activity by replacement of individual Cys^{417} or Cys^{420} Abolished G_i coupling and inhibition of Adenylyl Cyclase activity by double mutant Decrease in receptor-dependent activation of ERK (Papoucheva *et al.*, 2004)
CCR_5 receptor	Shorter protein half-life Decreased membrane expression No effect on Ca^{2+} mobilisation No effect on inhibition of Adenylyl Cyclase (Percherancier *et al.*, 2001) Impaired trafficking (Percherancier *et al.*, 2001 ; Blanpain *et al.*, 2001) May affect duration of functional response May decrease G protein activation (from GTPγS binding studies) (Blanpain *et al.*, 2001) Profoundly reduces PMA-induced receptor phosphorylation, homologous desensitisation and internalization. (Kraft *et al.*, 2001).

Roles for GPCR palmitoylation have been suggested in the creation of a fourth intracellular loop, cell surface expression, sub-domain targeting, agonist regulation of receptor function, G protein interactions, phosphorylation and desensitisation, sequestration and internalisation, as well as down-regulation. The importance of palmitoylation in each of these processes is discussed in the following paragraphs.

Creation of a Fourth Intracellular Loop

The position of the palmitoylated cysteine residues (15-20 residues from the proposed membrane-cytosol border) of many GPCRs, led to the hypothesis that a fourth intracellular loop could be created if the palmitate moiety were to be inserted in the bilayer (Ovchinnikov *et al.*, 1988). The first demonstrations of palmitate insertion into the membrane (Moench *et al.*, 1994) and the existence of the fourth loop (Ganter *et al.*, 1992) were then shown for rhodopsin. Further evidence for the existence of a fourth intracellular loop arose from the X-ray crystallographic structure of inactive bovine rhodopsin (Palczewski *et al.*, 2000). This high-resolution three-dimensional structural information indicated an eighth helix in addition to the expected bundle of seven TM helices. This eighth helix is thought to have arisen by palmitate insertion into the bilayer. Several studies have suggested a role for this region in G protein activation (Altenbach *et al.*, 2001; Krishna *et al.*, 2002; Okuno *et al.*, 2003). For some GPCRs, regions of the fourth intracellular loop are thought to be involved in GPCR-G protein interactions. In one study (Konig *et al.*, 1989), a synthetic peptide from the fourth intracellular loop of rhodopsin was capable of interacting with transducin.

Cell Surface Expression

Receptor intracellular trapping has been reported upon mutation of palmitoylation sites in the LH/hCG receptor (Zhu *et al.*, 1995), the CCR_5 receptor (Percherancier *et al.*, 2001), and the vasopressin V_2 receptor (Schulein *et al.*, 1996; Sadeghi *et al.*, 1997). Therefore for these proteins, palmitoylated cyteines appear to play a role in the normal processing of GPCRs.

Sub-Domain Targeting

It has been reported for the m_2 muscarinic receptor that palmitoylation may be important for the targeting of this GPCR to specialised subdomains of the plasma

membrane, known as caveolae (Feron *et al.*, 1997). This targeting is believed to promote interaction of the GPCR with signalling complexes located in these subdomains, thereby promoting signal transduction.

Agonist Regulation of Receptor Function

Altered, usually increased, incorporation of ^{3}H palmitate into various GPCRs has been observed upon agonist stimulation (Mouillac *et al.*, 1992; Loisel *et al.*, 1996; Ponimaskin *et al.*, 2001). Later, this effect was attributed to an increased turnover rate of the receptor-bound palmitate upon receptor activation (Loisel *et al.*, 1996, 1999). This increased turnover rate of palmitate on GPCRs was observed for the β_2 and α_{2A}-adrenergic receptors (Loisel *et al.*, 1996; Kennedy and Limbird, 1994). In addition agonist-stimulated incorporation of palmitate has been reported for the muscarinic m_2 receptor (Hayashi *et al.*, 1997) and the 5-hydroxytryptamine$_{4A}$ receptor (Ponimaskin *et al.*, 2001). However, increased palmitate incorporation upon agonist stimulation is not always observed. In the work by Loisel *et al.* (1996), the agonist stimulated increase in ^{3}H palmitate incorporation was found to only occur transiently and after prolonged agonist stimulation of the β_2AR a decrease in incorporation of ^{3}H palmitate was actually observed. In this study, reduction of the incorporation of ^{3}H palmitate in response to agonist pre-treatment was also observed. These findings led the authors to suggest that upon prolonged agonist stimulation receptor repalmitoylation was inhibited, possibly implying a role for palmitoylation status in desensitisation. There are also GPCRs for which no change in incorporation of ^{3}H palmitate is observed upon agonist stimulation, such as the adenosine A_1 receptor (Gao *et al.*, 1999). As well as other GPCRs, which exhibit slight decreases in incorporation of ^{3}H palmitate upon stimulation, such as the vasopressin V_2 receptor (Sadeghi *et al.*, 1997). It is important to re-emphasise at this point that results obtained by pulse-labelling studies are not necessarily reflective of an overall difference in palmitoylation state, they are the combined effect of the rates of both palmitoylation and depalmitoylation. Thereby, an agonist-promoted increase in palmitate turnover could give rise to either a decrease or an increase in palmitate incorporation, depending on the kinetics and experimental conditions. Receptor agonist is not the only moiety shown to be able to regulate palmitoylation. Adam *et al.* (1999) have reported that nitric oxide (NO) could suppress the incorporation of ^{3}H palmitate

into the β_2-adrenoceptor, both in the basal and activated states. They also reported that NO could reduce agonist stimulated adenylyl cyclase activity by this receptor and therefore proposed that these effects may be a consequence of depalmitoylation of the receptor.

G Protein Interactions

Mutation of palmitoylation sites in the β_2-adrenoceptor was reported to reduce the efficiency of receptor-G protein interaction since this mutation resulted in decreased adenylyl cyclase activity (O'Dowd *et al.*, 1989). Later, it was realised this abrogation of GPCR-G protein interactions was indirectly brought about by the increased basal phosphorylation of palmitoylation-site mutants (Moffet *et al.*, 1996). Effects on signalling by mutating palmitoylated cysteines have not been observed for all GPCRs tested. In fact, there seem to be rather more examples of receptors that can still signal successfully despite palmitoylation-site mutations. This has been observed for the α_{2A}-adrenoceptor (Kennedy and Limbird, 1994), the LH/hCG receptor (Kawate and Menon, 1994; Kawate *et al.*, 1997), the dopamine D_1 receptor (Jin *et al.*, 1997), the A_1 adenosine receptor (Gao *et al.*, 1999) and the TRH receptor (Tanaka *et al.*, 1998).

Phosphorylation and Desensitisation

Phosphorylation by a number of kinases, including PKA and GRKs, initiates events leading to receptor desensitisation. Palmitoylation is now believed to play a role in these events. This view has resulted from a number of studies. Firstly, as outlined previously, the β_2-adrenoceptor undergoes increased basal phosphorylation upon mutation of palmitoylation sites (Moffett *et al.*, 1996), a phenomenon also reported for the adenosine A_3 receptor (Palmer and Stiles, 2000). The work by Moffett *et al.* (1996) involved the creation of palmitoylation-deficient mutants ($Cys^{341}Gly$) also containing mutations in two phosphorylation sites ($Ser^{345}Ala$ and $Ser^{346}Ala$). Triple mutants were observed to have normal phosphorylation and receptor coupling. From this work and the results of further studies by Moffett *et al.* (2001), it was therefore concluded that palmitoylation restrained the access of various kinases to the GPCR phosphorylation sites. These studies would appear to suggest that in order for phosphorylation, functional uncoupling and desensitisation of the β_2AR to occur, this GPCR must first

undergo depalmitoylation. There are also examples where palmitoylation deficient mutants exhibited decreased agonist-stimulated phosphorylation (V_{1a} vasopressin receptor, Hawtin *et al.*, 2001), and reduced internalisation and desensitisation (CCR_5 chemokine receptor, Kraft *et al.*, 2001).

Sequestration and Internalisation

For many receptors internalisation occurs closely after phosphorylation. Internalisation has been implicated in both desensitisation and resensitisation events (Ferguson and Caron, 1998). In addition to the CCR_5 chemokine receptor already discussed, the internalisation properties of other GPCRs are also affected by the ability of the receptor to undergo palmitoylation. This has been reported for the vasopressin V_2 (Schulein *et al.*, 1996) and TRH (Groarke *et al.*, 2001) receptors. In these two receptors, as for the CCR5 receptor, mutating palmitoylation sites leads to decreased internalisation properties. The decreased agonist-promoted internalisation of the TRH receptor was shown to be as a result of decreased interaction of this GPCR with arrestin. Therefore, it is thought that palmitoylation may play a regulatory role in this interaction (Groarke *et al.*, 2001). A contrasting example for the role of GPCR palmitoylation in internalisation is that of the LH/hCG receptor. Studies of this receptor showed that mutation of palmitoylation sites resulted in double the rate of internalisation for the mutants compared with the wild type (Kawate and Menon, 1994). These authors also noted some link between palmitoylation and arrestin, whereby the internalisation rate of both the wild type and mutant receptor were enhanced by arrestin overexpression, but this phenomenon was especially pronounced for the palmitoylation-mutant receptor.

Down-regulation

In addition to the role of palmitoylation in phosphorylation and internalisation events, it has also been implicated in the process of desensitisation. The mutation of the palmitoylation sites in the LH/hCG receptor, which were observed to result in increased internalisation of the receptor, were additionally found to enhance the down-regulation of this receptor (Kawate *et al.*, 1997). An enhanced down-regulation of palmitoylation mutants was also observed for the histamine H_2 receptor (Fukushima *et al.*, 2001). The opposite effect was however observed for the α_{2A}-adrenoceptor. In this case, no down-regulation was observed for a palmitoylation-mutant receptor. Interestingly, the

phenotype in-terms of down-regulation for this particular α_{2A}-adrenoceptor mutation ($Cys^{442}Phe$), correlates with that of the α_{2C}-adrenoceptor. The α_{2C}-adrenoceptor lacks a palmitoylation site (having a phenylalanine residue naturally at the corresponding position in its sequence), and fails to undergo agonist-induced down-regulation (Eason and Liggett, 1992; 1993).

In summary, if it is taken into consideration that various aspects of protein structure and function can be influenced by palmitoylation, then it is perhaps not surprising that such a diverse array of functional effects are observed. GPCR-specific roles for palmitoylation may therefore reflect the unique nature of each GPCR and the specific processes it regulates. Therefore, a considerable amount of further research is required before any definite conclusions can be drawn concerning the exact role(s) of palmitoylation for the GPCR family.

1.5.8 G Protein Palmitoylation

Many Gα proteins, such as $G_s\alpha$ (Degtyarev *et al.*, 1993), $G_q\alpha$ (Hepler *et al.*, 1996) and $G_{o1}\alpha$ (Grassie *et al.*, 1994), have been shown to undergo palmitoylation near their N-terminus. This lipid modification has been implicated in various aspects of G protein function such as membrane association, subdomain localisation, efficient protein-protein interactions, signalling efficiency and agonist regulated G protein activation. The current evidence supporting each of these roles is outlined in the following paragraphs.

Membrane Association

G protein heterotrimers are peripheral membrane proteins which require access to the inner surface of the plasma membrane to perform their function. These proteins gain this access via a number of strategies such as lipid modifications and protein-protein interactions (Chen and Manning, 2001). The presence of palmitate on G proteins seems to be required for membrane anchoring, although other hydrophobic modifications (such as myristoylation and prenylation) are also involved (Resh, 1994). In support of this anchorage role various studies indicate mutations in palmitoylation sites result in more soluble or mislocalised Gα subunits (Wedegaertner *et al.*, 1993; Grassie *et al.*,

1994). Furthermore, G_s activation and depalmitoylation led to its redistribution to the cytosol (Wedegaertner and Bourne, 1994). However, such a cytosolic shift has not been observed in all cases. An interesting, contradictory observation concerning the consequences of G_s depalmitoylation has been reported (Huang *et al.*, 1999), where direct enzymatic depalmitoylation failed to modify membrane attachment of this Gα protein. Although some relationship between membrane association of the Gα proteins and palmitoylation has been suggested, the reason for this requirement is still speculative. It is thought that membrane association may target the protein to a region where the enzymes of the palmitoylation cycle and/or an abundant supply of palmitate donor may reside, promoting palmitate cycling (Qanbar and Bouvier, 2003).

Subdomain Localisation

Palmitoylation can contribute to targeting of Gα subunits to specific subcellular compartments such as the Golgi complex, the plasma membrane or caveolae (Mumby, 1997). The specific subcellular distribution of Green Fluorescent Protein (GFP)-fused constructs was shown to be affected by palmitoylation status (McCabe and Berthiaume, 1999). A number of G proteins have now been shown to localise to the special membrane invaginations known as caveolae. One such example is $G_{i1}\alpha$, whose partitioning into this subdomain increased four-fold upon palmitoylation of an already myristoylated α subunit (Song *et al.*, 1997).

Protein-Protein Interactions

The presence of palmitate on Gα subunits has been shown to affect its association/interaction with a number of proteins. In one study, it was shown that palmitoylated Gα associated more tightly with Gβγ than depalmitoylated Gα (Iiri *et al.*, 1996). Palmitoylation status also plays a regulatory role in the functional interactions between Gα subunits and RGS proteins (Tu *et al.*, 1997). Mutations in either the Gα protein or the RGS protein palmitoylation sites (present in the RGS box, containing the GTPase activating domain) can result in altered ability of the GAP protein to accelerate the GTPase reaction (Chen and Manning, 2001).

Signalling Efficiency

For most Gα subunits, loss of palmitoylation leads to loss of ability to signal effectively. This has been shown for $G_s\alpha$ (Wedegaertner et al., 1993) and $G_q\alpha$ (Edgerton et al., 1994). Also, a constitutively active mutant of $G_{12}\alpha$ was found to lose its transforming activity if its palmitoylation site was mutated (Jones and Gutkind, 1998). However, in some cases, an apparent loss of ability to signal has been attributed to absence of membrane localisation, and was recovered upon fusion of the Gα subunit to a receptor. This has been reported for both the $G_{i1}\alpha$ protein (Wise and Milligan, 1997) and $G_s\alpha$ (Ugur *et al.*, 2003).

Agonist Regulated G Protein Palmitoylation

Agonist regulation of G protein palmitoylation, already briefly discussed, indicates that this modification is indeed important for G protein function. This phenomenon has been observed for a number of G proteins including, G_s via the β_2AR (Wedegaertner and Bourne, 1994) and G_i via the 5-HT_{1A} receptor (Chen and Manning, 2000). The effects of ^{3}H palmitate incorporation (from metabolic labelling studies) were found to vary, dependent on the duration of stimulation. Most studies (Wedegaertner and Bourne, 1994; Chen and Manning, 2000; Stanislaus *et al.*, 1997; Bhamre *et al.*, 1998) observed an increase in the incorporation of ^{3}H palmitate in response to transient agonist stimulation; thought to reflect an increase in turnover of palmitate attached to GTP-bound Gα (Loisel *et al.*, 1999). In support of this, pulse chase palmitoylation assays often show a slightly more rapid depalmitoylation of Gα in response to agonist (Mumby *et al.*, 1994). However, upon long-term agonist treatment, decreases in incorporation of ^{3}H palmitate are often observed (Ammer and Schulz, 1997; Seassholtz *et al.*, 1997). These responses to chronic stimulation, are thought to be linked to desensitisation mechanisms (Ammer and Schulz, 1997).

In summary, as for the GPCRs, there are a number of proposed functional roles for G protein palmitoylation. However, unlike GPCRs, there seem to be more shared functional roles, such as membrane association and protein-protein interactions, for palmitoylation of the heterotrimeric G proteins. Nevertheless, just as is the case for

GPCRs, there is still a lot of work to be done before a clear understanding of the role(s) of this modification are fully appreciated.

1.6 Research Aims

With the implication of the role of palmitoylation in various processes, as well as the demonstration that for some receptors palmitoylation is a dynamic process, the stage has been set for further study of palmitoylation. The regulation of this post-translational modification, and the role it plays is studied herein for two particular types of GPCR-G protein fusion proteins. The α_{2A}-adrenoceptor and the 5-HT_{1A}-receptor are both GPCRs belonging to the rhodopsin-like family. These receptors are not markedly different structurally and both are thought to bind their small monoamine ligands by a similar mechanism. In addition, these two receptors both bind to the $G_{i/o}$ class of Gα proteins, producing some similar downstream effects. Therefore it was decided to study various features of palmitoylation by the use of fusion proteins between the $G_{o1}\alpha Cys^{351}Ile$ protein and each of these two receptors. The presence of potential palmitoylation sites in the GPCRs and in the G protein meant that a variety of different fusion constructs were created, each with varying ability to be palmitoylated. The α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ fusion construct has 2 potential palmitoylation sites, one in the receptor and one in the G protein, therefore four palmitoylation variant α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ constructs were created. The 5-HT_{1A}-receptor-$G_{o1}\alpha Cys^{351}Ile$ fusion construct has 3 potential palmitoylation sites, two in the receptor and one in the G protein, therefore eight palmitoylation variant 5-HT_{1A}-receptor-$G_{o1}\alpha Cys^{351}Ile$ constructs were created for this fusion. A fusion protein approach was employed in the current study in order that we could profit from a number of the beneficial features these constructs offer. Firstly, GPCR-G protein fusion proteins have been demonstrated to function as agonist activated GTPases with Michaelis-Menten kinetics (Wise *et al.*, 1997c), a beneficial feature for functional assays. Secondly, expression of the palmitoylation deficient $G_{o1}\alpha Cys^{351}Ile$ protein at the plasma membrane (allowing interaction with GPCRs) should be ensured upon fusion to a GPCR (Wise and Milligan, 1997). Thirdly, in most cell systems there is a large quantitative excess of G protein

over receptor, meaning agonist regulation of receptor would presumably only regulate a small fraction of the G protein population (Milligan, 2000). By studying palmitoylation in a fusion protein system we should be able to immunoprecipitate both the receptor and the interacting G protein partner, away from the endogenous G protein, avoiding any potential "dilution" of observed effects by non-activated G proteins. These advantages of fusion proteins offer an attractive approach by which to address the role(s) of palmitoylation on GPCRs and G proteins.

The aim of this work was to investigate two main features of palmitoylation of GPCRs and G proteins. The first area explored was the dynamic regulation of palmitoylation in the fused GPCRs and G proteins and the second area involved a study of the functional implications of palmitoylation status in these fusion proteins. In order to do this, the following four specific objectives were set for this study.

1. To analyse the palmitoylation status of defined GPCR-G protein pairs
2. To understand how post-translational acylation of either partner is regulated by agonist ligands.
3. To monitor the function and efficiency of interaction between the protein partners of these fusion constructs using agonist stimulation of high affinity GTPase activity and GTPγS binding.
4. To determine the importance of receptor and G protein palmitoylation for the effectiveness of the regulator of G protein signalling, RGS16.

Chapter 2

Materials and Methods

2.1 Materials

The materials used were obtained from the following suppliers:

American Tissue Culture Collection, Rockville, USA
Human embryonic kidney large T antigen (HEK293T) cells.

Amersham Pharmacia Biotech UK Ltd., Buckinghamshire, UK
[^{3}H]-WAY100635, [^{3}H]-RS-79948-197, [9,10(n)-^{3}H] Palmitic Acid, Glutathione Sepharose 4B, full range Rainbow™ molecular weight marker, anti rabbit IgG-horseradish peroxidase linked antibody (from Donkey), Hyperfilm™ MP autoradiography film, ECL$^+$Plus western blotting detection system.

BDH, Lutterworth, Leicestershire, UK
Glacial acetic acid, orthophosphoric acid, methanol, isopropanol, ethanol, trichloroacetic acid

Becton Dickinson UK Ltd, Oxford, UK
BD Plastipak™ 1ml sterile syringes, Microlance™3 25GA$^{5/8}$ and 26GA$^{3/8}$ needles, Falcon 60mm dishes

Bibby Sterilin Ltd, Staffs, UK
30ml sterilins, 50ml sterilins

BloWhittaker Molecular Applications, Rockland, ME, USA
SeaKem LE Agarose

CN Biosciences, Nottingham, UK
Calbiochem Pansorbin cells

Costar, Cambridge, MA, USA
Cryovials, 5, 10, and 25ml graduated sterile tissue culture plastic pipettes, cell scrapers.

Duchefa, Haarlem, The Netherlands
Tryptone, yeast extract, micro-agar

Eppendorf, Hamburg, Germany
96 well Deepwell plates, Filtertips 100µl

Fisher scientific, Loughborough, Leicestershire, UK
HEPES, EDTA, DMSO, concentrated HCl, pyrex borosilicate glass binding tubes, Glycine, SDS, Ammonium sulphate, sucrose, potassium acetate, potassium di-hydrogen orthophosphate, calcium chloride, sodium bicarbonate, manganese chloride.

GIBCO^{BRL} Life Technologies, Paisley, UK
Lipofectamine™ transfection reagent, OPTIMEM-1, L-glutamine, NBCS, DMEM, competent cells.

Greiner Bio-One Ltd., Gloucestershire, UK
7ml bijous

Helena Biosciences (Distributor for MBI Fermentas), Tyne & Wear, UK
Restriction enzymes

ICN Biomedicals Inc, Aurora, OH
Linbro plate sealer with adhesive back

Invitrogen BV, Groningen, Netherlands
NuPage® Novex high-performance pre-cast Bis-Tris gels, NuPAGE™ MOPS SDS Running Buffer (20x), XCell Surelock™ mini-cell gel tank, XCell II™ blot module

Iwaki, Scitech Division, Asahi, Japan
75cm^2 and 150cm^2 tissue culture flasks, 60mm and 100mm tissue culture dishes, 96 well/flat bottom Elisa plates

Konica Europe, Hohenbrunn, Germany
X-ray film

Melford Laboratories, Suffolk, UK
DTT, IPTG

Millipore Corporation, Bedford, MA
Immobilion™-P PVDF

NEN Life Science Products Inc., Boston, USA
[γ^{32}P]-Guanosine 5'-triphosphate, 250μCi (6000Ci/mMole)

Novagen, Madison, WI, USA

Benzonase Nuclease, bugbuster™ protein extraction reagent

Pierce, Perbio Science UK Ltd., Tattenhall, Cheshire, UK
EZ-Link™ Biotin-LC-Hydrazide, Streptavidin-HRP conjugate.

Promega UK Ltd., Southampton, UK
Restriction endonucleases, pfu polymerase, calf intestinal alkaline phosphatase, DNA purification kits: Wizard™ Plus SV Minipreps and Wizard™ Plus SV Maxipreps systems.

Packard Instruments BV, Netherlands
Ultima Gold XR liquid scintillation cocktail, Optiplate™ 96 well plates, microscint™20, Unifilter-96 GF/C, TopSeal™-A: 96well microplates

Premier Brands UK Ltd., Merseyside, UK
Marvel

Qiagen, Crawley, West Sussex, UK
Qiagen plasmid maxiprep kit, QIAquick Gel Extraction Kit, QIAquick PCR Purification Kit

Roche Diagnostics Ltd., Lewes, East Sussex, UK
12CA5 monoclonal mouse IgG (binds to haemagglutinin (HA) epitope-tagged proteins). Complete™ (Mini/EDTA free) protease inhibitor cocktail tablets, T4 DNA ligase, bovine serum albumin (fraction V), App[NH]p, creatine phosphate, creatine kinase, GTP, DNA molecular weight marker X (0.07-12.2kbp), restriction enzymes

Robinson Healthcare, Chesterfield, UK

Cotton wool

Sigma-Aldrich Company Ltd., Poole, Dorset, UK
$RbCl_2$, $Na_2H_2P_2O_7$, NaH_2PO_4, $NaH_2PO_4.2H_2O$, KOH, KCl, NaCl, $MgCl_2$, $CaCl_2$, $MnCl_2$, NaCl, NaOH, $C_2H_3O_2Na$, $C_2H_3O_2K$, DTT, EDTA, ATP, BSA (essentially globulin-free), MOPS, DMSO, glycerol, Triton X-100, Tween 20, bromophenol blue, ethylene glycol, ampicillin, agarose, ethidium bromide, Protein G-Sepharose, Protein A-Sepharose, mineral oil, sodium m-periodate, DMEM, 0.25% Trypsin-EDTA, Poly-D-Lysine, NBCS, 5-HT, WAY 100635, oubain, pertussis toxin, coomassie blue, activated charcoal, ascorbic acid, glutathione, bicinchoninic acid, trizma base, pyruvic acid (sodium salt), palmitic acid (sodium salt)

Sarstedt, Numbrecht, Germany
1.5ml tubes, 0.5ml tubes, yellow and blue tips

Schleicher and Schuell, Dassel, Germany
Protran nitrocellulose transfer membrane

Thermo Hybaid Interactiva Division, Ulm, Germany
Oligonucleotides for PCR reactions.

ThermoLabsystems, Thermo Life Sciences, Basingstoke, UK
Finntip 250 universal

Thistle Scientific Ltd, Glasgow, UK
EA wax

Tocris, Bristol, UK
8-OH-DPAT hydrochloride, 8-OH-DPAT hydrobromide

Whatman International Ltd., Maidstone, UK
3MM chromatography paper, 3MM filter paper, GF/C Glass fibre filters

ON1 antibody was generated against a synthetic peptide corresponding to Goα amino acids 1-16 of the mature polypeptide (Mullaney and Milligan, 1989).
OC2 antibody was generated against a synthetic peptide corresponding to Goα amino acids 345-354 of the mature polypeptide (Mullaney and Milligan, 1989)

2.2 General Buffers

Phosphate Buffered Saline (10x)

137mM NaCl, 2.7mM KCl, 1.5 mM KH_2PO_4, 10.2mM Na_2HPO_4, pH 7.4

Diluted 1 in 10 to make a 1x stock (stored at 4°C).

Tris-EDTA (TE) buffer for membranes

10mM Tris, 0.1mM EDTA, pH 7.5

Stored at 4°C.

Tris-EDTA (TE) buffer for radioligand binding assay

75mM Tris, 5mM EDTA, pH 7.5

Stored at 4°C.

Tris-EDTA-Magnesium (TEM) buffer for radioligand binding assay

75mM Tris, 5mM EDTA, 12.5mM $MgCl_2$, pH 7.5

Stored at 4°C.

Laemmli buffer (2x)

0.4M DTT, 0.17M SDS, 50mM Tris, 5M Urea, 0.01%(w/v) Bromophenol Blue.

Stored in aliquots at -20°C.

TAE buffer (50x)

40mM Tris-acetate, 1mM EDTA, glacial acetic acid pH 8.0

Diluted 1 in 50 prior to use.

DNA loading buffer (6x)

0.25% bromophenol blue, 0.25% xylene cyanol FF and 15% Ficoll (type 400: Pharmacia).

Store at room temperature.

Liquid Broth (LB)

1% (w/v) bactotryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl, pH 7

Autoclave sterilised at 126°C, stored at room temperature.

BCA assay buffers

BCA reagent A:

1% (w/v) BCA, 2% (w/c) Na_2CO_3, 0.16% (w/v) sodium tartrate, 0.4% NaOH, 0.95% $NaHCO_3$, pH 11.25

BCA reagent B:

4% $CuSO_4$

The two reagents are mixed A:B in a 49:1 ratio prior to use.

Competent cell buffers

Competent cell buffer 1:

0.03M $C_2H_3O_2K$, 0.1M $RbCl_2$, 0.01M $CaCl_2$, 0.05M $MnCl_2$, 15% glycerol, pH 5.8 with acetic acid, filter sterilised and stored at 4°C

Competent cell buffer 2:

10mM MOPS pH 6.5, 0.075M $CaCl_2$, 0.01M $RbCl_2$, 15% glycerol, pH 6.5 with concentrated HCl, filter sterilised and stored at 4°C

2.3 General Molecular Biology

2.3.1 Preparation of LB Ampicillin Agar Plates

LB agar (1% (w/v) bactotryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl, and 1.5% (w/v) agar) was autoclaved and allowed to cool before the addition of ampicillin (50μg/ml). The liquid LB agar was decanted into 100mm Petri dishes and allowed to solidify prior to storage at 4°C until required.

2.3.2 Preparation of XL1 Blue Competent Bacterial Cells

An overnight culture of XL1 Blue bacteria was grown in 5ml of LB broth. The following day the culture was used to inoculate 100ml of LB broth that was grown with aeration until the optical density at 550nm reached 0.48. The culture was chilled on ice for 10 minutes then spun at 3K for 10 minutes at 4°C in sterile 2 x 50ml disposable

plastic tubes. The supernatant was removed and the cells resuspended in 20ml of competent cell buffer 1. The suspension was chilled on ice for 5 minutes then spun at 3K for 10 minutes at 4°C. Following removal of supernatant cell pellets were resuspended in 2ml of competent cell buffer 2. After incubation on ice for 15 minutes the samples were aliquoted and stored at –80°C until required.

2.3.3 Transformation of Competent XL1 Blue Bacterial Cells with Plasmid DNA

To an aliquot of 50µl competent bacteria that had been allowed to thaw on ice, 10ng of DNA was added, and incubated on ice for 15 minutes. Cells were then incubated at 42°C for 90 seconds and returned to ice for 2 minutes. 450µl of LB broth was added to the reaction before incubation at 37°C for 45 minutes in a shaking incubator. 200µl of the reaction was spread onto LB ampicillin agar plates and incubated inverted overnight at 37°C. Transformed colonies were selected the following day.

2.3.4 Preparation of Plasmid DNA

Colonies transformed using XL1 Blue bacteria were picked and grown overnight in 5ml of LB broth containing ampicillin (50µg/ml). Plasmid DNA was prepared using the Promega™ Wizard Plus SV Miniprep purification system, as per manufacturers instructions, to obtain a typical yield of 100µl of 0.05-0.4µg/µl plasmid DNA. Preparation of larger quantities of DNA was accomplished by transferring the 5ml overnight culture into 500ml of LB broth containing ampicillin (50µg/ml) and allowing a further overnight period of growth. The DNA was purified using the Promega™ Wizard Maxiprep purification system, as per the manufacturer's instructions, to obtain a typical yield of 1ml of 0.5-2µg/µl plasmid DNA.

2.3.5 Quantification of DNA

The concentration of plasmid DNA generated from maxipreps and minipreps was determined by measurement of the absorbance at 260nm of a 1:50 dilution of the DNA sample. An A_{260} value of 1 unit was assumed to be equivalent to 50µg/ml of double stranded DNA. The A_{280} value of the solution was also measured to assess the purity of

the DNA solution. A DNA solution with an A_{260}/A_{280} ratio of between 1.7 and 2.0 was considered pure enough for use.

2.3.6 Digestion of DNA with Restriction Endonucleases

Using the appropriate restriction enzymes (1-2 units) and buffer, 1μg of DNA was digested in a 10μl volume for a minimum of 2 hours at 37°C (or as per manufacturers instructions).

2.3.7 DNA Gel Electrophoresis

Digested DNA fragments between 0.25 and 6kb were diluted 1 in 6 with 6x loading buffer and separated on 1% (w/v) agarose gels containing TAE buffer and 2.5mg/ml ethidium bromide. The gels were run, immersed in 1 x TAE buffer, at 100 volts for 20-30 minutes in horizontal gel tanks. Ultraviolet light was used to analyse the separated DNA fragments on the gels. The size of each DNA fragment was calculated by comparison with a 1kb ladder.

2.3.8 DNA Purification from Agarose Gels

After excision of DNA fragments from the gel using a sterile razorblade, purification of DNA fragments from agarose gels was carried out using the Quiagen QIAquick gel extraction kit as per the manufacturer's instructions. DNA was eluted from the purification column using 30μl sterile water.

2.3.9 Alkaline Phosphatase Treatment of Plasmid Vectors

This treatment of digested plasmid vectors minimised re-ligation of the vector to itself. The 5′ phosphate group was removed by incubation of 200ng of digested vector with 2 units of alkaline phosphatase for 2 hours at 37°C. The treated plasmid was then isolated from the reaction mixture by agarose gel electrophoresis and gel extraction as described previously.

2.3.10 DNA Ligations

Ligations of cDNA inserts into vector DNAs were performed using a vector to insert ratio of 1:3. Reactions were performed at 4°C for 16 hours in a total volume of 10µl: containing 1 unit of T4 ligase in the appropriate buffer. Ligation products were then transformed as described in 2.3.3.

2.4 Mutation of GPCR-G Protein Fusions to Produce Palmitoylation-Deficient Variants

GPCR-G protein fusion constructs in pcDNA3 vector were used as DNA templates for a series of PCR reactions. These reactions introduced mutations, which remove potential palmitoylation sites from these constructs. The presence of multiple potential palmitoylation sites in the fusions meant that all possible constructs had to be created, each with different ability to be palmitoylated. The fusion proteins utilised in this study were between the rat $G_{o1}\alpha Cys^{351}Ile$ protein and either the porcine α_{2A}-adrenoceptor or the human 5-HT_{1A}-serotonin receptor. The α_{2A}-adrenoceptor- $G_{o1}\alpha Cys^{351}Ile$ fusion construct has 2 potential palmitoylation sites, one in the receptor and one in the G protein, therefore four palmitoylation variant constructs are possible. The 5-HT_{1A}-receptor-$G_{o1}\alpha Cys^{351}Ile$ fusion construct has 3 potential palmitoylation sites, two in the receptor and one in the G protein, therefore eight palmitoylation variant constructs are possible for this fusion. Regardless of which fusion construct was used as the template DNA, the general PCR method was the same. This method is detailed in Figure 2.1.

2.4.1 Polymerase Chain Reaction

PCR reactions were carried out on a Hybaid Omnigene thermal cycler in a total volume of 100µl containing 100ng of DNA template, 0.25mM dNTPs (dATP, dCTP, dGTP, dTTP), 50pmol of sense and anti-sense oligonucleotide primers, 1x *Pfu* thermophilic buffer, and 2.5 units of *Pfu* polymerase. In all reactions *Pfu* enzyme was added after the first denaturation step.

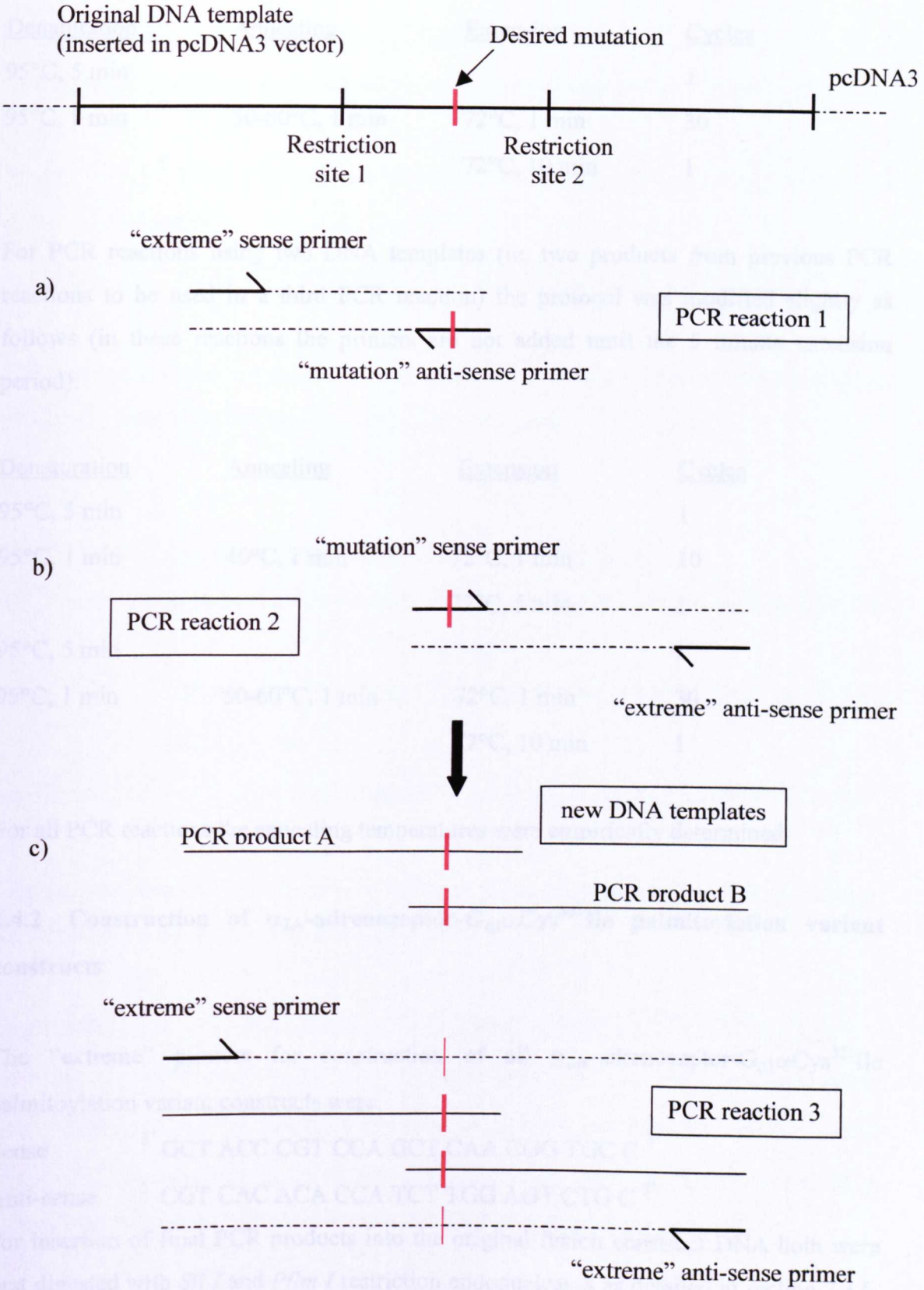
Figure 2.1

PCR method used in this study

This method of introducing mutations into constructs used a set of central "mutation" primers (sense and anti-sense) to incorporate the desired mutation, as well as a set of "extreme" primers (sense and anti-sense). It was necessary to identify restriction sites for isolation of the mutated region. To create the mutation required a series of three PCR reactions.

a) The first PCR reaction used the original DNA template, the "extreme" sense primer and the "mutation" anti-sense primer. The product of this reaction was PCR fragment A.
b) The second PCR reaction used the original DNA template, the "extreme" anti-sense primer and the "mutation" sense primer. The product of this reaction was PCR fragment B.
c) The third PCR reaction used the PCR products A and B as templates and both "extreme" primers, sense and anti-sense, to create the final PCR product. The final PCR product was then digested with restriction endonucleases 1 and 2 before ligation into the original DNA template (previously digested with the same two endonucleases).

Figure 2.1


Original DNA template
(inserted in pcDNA3 vector)
Desired mutation
pcDNA3
Restriction
site 1
Restriction
site 2
"extreme" sense primer
a)
PCR reaction 1
"mutation" anti-sense primer
"mutation" sense primer
b)
PCR reaction 2
"extreme" anti-sense primer
new DNA templates
PCR product A
c)
PCR product B
"extreme" sense primer
PCR reaction 3
"extreme" anti-sense primer

For PCR reactions using a single DNA template, the following general protocol was used:

Denaturation	Annealing	Extension	Cycles
95°C, 5 min			1
95°C, 1 min	50-60°C, 1 min	72°C, 1 min	30
		72°C, 10 min	1

For PCR reactions using two DNA templates (ie, two products from previous PCR reactions to be used in a third PCR reaction) the protocol was modified slightly as follows (in these reactions the primers are not added until the 5 minute extension period):

Denaturation	Annealing	Extension	Cycles
95°C, 5 min			1
95°C, 1 min	40°C, 1 min	72°C, 1 min	10
		72°C, 5 min	1
95°C, 5 min			1
95°C, 1 min	50-60°C, 1 min	72°C, 1 min	30
		72°C, 10 min	1

For all PCR reactions the annealing temperatures were empirically determined.

2.4.2 Construction of α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ palmitoylation variant constructs

The "extreme" primers for construction of all α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ palmitoylation variant constructs were:

Sense $^{5'}$ GCT ACC CGT CCA GCT CAA CGG TGC C $^{3'}$

Anti-sense $^{5'}$ CGT CAC ACA CCA TCT TGG AGT CTG C $^{3'}$

For insertion of final PCR products into the original fusion construct DNA both were first digested with *Sfi I* and *Pflm I* restriction endonucleases as detailed in section 2.3.6.

The digestion products were then ligated together as outlined in section 2.3.7 to produce constructs containing the desired mutations.

Construction of α_{2A}-adrenoceptor-Cys^{442}Ala-$G_{o1}\alpha$Cys^{351}Ile (**C^{442}A**)

The "mutation" primers for construction of α_{2A}-adrenoceptor-Cys^{442}Ala-$G_{o1}\alpha$Cys^{351}Ile were:

Sense $^{5'}$ GCC TTC AAG AAG ATC CTC ***GCA*** CGT GGG GAC AGG AAA CGG $^{3'}$

Anti-sense $^{5'}$ CCG TTT CCT GTC CCC ACG ***TGC*** GAG GAT CTT CTT GAA GGC $^{3'}$

The mutated residues are shown in bold italics in the above sequences and the position of a newly created restriction site, *Eco72I*, is underlined.

Construction of α_{2A}-adrenoceptor-$G_{o1}\alpha$Cys^{3}Ser, Cys^{351}Ile (**C^{3}S**)

The "mutation" primers for construction of α_{2A}-adrenoceptor-$G_{o1}\alpha$Cys^{3}Ser, Cys^{351}Ile were:

Sense $^{5'}$ GGA AAC GGA TCG CCA TGG GA***A*** GTA CTC TGA GCG CAG AGG AGA GA $^{3'}$

Anti-sense $^{5'}$ TCT CTC CTC TGC GCT CAG AGT AC***T*** TCC CAT GGC GAT CCG TTT CC $^{3'}$

The mutated residue is shown in bold italics in the above sequences and the position of a newly created restriction site, *Sca I*, is underlined.

Construction of α_{2A}-adrenoceptor-Cys^{442}Ala-$G_{o1}\alpha$Cys^{3}Ser, Cys^{351}Ile (**C^{442}A, C^{3}S**)

To create α_{2A}-adrenoceptor-Cys^{442}Ala-$G_{o1}\alpha$Cys^{3}Ser, Cys^{351}Ile, the α_{2A}-adrenoceptor-Cys^{442}Ala-$G_{o1}\alpha$Cys^{351}Ile construct was used as the DNA template. The primers used to create α_{2A}-adrenoceptor-$G_{o1}\alpha$Cys^{3}Ser, Cys^{351}Ile were then employed as before to incorporate the second mutation. Mutant contains both *Eco72I*, and *Sca I*.

2.4.3 Construction of 5-HT_{1A}-receptor-$G_{o1}\alpha Cys^{351}$Ile palmitoylation variant constructs

The "extreme" primers for construction of all 5-HT_{1A}-receptor-$G_{o1}\alpha Cys^{351}$Ile palmitoylation variant constructs were:

Sense $^{5'}$ CGA GAG AGG AAG ACA GTG AAG ACG C $^{3'}$

Anti-sense $^{5'}$ GCA CAA TGG CTG CCA GAG ACT GGA TGG $^{3'}$

For insertion of PCR products into the original fusion construct DNA both were first digested with *Mlu I* and *Pflm I* restriction endonucleases as detailed in section 2.3.6. The digestion products were then ligated together as outlined in sections 2.3.7 and 2.3.9 to produce constructs containing the desired mutations.

Construction of 5-HT_{1A}-receptor Cys^{417}Ser-$G_{o1}\alpha Cys^{351}$Ile (**$C^{417}S$**)

The "mutation" primers for construction of 5-HT_{1A}-receptor Cys^{417}Ser-$G_{o1}\alpha Cys^{351}$Ile were:

Sense $^{5'}$ GAA GAT CAT TAA GT***C*** TAA GTT CTG CCG C $^{3'}$

Anti-sense $^{5'}$ GCG GCA GAA CTT A***G***A CTT AAT GAT CTT C $^{3'}$

The mutated residue is shown in bold italics in the above sequences.

Construction of 5-HT_{1A}-receptor Cys^{417}Ser-$G_{o1}\alpha Cys^{3}$Ser, Cys^{351}Ile (**$C^{417}S$, $C^{3}S$**)

The "mutation" primers for construction of 5-HT_{1A}-receptor Cys^{417}Ser-$G_{o1}\alpha Cys^{3}$Ser, Cys^{351}Ile were:

Sense $^{5'}$ GAA GAT CAT TAA GT***C*** TAA GTT CTG CCG CCA GGG ATC TAT GGG A***A***G TAC TCT GAG CGC AGA G $^{3'}$

Anti-sense $^{5'}$ CTC TGC GCT CAG AGT A***G***A TCC CAT AGA TCC CTG GCG GCA GAA CTT AC***T*** CTT AAT GAT CTT C $^{3'}$

The mutated residues are shown in bold italics in the above sequences.

Construction of 5-HT_{1A}-receptor Cys^{420}Ser-$G_{o1}\alpha Cys^{351}$Ile (**$C^{420}S$**)

The "mutation" primers for construction of 5-HT_{1A}-receptor Cys^{420}Ser-$G_{o1}\alpha Cys^{351}$Ile were:

Sense $^{5'}$ CAT TAA GTG TAA GTT CT***C*** CCG CCA GGG ATC TAT G $^{3'}$

Anti-sense $^{5'}$ CAT AGA TCC CTG GCG G***G***A GAA CTT ACA CTT AAT G $^{3'}$

The mutated residue is shown in bold italics in the above sequences.

Construction of 5-HT_{1A}-receptor Cys^{420}Ser-G_{o1}αCys^{3}Ser, Cys^{351}Ile (**$C^{420}S$, $C^{3}S$**)

The "mutation" primers for construction of 5-HT_{1A}-receptor Cys^{420}Ser-G_{o1}αCys^{3}Ser, Cys^{351}Ile were:

Sense $^{5'}$ CAT TAA GTG TAA GTT CT***C*** CCG CCA GGG ATC TAT GGG A***A***G TAC TCT GAG CGC AGA G $^{3'}$

Anti-sense $^{5'}$ CTC TGC GCT CAG AGT AC***T*** TCC CAT AGA TCC CTG GCG G***G***A GAA CTT ACA CTT AAT G $^{3'}$

The mutated residues are shown in bold italics in the above sequences.

Construction of 5-HT_{1A}-receptor Cys^{417}Ser, Cys^{420}Ser-G_{o1}αCys^{351}Ile (**$C^{417}S$, $C^{420}S$**)

The "mutation" primers for construction of 5-HT_{1A}-receptor Cys^{417}Ser, Cys^{420}Ser-G_{o1}αCys^{351}Ile were:

Sense $^{5'}$ GAA GAT CAT TAA GT***C*** TAA GTT CT***C*** CCG CCA GGG ATC TAT G $^{3'}$

Anti-sense $^{5'}$ CAT AGA TCC CTG GCG G***G***A GAA CTT A***G***A CTT AAT GAT CTT C $^{3'}$

The mutated residues are shown in bold italics in the above sequences

Construction of 5-HT_{1A}-receptor-G_{o1}αCys^{3}Ser, Cys^{351}Ile (**$C^{3}S$**)

The "mutation" primers for construction of 5-HT_{1A}-receptor-G_{o1}αCys^{3}Ser, Cys^{351}Ile were:

Sense $^{5'}$ GGA TCT ATG GGA ***A***GT ACT CTG AGC GCA GAG $^{3'}$

Anti-sense $^{5'}$ CTC TGC GCT CAG AGT AC***T*** TCC CAT AGA TCC $^{3'}$

The mutated residue is shown in bold italics in the above sequences

Construction of 5-HT_{1A}-receptor $Cys^{417}Ser$, $Cys^{420}Ser$-$G_{o1}\alpha Cys^{3}Ser$, $Cys^{351}Ile$ ($C^{417}S$, $C^{420}S$, $C^{3}S$)

The "mutation" primers for construction of 5-HT_{1A}-receptor $Cys^{417}Ser$, $Cys^{420}Ser$-$G_{o1}\alpha Cys^{3}Ser$, $Cys^{351}Ile$ were:

Sense $^{5'}$ GAA GAT CAT TAA GT***C*** TAA GTT CT***C*** CCG CCA GGG ATC TAT GGG A***A***G TAC TCT GAG CGC AGA G $^{3'}$

Anti-sense $^{5'}$ CTC TGC GCT CAG AGT A***C***T TCC CAT AGA TCC CTG GCG G***G***A GAA CTT A***G***A CTT AAT GAT CTT C $^{3'}$

The mutated residues are shown in bold italics in the above sequences

2.5 Cell Culture

2.5.1 Cell Growth

HEK293T cells were grown in DMEM supplemented with 10% NBCS and 1% L-glutamine in a 37°C humidified 5% CO_2 atmosphere.

2.5.2 Passage of Cells

Confluent 75cm^2 flasks of cells were passaged by the addition of 2ml of sterile 0.25% trypsin-EDTA solution to cells after removal of growth media. After detachment, cells were resuspended using a further 8ml of fresh media. This suspension was split into flasks and dishes as required (1:10 for routine passage).

2.5.3 Coating Plates with Poly-D-Lysine

50mg of poly-D-lysine was diluted with 50ml of sterile water to make a 1mg/ml stock solution. Tissue culture plates and coverslips were coated with a 1:10 dilution of the stock solution for 30 minutes before removal of the solution and air-drying.

2.5.4 LipofectAMINE-Based Transient Transfections for Cell Harvesting

HEK293T cells were transiently transfected at approximately 70-80% confluency in 10cm dishes. 4-6μg of DNA and 25μl of LipofectAMINE reagent were mixed gently with enough OptiMEM to give a total volume of 1200μl. This mix was then incubated for 30 minutes at room temperature. During this period, the cells were washed with OptiMEM. Next, 4800μl of OptiMEM was added to the DNA-LipofectAMINE mix before drop-wise addition of the full 6000μl to each dish. Cells were incubated at 37°C in 5% CO_2 for 4 hours, before replacement of the transfection media with fresh growth media. Cells were routinely harvested after 24-48 hours following 16 hours incubation with pertussis toxin (50ng/ml) to inactivate endogenous G_i class G proteins.

2.5.5 LipofectAMINE-Based Transient Transfections for Palmitoylation Assay

HEK293T cells were transiently transfected at approximately 70-80% confluency in 150cm^2 flasks. 8-12μg of DNA and 50μl of LipofectAMINE reagent were mixed gently with enough OptiMEM to give a total volume of 4800μl. This mix was then incubated for 30 minutes at room temperature. During this period, the cells were washed with OptiMEM. Next, 15200μl of OptiMEM was added to the DNA-LipofectAMINE mix before addition of the full 20ml to each flask. Cells were incubated at 37°C in 5% CO_2 for 4 hours, before replacement of the transfection media with fresh growth media. 24 hours later each 150cm^2 flask of cells was split into the appropriate number (assay dependent) of 6cm dishes; to ensure equal expression level for the construct in each 6cm dish. Cells were then incubated at 37°C in 5% CO_2 for a further 24 hours prior to commencement of the palmitoylation assay. Note that radioligand binding assays ensured equalised construct expression of multiple constructs used in parallel.

2.5.6 LipofectAMINE-Based Transient Transfections for Receptor Internalisation Assay

HEK293T cells were transiently transfected at approximately 70-80% confluency in a 10cm dish as described in 2.5.4 except that after 24 hours, each 10cm dish of cells was split as appropriate into a 6 well plate; to ensure equal expression level in each well.

Cells were then incubated at 37°C in 5% CO_2 for a further 24 hours prior to commencement of the receptor internalisation assay. Note that for the purposes of this study, equalised expression across constructs is not required, only equalised expression for duplicate wells of each construct is necessary.

2.5.7 Cell Harvesting

Cells were grown to confluency in 75cm^2 or 150cm^2 flasks and the media discarded before harvesting by scraping using disposable cell scrapers. Cells were washed using 2 x 10ml of ice cold PBS and centrifuged for 5 minutes at 1811 x g in a refrigerated centrifuge. After discarding the supernatant, the pellet can be frozen at -80°C until required.

2.6 Protein Biochemistry

2.6.1 BCA Assay to Determine Protein Concentration

Protein concentration was determined using a BCA assay. The principles behind this assay are herein described. Two solutions, BCA reagent A (which contains Bicinchoninic Acid) and BCA reagent B (copper sulphate) are mixed in a 49:1 ratio and 200µl is added to 10µl of protein sample/standard in a 96 well plate. After incubation at 37°C for 30 min, the absorbance at 492nm is read. In this assay proteins reduce Cu^{2+} ions to Cu^{1+} in a concentration-dependent manner and BCA forms a complex with Cu^{1+} ions to form a purple coloured solution with absorbance at 492nm. The A_{492} value of the solution is therefore directly proportional to the protein concentration and is determined by comparison to a BSA standard curve (0.2-2mg/ml).

2.6.2 Preparation of Cell Membranes

Harvested cell pellets were thawed and resuspended in 1ml of TE buffer before cell rupture by 30 strokes of a chilled glass-on-glass Dounce homogeniser and 20 passages through a 25-gauge syringe needle. After a 10 minute, 199 x g centrifugation at 4°C; to remove unbroken cells and nuclei; the supernatant was collected and ultracentrifuged at

50K for 30 minutes in a Beckman Optima TLX Ultracentrifuge (Palo Alto, CA) with a TLA 100.2 rotor. The resulting pellet was resuspended in 300µl of TE buffer and, following determination of protein concentration, diluted to 1µg/µl, aliquoted and stored at –80°C until required.

2.6.3 Sodium Dodecyl Sulphide Polyacrylamide Gel Electrophoresis

Samples were boiled at 100°C for five minutes and loaded onto precast NuPage® Novex Bis-Tris 4-12% gradient gels alongside full range Rainbow™ molecular weight markers. NuPage® MOPS SDS buffer was used to run gels at 200 volts constant in the XCell Surelock™ mini-cell gel tank (Invitrogen BV) until the dye front reached the foot of the gel. Subsequent Coomassie Blue staining, semi-dry transfer or standard western blotting was then carried out.

2.6.4 Semi-Dry Protein Transfer & Autoradiography (For Palmitoylation Assay)

Following sample separation as in section 2.6.3, proteins were electrophoretically transferred onto PVDF membrane. A semi-dry transfer procedure was carried out using Novablot transfer apparatus. The assembly was constructed as follows, from base to lid; 6 pieces of filter paper, the PVDF, the gel, then 6 pieces of filter paper. Before use the PVDF was pre-wet with methanol and all pieces of the assembly were pre-soaked in transfer buffer (0.2M glycine, 25mM tris, and 20% (v/v) methanol). Gels were transferred for 2 hours by a constant current of 75mA. After transfer the membrane was washed in distilled water, air-dried, coated with EA wax then placed at -80°C to undergo autoradiography for a typical period of 4 weeks.

2.6.5 Western Blotting

Following sample separation as in section 2.6.3, proteins were electrophoretically transferred onto nitrocellulose using the XCell II™ blot module (Invitrogen BV). Gels were transferred at 30V, ~140mA, for 1 hour in transfer buffer (0.2M glycine, 25mM tris, and 20% (v/v) methanol). The nitrocellulose was blocked in 5%(w/v) Marvel in PBS/0.1%(v/v) Tween 20, for 1 hour at room temperature and washed 3 times with

PBS/0.1% Tween 20, over a 30 minute period. Incubation with the primary antibody was in 1% Marvel prepared in PBS/0.1% Tween 20, for 1 hour at room temperature, followed by 5 washes in PBS/0.1% Tween 20, over a 30-minute period. Incubation with secondary antibody was then done in 1% Marvel-PBS/0.1% Tween 20, for 1 hour at room temperature, once again followed by 5 washes over 30 minutes with PBS/0.1% Tween 20. The nitrocellulose was incubated with a 50:50 (v/v) mixture of ECL reagents for 2 minutes prior to exposure to and development of X-ray film.

For Western blot analysis the following antibody incubations were used:

1° Antibody	Dilution	2° Antibody	Dilution
ON1	1:2000	Anti-rabbit IgG	1:10 000
OC1	1:2000	Anti-rabbit IgG	1:10 000

2.6.6 GST Fusion Protein Preparation

Colonies transformed using XL1 Blue bacteria were picked and grown overnight in 10ml of LB broth containing ampicillin (50μg/ml). The following morning, this culture was added to 500ml of LB broth containing ampicillin (50μg/ml) and grown with aeration until an OD_{600} of 0.2 was reached. 500μl of 1M IPTG was added and the culture grown for a further 4 hours with aeration after which the sample was cooled on ice for 10 minutes. The large culture was centrifuged for 15 minutes at 5524 x g to pellet the cells and the supernatant was discarded. The pellet was resuspended in bugbuster™ protein extraction reagent (5ml per gram of wet pellet) then 2-4μl of benzonase nuclease enzyme was added. The resuspended pellet was then left on ice for 1 hour, sonicated 2 x 30 seconds at 60kHz using a probe sonicator then centrifuged at 20817 x g for 30 minutes. The supernatant was then transferred to a sterile 50ml tube containing 300μl of glutathione sepharose 4B gel (pre-washed 2 x 1ml with sterile PBS). One protease inhibitor tablet as well as DTT, to a final concentration of 5mM, was added and the sample was placed on a rotary wheel overnight at 4°C. Next day the mixture was centrifuged for 5 minutes at 500 x g before removal of the supernatant

(stored at –80°C) and washing of the glutathione sepharose 4B gel twice with 5ml sterile PBS. The pellet was resuspended in 300µl of 10mM glutathione, mixed by gentle inversion and left on ice for 5 minutes. The sample was then centrifuged for 3 minutes at 500 x g before the supernatant was removed and kept on ice. The addition of glutathione and its removal was repeated five times. Samples for SDS PAGE were collected throughout the procedure.

2.7 Assays

2.7.1 Radioligand-Binding Assays: One Near-Saturating Concentration of Radioligand

Radioligand-binding assays using one near-saturating concentration of radioligand were performed in order to give an estimation of the expression level of receptor-G protein fusion constructs. This approach was used in preliminary studies, as well as during palmitoylation assays involving multiple constructs. Triplicate reaction mixtures were set up containing 0.5µg of protein and ~5nM radioligand (final), with non-specific binding being determined by the inclusion of 100µM idazoxan. The samples were incubated at 30°C for 45 minutes and subsequently passed through a Brandel GF/C glassfibre filter using a Brandel cell harvester in TE buffer (75mM Tris, 5mM EDTA pH 7.5). Filters were washed to remove unbound radioligand from the membrane and were then inserted into vials containing 5ml liquid scintillant. Vials were counted in a Beckman LS6500 scintillation counter, using the [^{3}H] counting channel. Specific binding was determined by the subtraction of non-specific counts from the total counts. Expression level (fmol/mg) was calculated from these results by using the known specific activity of the radioligand and the amount of membranes added in each reaction. An example of this calculation can be found in the Appendix (section 8.5).

2.7.2 Radioligand-Binding Assays: Various Concentrations of Radioligand

A more accurate determination of the receptor expression level was obtained by performing a radioligand-binding assay using a range of concentrations of radioligand (~0.02nM to ~5nM). The procedure is essentially identical to that for a one-point

radioligand-binding assay, with only the concentration of radioligand differing. Using the data analysis package Graphpad Prism (San Diego, CA), saturation-binding data were fitted to non-linear regression curves to determine total receptor expression (B_{max}) and the equilibrium dissociation constant (K_d) of the ligands for the binding sites.

2.7.3 Competition Radioligand Binding Assays

Radioligand binding was also assayed in competitive binding experiments. In these experiments increasing concentrations of non-radioactive ligand are used to compete for binding with a single concentration of a tritiated radioligand. Triplicate reaction mixtures were set up containing 0.5μg of protein, radioligand at a concentration equivalent to the K_d for the receptor (~0.32nM), and a range of concentrations of competing non-radioactive ligand; usually from 10^{-12} – 10^{-3}M. Again, non-specific binding was determined by the inclusion of 100μM idazoxan. The samples were incubated at 30°C for 45 minutes prior to filtration as in 2.7.1. Using the data analysis package Graphpad Prism (San Diego, CA), competition-binding data was plotted as % of radioligand binding against log non-radioactive ligand concentration and an IC_{50} value was determined using nonlinear regression. The equilibrium dissociation constant for the binding of the competing "cold" drug (K_i) was calculated using the Cheng-Prusoff equation (Cheng and Prusoff, 1973). See appendix (Section 8.6).

2.7.4 *In vivo* Palmitoylation Assays

Cells were labelled with 0.5mCi/ml [9,10(n)-^{3}H] palmitic acid in DMEM supplemented with 2mM L-glutamine, 5% (v/v) dialysed NBCS, 0.1mM ascorbic acid, and 5mM pyruvic acid at 37°C in a 5% CO_2 humidified atmosphere. After incubation for the appropriate time in the presence or absence of agonist, reactions were terminated by the addition of 200μl of 1% (w/v) SDS. Proteins were denatured by passage through a 25-gauge needle followed by 5-min incubation at 100°C. After chilling to 4°C, 800μl of Kahn solubilisation buffer (1%(v/v) Triton X-100, 10mM EDTA, 100mM NaH_2PO_4, 10mM NaF, 50mM HEPES (pH 7.2)) was added, and the samples were pre-cleared by incubation at 4°C for 1h with 100μl of Pansorbin. The pre-cleared supernatants were then incubated at 4°C for 16h with protein A-sepharose and 10μl of antiserum ON1.

Immune complexes were isolated by centrifugation, washed three times with Kahn immunoprecipitation wash buffer (1%(v/v) Triton X-100, 100mM NaCl, 50mM NaH_2PO_4, 100mM NaF, 50mM HEPES (pH 7.2) plus 0.5% SDS), and eluted from the protein A-sepharose by addition of electrophoresis buffer containing 20mM DTT and heating to 80°C for 3min. Analysis was by SDS-PAGE as described in 2.6.3, followed by transfer and autoradiography as described in 2.6.4.

2.7.5 High Affinity GTPase Assays

High affinity GTPase assays were performed as per Wise *at al.* (1997a,b,c) with modifications for 96 well plate use. Each reaction was performed in triplicate using 1.5μg of protein in a 100μl total volume. An incubation period of 40 minutes at 37°C preceded reaction termination by the addition of 900μl activated charcoal slurry (5g activated charcoal, 10ml of 100mM orthophosphoric acid per 100ml). The reaction mixture volume of 100μl contained 20mM creatine phosphate, 0.1u/μl creatine kinase, 0.2mM App[NH]p, 2mM ATP, 2mM oubain, 200mM NaCl, 10mM $MgCl_2$, 4mM DTT, 0.2mM EDTA, 80mM Tris/HCl and [γ^{32}P]-GTP for 50,000cpm per reaction. Following centrifugation at 3220 x g for 10 minutes a 300μl sample of supernatant was counted using a Packard Topcount NXT™ microplate scintillation counter.

High affinity GTPase activity was determined over a range of GTP concentrations (25-3000nM) to allow calculation of V_{max} for GTP hydrolysis and the K_m for GTP. This was measured in the absence and presence of agonist (100μM) and 1μM RGS protein. The data was analysed and plotted using Graphpad Prism as V (pmol/mg/min) against [GTP] and also as V (pmol/mg/min) against V/S. An example of these calculations can be found in the Appendix (section 8.7).

2.7.6 [^{35}S] GTPγS Binding Assays

[^{35}S] GTPγS binding experiments were initiated by the addition of membranes expressing 10fmol of fusion construct to an assay buffer (20mM HEPES (pH 7.4), 3mM

$MgCl_2$, 100nM NaCl, 1μM guanosine 5'-diphosphate, 0.2mM ascorbic acid, 50nCi of [^{35}S] GTPγS) containing 100μM agonist. Non-specific binding was determined in the same conditions but in the presence of 100μM GTPγS. Reactions were incubated for 2.5 minutes at 30°C and were terminated by the addition of 0.5ml of ice-cold GTPγS stop buffer (20mM HEPES (pH 7.4), 3mM $MgCl_2$ and 100mM NaCl). The samples were centrifuged at 16,000 x g for 15 minutes at 4°C, and the resulting pellets were resuspended in solubilisation buffer (100mM Tris, 200mM NaCl, 1mM EDTA, 1.25% Nonidet P-40) plus 0.2% SDS. Samples were pre-cleared with Pansorbin, followed by immunoprecipitation with ON1 antiserum. Finally, the immunocomplexes were washed twice with solubilisation buffer, and bound [^{35}S] GTPγS was estimated by liquid-scintillation spectrometry.

2.7.7 Receptor Internalisation Assay

HEK293T cells expressing the construct of interest were plated onto 6 well plates at a density of ~10^6 cells/well. The next day the cells were washed, and 1ml/well medium was applied. Cells were treated with agonist for the appropriate time-points ranging from 0 to 90 min. The reactions were terminated by the placing of plates on ice then washing the cells 3 times with ice cold PBS. The alcohol groups on the cell-surface glycoproteins were oxidised to aldehydes by 30 min incubation with 10mM sodium m-periodate. After the removal of periodate, cells were washed once with PBS and twice with 0.1M sodium acetate, pH 5.5, and incubated in the same buffer supplemented with 1mM biotin-LC-hydrazide. This reacts with the newly formed aldehyde groups, thereby labelling all cell surface glycoproteins with biotin. Labelling was terminated by removal of the biotin solution and washing the cells three times with PBS. Cells were then solubilised prior to immunoprecipitation with 12CA5 antibody (1μg/sample). This antibody recognises the N-terminal haemagglutinin (HA) tag present on all the fusion constructs used in this study. After SDS-PAGE and the transfer of the proteins onto nitrocellulose membranes, cell surface biotin-labelled receptors were identified by incubation of the membranes with 1μg/ml HRP-conjugated streptavidin in 5% (w/v) non-fat milk/PBS-T for 1 hour at room temperature. After several washes with PBS-T, reactive proteins were visualised by enhanced chemiluminescence. Agonist-mediated loss of cell surface receptors was quantified by densitometric scanning of blots.

Chapter 3

Regulation of palmitoylation in

α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ fusion proteins

3.1 Introduction

The α_{2A}-adrenoceptor used in this study is one member of the vast G protein coupled receptor (GPCR) family. These GPCRs are responsible for signal transduction across the plasma membrane via interaction with members of the G protein family of signalling proteins. The natural ligands for the α_{2A}-adrenoceptor are adrenaline and noradrenaline: catecholamine hormones released into the plasma at times of stress or increased energy need. Upon stimulation, this α_{2A}-adrenoceptor interacts with pertussis-toxin-sensitive members of the G_i/G_o class of heterotrimeric G proteins, leading to inhibition of adenylyl cyclase and L-type Ca^{2+} channels and activation of K^+ channels. The particular G protein used in this study is the G_{o1} G protein, whose main function is thought to be inhibition of neuronal calcium channel activity (Heschler *et al.*, 1987).

This study concerns the attachment of palmitate, a 16-carbon saturated fatty acid moiety, to both the α_{2A}-adrenoceptor and the $G_{o1}\alpha$ protein. A wide variety of cellular proteins are known to undergo palmitoylation: a modification usually but not exclusively (Kleuss and Krause, 2003) attached via thioester linkage to cysteine residues. Since thioester bonds are known to be labile (Magee *et al.*, 1987), such thioacylation by palmitate has the potential to be both dynamic and regulated (Mumby, 1997; Qanbar and Bouvier, 2003). Agonist-dependent regulation of palmitoylation status has been shown to occur for both GPCRs (Ponimaskin *et al.*, 2001; Ng *et al.*, 1994; Mouillac *et al.*, 1992) and G proteins (Wedegartner and Bourne, 1994; Chen and Manning, 2000).

GPCR and G protein palmitoylation has been implicated in the modulation of a number of functional properties such as GPCR-G protein interactions, RGS-G protein interactions, GPCR phosphorylation, GPCR desensitisation and down-regulation, as well as caveolar targeting and membrane localisation of many proteins. The observation that agonist can regulate this modification of these two protein classes (Mumby *et al.*, 1994, Wedegaertner and Bourne, 1994, Loisel *et al.*, 1999, Stevens *et al.*, 2001) is therefore very interesting indeed. It would be very informative to ascertain whether

regulation of GPCR and G protein acylation is co-ordinated and if acylation of one partner is able to modulate palmitoylation of the other.

One efficient way to study the interactions between GPCRs and G proteins is to make use of fusion proteins in which the two polypeptides are expressed from a single open reading frame (Milligan, 2000). These fusion proteins have proved very useful in a number of studies of GPCR-G protein interactions (Bertin *et al.*, 1994; Wise and Milligan, 1997; Seifert *et al.*, 1998; Guo *et al.*, 2001). In addition, preservation of basic features and regulation of interactions between GPCRs, G protein α-subunits and their interacting proteins (e.g. the G protein βγ-complex) have also been demonstrated for these fusion proteins (Bertaso *et al.*, 2003; Wise and Milligan, 1997; Cavalli *et al.*, 2000).

A fusion protein approach offers a number of specific advantages when studying palmitoylation in GPCRs and G proteins. The nature of the fusion protein means that there is defined stoichiometry of expression of the two substituent entities as 1:1 and also ensures their co-localisation following expression. One advantage of co-localisation is the ability to isolate only the interacting (i.e. activated) G protein. Another advantage is ensured membrane expression (and subsequent receptor interaction) of acylation-deficient G proteins. One further advantage of the GPCR-G protein fusion protein approach in the study of palmitoylation is in the immunoprecipitation of proteins. The fusion protein approach means the same antibody can be used to immunoprecipitate all possible acylation-variant fusion proteins within one study. Fusion protein approaches to studying palmitoylation have already been used successfully by a number of groups (Loisel *et al.*, 1999; Stevens *et al.*, 2001).

Both the α_{2A}-adrenoceptor (Kennedy and Limbird, 1993) and the $G_{o1}\alpha$ protein (Grassie *et al.*, 1994) used in this study have previously been shown to undergo palmitoylation. In the work of Kennedy and Limbird (1993, 1994) the measured half-life of [^{3}H] palmitate on this GPCR was many hours and was not substantially different than the half-life of the protein. In addition, although the presence of agonist was reported to enhance turnover of palmitate, the effect was modest and de-acylation remained a slow process. These observations for the α_{2A}-adrenoceptor appear in contrast to those for

many other protein targets for thio-acylation where rapid cycles of palmitoylation and depalmitoylation are thought to occur (Qanbar and Bouvier, 2003). Given that these limited studies of palmitoylation of the α_{2A}-adrenoceptor were performed in excess of ten years ago and also that no study of the dynamics of $G_{o1}\alpha$ palmitoylation have been performed, we considered these proteins well suited for the current investigation. With the advent of the usefulness of fusion proteins as a tool for the study of palmitoylation of GPCRs and G proteins, it was thought appropriate to re-address the study of palmitoylation in the α_{2A}-adrenoceptor and the G_{o1} protein α-subunit by the use of fusions, in an attempt to see whether regulated acylation is co-ordinated in these two proteins.

Herein, the four possible palmitoylation–variant α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ fusion proteins (α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ **(WT)**, $\alpha_{2A}Cys^{442}Ala$-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ **($C^{442}A$)**, α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{3}Ser,Cys^{351}Ile$ **($C^{3}S$)** or $\alpha_{2A}Cys^{442}Ala$-adrenoceptor-$G_{o1}\alpha Cys^{3}Ser$, $Cys^{351}Ile$ **($C^{442}A$, $C^{3}S$)**) were created and subjected to a series of palmitoylation assays. The specific objective for the work contained in this chapter was to investigate the regulation of palmitate attached to the GPCR and G protein parts of these fusions.

3.2 Results

Construction and expression of α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ fusion proteins

PCR was used to mutate a previously existing α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ fusion protein, available in-house, in order to remove the predicted palmitoylation sites from the GPCR (Kennedy and Limbird, 1993) or the G protein (Grassie *et al.*, 1994) or both parts of the fusion protein. These cDNA constructs were transiently transfected into HEK293T cells and a number of palmitoylation assays were performed to investigate the regulation of GPCR and G protein acylation in the fusion proteins. In order to assure equal construct expression levels in all 6cm dishes used within one palmitoylation assay, it was necessary to transfect one initial plate of HEK 293T cells with each desired fusion construct DNA, then to split these transfected cells into multiple duplicate 6cm dishes to be used in the palmitoylation assay. In **Figure 3.1** it is shown that such an approach led to equal expression levels of the wild type (**WT**) α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ construct. In this and in subsequent experiments, the expression levels of α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ constructs were determined by performing receptor binding studies using the tritiated antagonist [^{3}H]-RS-79948-197 (Wise *et al.*, 1997c). In these receptor binding studies the α_2-selective antagonist idazoxan was also used to allow calculation of non-specific binding (Langer and Hicks, 1984).

Confirmation of the Cys^{442} site on the GPCR and the Cys^3 site on the G protein as the sites for incorporation of [^{3}H] palmitic acid in the α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ fusion proteins

The first objective for the current study was to validate the Cys^{442} site on the GPCR and the Cys^3 site on the G protein as the sites for incorporation of palmitate. In order to do this a one time-point, pulse labelling palmitoylation assay was performed for all four palmitoylation-variant fusion proteins plus pcDNA3 control. The constructs were expressed transiently in HEK293T cells. [9,10(n)-^{3}H] palmitic acid was added to the cells in the presence or absence of 100μM adrenaline for 30 min. Following labelling of cells, immunoprecipitation using an antiserum (ON1) that identifies the N-terminal

region of $G_{o1}\alpha$ (Mullaney and Milligan, 1989), SDS-PAGE and autoradiography, the incorporation of [^{3}H] palmitate into bands with apparent molecular mass of some 89 kDa (corresponding to α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ fusions) was assessed. The α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ (**WT**), $\alpha_{2A}Cys^{442}Ala$-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ (**$C^{442}A$**) and α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{3}Ser,Cys^{351}Ile$ (**$C^{3}S$**) fusions but not the pcDNA3 control or $\alpha_{2A}Cys^{442}Ala$-adrenoceptor-$G_{o1}\alpha Cys^{3}Ser$, $Cys^{351}Ile$ (**$C^{442}A$, $C^{3}S$**) form of the fusion protein incorporated [^{3}H] palmitate (**Figure 3.2 a**, upper panel). The lack of incorporation of [^{3}H] palmitate into $\alpha_{2A}Cys^{442}Ala$-adrenoceptor-$G_{o1}\alpha Cys^{3}Ser$, $Cys^{351}Ile$ (**$C^{442}A$, $C^{3}S$**) confirmed that all the detected dynamic thio-acylation reflected modification at these two locations.

It was very interesting to note with the same period of exposure to [^{3}H] palmitate that the extent of incorporation of radioactivity into the **WT**, **$C^{442}A$** and **$C^{3}S$** constructs was not equal (**Figure 3.2 b, Table 3.1**), despite parallel immunoblots confirming that the loading of the individual constructs was the same (**Figure 3.2 a**, lower panel). Incorporation of [^{3}H] palmitate into the unstimulated α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{3}Ser,Cys^{351}Ile$ (**$C^{3}S$**) fusion protein was substantially lower (34.6 +/- 4.2%, mean +/- SEM, n=3) than for either the unstimulated α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ (**WT**) fusion protein (set as 100%, $p<0.05$) or the unstimulated $\alpha_{2A}Cys^{442}Ala$-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ (**$C^{442}A$**) fusion (96.7 +/- 9.6%, mean +/- SEM, n=3, $p<0.05$). The level of incorporation of [^{3}H] palmitate into the unstimulated α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ (**WT**) and the unstimulated $\alpha_{2A}Cys^{442}Ala$-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ (**$C^{442}A$**) were not significantly different ($p>0.05$) in three similar experiments (**Figure 3.2 b**).

It was also noted that the presence of adrenaline substantially reduced incorporation of [^{3}H] palmitate into both the α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ (**WT**) ($p<0.05$) and the $\alpha_{2A}Cys^{442}Ala$-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ (**$C^{442}A$**) ($p<0.05$) fusion proteins but did not do so for the α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{3}Ser,Cys^{351}Ile$ (**$C^{3}S$**) fusion protein ($p>0.05$) (**Figure 3.2 b**). The extent of inhibition of [^{3}H] palmitoylation of the $\alpha_{2A}Cys^{442}Ala$-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ (**$C^{442}A$**) fusion protein produced by adrenaline (66.0 +/- 5.5%, mean +/- SEM, n = 3) was significantly greater ($p<0.05$) than for the α_{2A}-

adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ (**WT**) fusion protein (30.8 +/- 12.1% mean +/- SEM, n = 3). To explore all these differences further, time courses of the incorporation of [^{3}H] palmitate into the α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ (**WT**), $\alpha_{2A}Cys^{442}Ala$-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ (**$C^{442}A$**) and α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{3}Ser,Cys^{351}Ile$ (**C^3S**) fusion proteins were performed.

Analysis of the time courses of incorporation of [^{3}H] palmitic acid in the α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ fusion proteins

The α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ (**WT**) fusion protein was expressed transiently in HEK293T cells. [^{3}H] palmitic acid was added to the cells in the presence or absence of the adrenoceptor agonist adrenaline (100μM) for times varying between 5-120 min. After cell lysate generation, immunoprecipitation, SDS-PAGE and autoradiography, in the absence of adrenaline, radioactivity was incorporated into a band with apparent molecular mass of approximately 89 kDa (**Figure 3.3 a**, upper panel). This occurred in a time-dependent manner with maximal incorporation being achieved between 60-120 min. In the presence of adrenaline, incorporation of [^{3}H] palmitate into the fusion protein was substantially reduced ($p<0.05$) over this time scale (**Figure 3.3 a**, upper panel, **Figure 3.3 b**). This effect was not attributable to unequal amounts of the fusion protein in each sample since, in parallel with these studies, samples of the cell lysates were resolved directly by SDS-PAGE and immunoblotted with antiserum ON1 (**Figure 3.3 a**, lower panel).

The time course of the incorporation of [^{3}H] palmitate into the $\alpha_{2A}Cys^{442}Ala$-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ (**$C^{442}A$**) fusion protein was also monitored and showed a similar pattern to that observed for the α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ (**WT**) construct. For $\alpha_{2A}Cys^{442}Ala$-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ (**$C^{442}A$**), radioactivity was again incorporated into a band with apparent molecular mass of approximately 89 kDa (**Figure 3.4 a**, upper panel). In the absence of adrenaline, this occurred in a time-dependent manner with maximal incorporation being achieved closer to 30 min. In the presence of adrenaline, incorporation of [^{3}H] palmitate into the fusion protein was substantially reduced ($p<0.05$) over this time scale (**Figure 3.4 a**, upper panel, **Figure 3.4 b**). Again this effect was not attributable to unequal amounts of the fusion protein in

each sample since, in parallel with these studies, samples of the cell lysates were resolved directly by SDS-PAGE and immunoblotted with antiserum ON1 (**Figure 3.4 a**, lower panel).

Upon analysis of the time course of incorporation of [^{3}H] palmitate into the α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^3Ser,Cys^{351}Ile$ (**C^3S**), the pattern observed was different to those for the other two α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ constructs. Again radioactivity was incorporated into a band with apparent molecular mass of approximately 89 kDa (**Figure 3.5 a**, upper panel). Incorporation occurred in a time-dependent manner with maximal incorporation not being achieved within the two-hour timescale used for the other two α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ fusion proteins. In the presence or absence of adrenaline, incorporation of [^{3}H] palmitate into the fusion protein was not significantly different ($p>0.05$) (**Figure 3.5 a**, upper panel, **Figure 3.5 b**). Immunoblots carried out in parallel once again ensured equal amounts of the fusion protein were present in each sample (**Figure 3.5 a**, lower panel).

It is useful, for clarity, to take the results of the time courses of the incorporation of [^{3}H] palmitate into the α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ (**WT**), $\alpha_{2A}Cys^{442}Ala$-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ (**$C^{442}A$**) and the α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^3Ser,Cys^{351}Ile$ (**C^3S**) fusion proteins together for interpretation (**Figure 3.6, Table 3.2**).

It can be seen that radio-labelling of the G protein element of the fusion (by use of the **$C^{442}A$** construct; $t½ = 8.2 +/- 1.3$ min, mean +/- SEM, $n = 3$) occurred significantly ($p<0.05$) more rapidly than incorporation of [^{3}H] palmitate into the receptor segment of the fusion (by use of the **C^3S** construct; $t½ = 27.4 +/- 2.9$ min, mean +/- SEM, $n = 3$).

In accord with the data of **Figures 3.2 a** and **b**, adrenaline did not alter the amount or rate ($t½ = 22.3 +/- 1.1$ min, mean +/- SEM, $n = 3$, $p>0.05$) of [^{3}H] palmitate incorporation into the α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^3Ser,Cys^{351}Ile$ (**C^3S**) fusion protein and although the amount of incorporation of [^{3}H] palmitate into the $\alpha_{2A}Cys^{442}Ala$-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ (**$C^{442}A$**) fusion was significantly reduced at all times points measured, the presence of adrenaline did not alter the rate ($t½ = 8.3 +/- 2.0$ min, mean +/- SEM, $n = 3$, $p>0.05$) of labelling (**Figure 3.6**).

When the results of the α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ (**WT**) fusion are compared to those of the other two fusions it is seen that the agonist-dependent differences in incorporation of palmitate for the **WT** are akin to those for the $\alpha_{2A}Cys^{442}Ala$-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ (**$C^{442}A$**) construct. However, the kinetics of incorporation for α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ (**WT**) were more like those observed for α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{3}Ser,Cys^{351}Ile$ (**$C^{3}S$**) (where t½ **WT** plus agonist = 31.4 +/- 4.4 min, mean +/- SEM, n = 3 and t½ **WT** minus agonist = 26.8 +/- 2.2 min, mean +/- SEM, n = 3). Unfortunately, data for the wild type fusion protein was not of sufficient quality to estimate whether distinct rapid and less rapid phases were present that might correspond to incorporation into the G protein and receptor elements. It is therefore concluded that it is much clearer, particularly when studying kinetics of GPCR or G protein palmitoylation, to study the GPCR or G protein parts in isolation. This can be achieved by use of the two separate $\alpha_{2A}Cys^{442}Ala$-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ (**$C^{442}A$**) and α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{3}Ser,Cys^{351}Ile$ (**$C^{3}S$**) constructs.

These studies indicated clear differences in the characteristics of palmitoylation (and the effects of adrenaline on this) in the GPCR and G protein elements of the fusion proteins. These results for the effects of adrenaline, obtained by pulse-labelling experiments, could potentially represent altered rates of palmitoylation or de-palmitoylation of the G protein. In order to investigate this, the dynamics of de-palmitoylation of GPCR-G protein fusions were studied in experiments performed in pulse-chase format.

Analysis of the depalmitoylation rates of the α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ fusion proteins

Following transfection of HEK293T cells with the α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ (**WT**) fusion protein and labelling of the cells with [^{3}H] palmitate for 30 min, the radiolabel was removed and replaced with non-radioactive palmitate. Samples were taken for analysis at times up to 180 min. [^{3}H] palmitate was removed from the immunoprecipitated fusion protein with t½ = 34.8 +/- 9.2 min (mean +/- SEM, n = 3) (**Figure 3.7 a** and **b**). When the chase was conducted in the presence of adrenaline removal of [^{3}H] palmitate from the fusion protein was substantially more rapid, with t½

= 20.0 +/- 3.3 min (mean +/- SEM, n = 3, $p<0.05$), demonstrating that agonist enhanced de-palmitoylation of the fusion protein.

To assess the contribution of the G protein to these effects, HEK293T cells were transfected to express the $\alpha_{2A}Cys^{442}Ala$-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ (**$C^{442}A$**) fusion protein in which only the G protein element is a target for thio-acylation. Using the same protocol the rate of disappearance of [^{3}H] palmitate from immunoprecipitated samples was again rapid ($t½$ = 37.0 +/- 4.9 min, mean +/- SEM, n = 3) and accelerated ($t½$ = 17.1 +/- 2.1 min, mean +/- SEM, n = 3, $p<0.05$) by the presence of agonist (**Figure 3.8 a** and **b**). In both sets of experiments, parallel immunoblots of cell lysates confirmed equal loading of the gel lanes.

Equivalent experiments were then performed with the α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{3}Ser,Cys^{351}Ile$ (**C^3S**) fusion protein in which only the GPCR element can be a target for palmitoylation. Again, a time-dependent reduction in the presence of [^{3}H] palmitate was observed, indicating dynamic de-palmitoylation of the receptor with a similar half–life ($t½$ = 27.3 +/- 4.1 min, mean +/- SEM, n = 3) as noted for the G protein but by contrast, this was not altered ($t½$ = 28.9 +/- 2.0 min, mean +/- SEM, n = 3, $p>0.05$) by the presence of adrenaline (**Figure 3.9 a** and **b**). The depalmitoylation data for all three α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ constructs are summarised in **Table 3.3.**

Analysis of concentration-dependent effects of adrenaline on palmitoylation of the α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ fusion protein

When labelling of the α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ (**WT**) fusion protein with [^{3}H] palmitate was allowed to proceed for 30 min in the presence of varying concentrations of adrenaline, it was found that the effects of adrenaline on palmitate incorporation were concentration-dependent. Half-maximal reduction in incorporation of [^{3}H] palmitate into the immunoprecipitated fusion protein was obtained with 1.4 +/- 0.2 x 10^{-8} M adrenaline (mean +/- SEM, n = 3) (**Figure 3.10 a** and **b**). As the calculated affinity (corrected IC_{50} = 2.6 +/- 0.6 x 10^{-8} M, mean +/- SEM, n = 3) of adrenaline to bind to the α_{2A}-adrenoceptor-$(Cys^{351}Ile)G\alpha_{o1}$ fusion protein (**Figure 3.11**) was similar it suggests

that binding of the agonist to the receptor was directly responsible for the regulation of [^{3}H] palmitoylation.

Analysis of requirement for G protein to be activated in order to produce agonist-regulation of G protein palmitoylation

The α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ (**WT**) and $\alpha_{2A}Cys^{442}Ala$-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ (**$C^{442}A$**) fusion proteins were further modified to encode a **$Gly^{204}Ala$** mutation within the G protein element of the constructs. This Gly is totally conserved in the α subunit of heterotrimeric G proteins. Such Gly to Ala mutations prevent effective exchange of GTP for GDP and hence the G protein is unable to adopt the active conformation. As anticipated, addition of adrenaline was unable to cause binding of [^{35}S] GTPγS to the pcDNA3 control, the α_{2A}-adrenoceptor-$G_{o1}\alpha Gly^{204}AlaCys^{351}Ile$ (**$Gly^{204}Ala$**) and $\alpha_{2A}Cys^{442}Ala$-adrenoceptor-$G_{o1}\alpha Gly^{204}AlaCys^{351}Ile$ (**$Cys^{442}Ala$, $Gly^{204}Ala$**) constructs. [^{35}S] GTPγS binding in response to adrenaline was observed however, for the α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ (**WT**) and $\alpha_{2A}Cys^{442}Ala$-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ (**$Cys^{442}Ala$**) constructs (**Figure 3.12**). We therefore used these forms of the fusions to assess if activation was required to produce agonist-regulation of G protein palmitoylation. Incorporation of [^{3}H] palmitate into an 89kDa band was observed for the α_{2A}-adrenoceptor-$G_{o1}\alpha Gly^{204}AlaCys^{351}Ile$ (**$Gly^{204}Ala$**) and $\alpha_{2A}Cys^{442}Ala$-adrenoceptor-$G_{o1}\alpha Gly^{204}AlaCys^{351}Ile$ (**$Cys^{442}Ala$, $Gly^{204}Ala$**) constructs in addition to the **WT** and **$Cys^{442}Ala$** fusion proteins (already shown previously in **Figure 3.2**). However, unlike for the α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ (**WT**) and $\alpha_{2A}Cys^{442}Ala$-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ (**$Cys^{442}Ala$**) fusion proteins, there was no effect of adrenaline on the palmitoylation status of the two **$Gly^{204}Ala$** fusions (**Figure 3.13 a** and **b**), indicating that G protein activation is required to produce agonist regulation of G protein palmitoylation.

Analysis of the incorporation of [^{3}H] palmitic acid into endogenously expressed $G_{o1}\alpha$ proteins

In order to assess how agonist-regulated G protein palmitoylation results obtained using the $\alpha_{2A}Cys^{442}Ala$-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ (**$C^{442}A$**) fusion protein compared with those for the non-fused G protein, we examined the effects of adrenaline on the palmitoylation status of the small amount of $G_{o1}\alpha$ that is expressed endogenously in HEK293 cells. It has previously been noted that when expressed at high levels the α_{2A}-adrenoceptor element of GPCR-G protein fusions can activate endogenous G proteins as well as the G protein fused to it (Burt *et al.*, 1998). HEK293T cells were transfected with the α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ (**WT**) fusion protein and incubated with [^{3}H] palmitate in the absence or presence of adrenaline. After cell lysate production, immunoprecipitation with antiserum ON1, SDS-PAGE and autoradiography, the incorporation of [^{3}H] palmitate into a band with apparent molecular mass of some 40 kDa (corresponding to endogenous $G_{o1}\alpha$) was assessed (**Figure 3.14**, upper panel). Expression levels of endogenous $G_{o1}\alpha$ were also assessed by parallel immunoblots with antiserum ON1 (**Figure 3.14**, lower panel). The endogenous $G_{o1}\alpha$ incorporated [^{3}H] palmitate in a time-dependent manner but in contrast to the fused $G_{o1}\alpha$, the presence of adrenaline enhanced labelling, suggesting some inherent difference for the receptor-linked G protein.

Analysis of the ability of the $\alpha_{2A}Cys^{442}Ala$-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ ($C^{442}A$) fusion protein to be repalmitoylated

When the agonist-promoted regulation of palmitoylation is compared for GPCR-G protein fusion proteins and their non-fused components some differences have been observed (current study and Loisel *et al.*, 1999). In the work of Loisel *et al.* (1999), the differences in agonist-promoted palmitate regulation observed for the β_2-adrenoceptor-$G_s\alpha$ fusion protein as opposed to the non-fused GPCR and G protein were thought to be as a result of the inability of the fusion protein to be repalmitoylated. For comparison with the findings of Loisel *et al.* (1999), the ability of the $\alpha_{2A}Cys^{442}Ala$-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ ($C^{442}A$) fusion protein to be repalmitoylated was assessed. HEK293T

cells were transfected with the $\alpha_{2A}Cys^{442}$Ala-adrenoceptor-$G_{o1}\alpha Cys^{351}$Ile (**$C^{442}A$**) fusion protein and incubated for 30 min with [^{3}H] palmitate in the absence of agonist (to allow approximately steady-state levels of [^{3}H] palmitate incorporation to be reached). Subsequent to this, cells were incubated with [^{3}H] palmitate in the absence or presence of adrenaline. After cell lysate production, immunoprecipitation with antiserum ON1, SDS-PAGE and autoradiography, the incorporation of [^{3}H] palmitate into a band of some 89kDa was monitored (**Figure 3.15 a** and **b**). In the absence of agonist the levels of [^{3}H] palmitate incorporated into the $\alpha_{2A}Cys^{442}$Ala-adrenoceptor-$G_{o1}\alpha Cys^{351}$Ile (**$C^{442}A$**) fusion protein remained relatively unchanged (**Figure 3.15 a** lanes 1, 2 and 4). In contrast, in the presence of adrenaline (**Figure 3.15 a** lanes 3 and 5) the levels of [^{3}H] palmitate incorporated into the $\alpha_{2A}Cys^{442}$Ala-adrenoceptor-$G_{o1}\alpha Cys^{351}$Ile (**$C^{442}A$**) fusion protein were markedly reduced ($p<0.05$). These results show that in the presence of adrenaline, depalmitoylation of the $\alpha_{2A}Cys^{442}$Ala-adrenoceptor-$G_{o1}\alpha Cys^{351}$Ile (**$C^{442}A$**) fusion occurs and that subsequent repalmitoylation of this fusion is inhibited (as reflected by the decrease in overall [^{3}H] palmitate incorporated into this protein).

Figure 3.1

Analysis of expression levels of an α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ fusion protein after sub-culture into multiple duplicate culture plates (determined from binding of a 5nM concentration of [^{3}H]-RS-79948-197 to membranes expressing the α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ fusion protein)

HEK293T cells were transfected to express α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ fusion protein. Membranes were prepared and binding of a 5nM concentration of [^{3}H]- RS-79948-197 to membranes expressing the α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ fusion protein was assessed for multiple duplicate culture plates (labelled 1-6). Results are from triplicate determinations. Analysis is representative of three similar experiments.

Figure 3.1

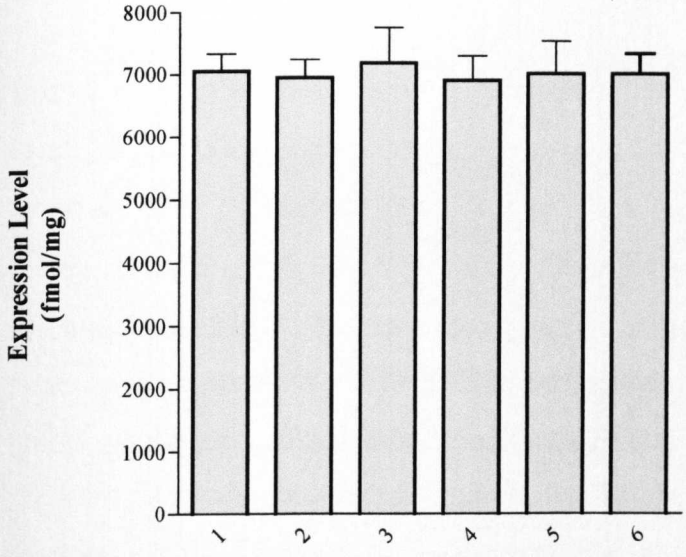

Expression Level
(fmol/mg)
8000
7000
6000
5000
4000
3000
2000
1000
0
1
2
3
4
5
6

Figure 3.2

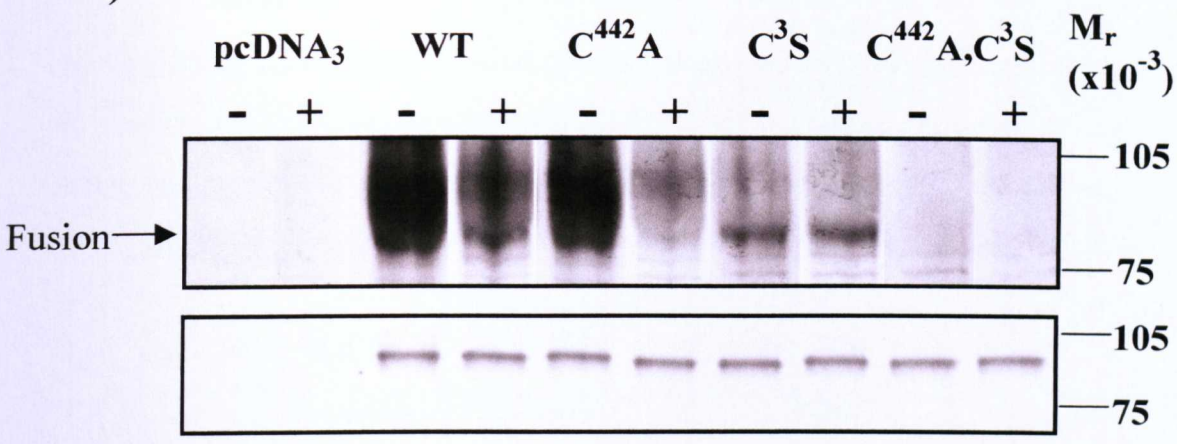
Incorporation of [^{3}H] palmitate into the palmitoylation-variant α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ fusion proteins in the presence and absence of adrenaline.

HEK293T cells were transfected with empty vector (**pcDNA3**) or to express α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ (**WT**), $\alpha_{2A}Cys^{442}Ala$-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ (**$C^{442}A$**), α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{3}Ser$, $Cys^{351}Ile$ (**C^3S**) or $\alpha_{2A}Cys^{442}Ala$ -adrenoceptor-$G_{o1}\alpha Cys^{3}Ser$, $Cys^{351}Ile$ (**$C^{442}A$, C^3S**) fusion proteins. Cells were incubated with [^{3}H] palmitate for 30min in the absence (-) or presence (+) of 100μM adrenaline. Samples were harvested and cell lysates produced. These were either immunoprecipitated with antiserum ON1 prior to SDS-PAGE and autoradiography for 1 month (**a**, upper panel) or resolved directly by SDS-PAGE and immunoblotted with antiserum ON1 (**a**, lower panel). **Figure 3.2** (**a**) shows one representative palmitoylation experiment with corresponding western blot analysis. Similar results were obtained for three separate experiments.

Autoradiographs as in the upper panel of **a** were scanned and signals quantitated (**b**) in the area of the film shown. **pcDNA3**=black bars, **WT**=blue bars, **$C^{442}A$**=green bars, **C^3S**=purple bars and **$C^{442}A$, C^3S**=brown bars. Results for three separate experiments were quantified and data is shown as mean +/- S.E.M., n= 3. In order to compare levels of incorporation from separate experiments it was necessary to express the levels of incorporation for each sample as a percentage of the incorporation observed for the unstimulated **WT** construct.

Figure 3.2

a)


pcDNA3
WT
C442A
C3S
C442A,C3S
Mr (x10-3)
- + - + - + - + - +
Fusion
105
75
105
75

b)

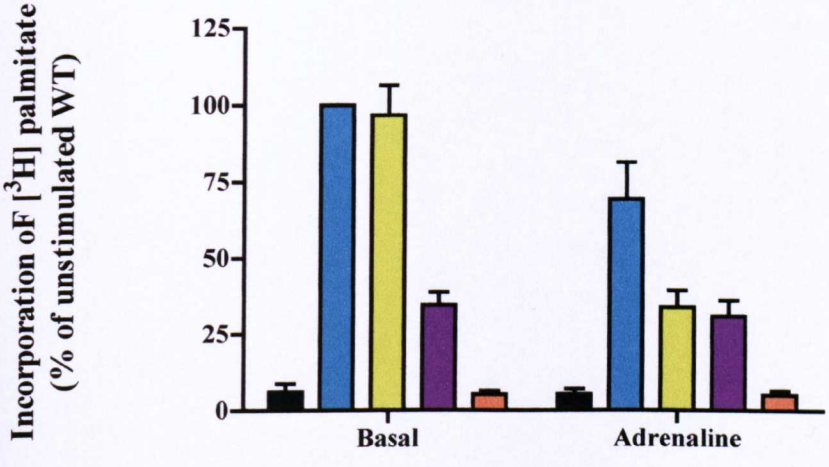

Incorporation oF [3H] palmitate (% of unstimulated WT)
125
100
75
50
25
0
Basal
Adrenaline

Table 3.1

Comparison of the incorporation of [^{3}H] palmitate into all four palmitoylation-variant fusion proteins in the presence and absence of adrenaline

The results from **Figures 3.2** were presented in a tabular form for clarity of comparison between the constructs. Incorporation level into each α_{2A}-adrenoceptor -$G_{o1}\alpha Cys^{351}Ile$ fusion protein in the presence and absence of adrenaline is given as a percentage (mean +/- SEM, n=3) of the incorporation observed for the unstimulated **WT** construct.

Table 3.1

Construct	Potential Site of [^{3}H] Palmitate Incorporation	Incorporation of [^{3}H] Palmitate in Absence of Adrenaline (%)	Incorporation of [^{3}H] Palmitate in Presence of Adrenaline (%)
α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ (**WT**)	GPCR Cys^{442} and G protein Cys^3 residue	100	69.2 +/- 12.1
$\alpha_{2A}Cys^{442}Ala$-adrenoceptor-$G_{o1}\alpha$ $Cys^{351}Ile$ (**$C^{442}A$**)	G protein Cys^3 residue	96.7 +/- 9.6	34.0 +/- 5.5
α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^3Ser$, $Cys^{351}Ile$ (**C^3S**)	GPCR Cys^{442} residue	34.6 +/- 4.2	31.1 +/- 5.2
$\alpha_{2A}Cys^{442}Ala$-adrenoceptor-$G_{o1}\alpha Cys^3Ser$, $Cys^{351}Ile$ (**$C^{442}A$, C^3S**)	None	None	None

Figure 3.3

Incorporation of [^{3}H] palmitate into the α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ (WT) fusion protein in the presence and absence of adrenaline.

An α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ fusion protein was expressed in HEK293T cells. Cells were incubated with [^{3}H] palmitate for the indicated times in the absence (left panels) or presence (right panels) of 100μM adrenaline. Samples were harvested and cell lysates produced. These were either immunoprecipitated with antiserum ON1 prior to SDS-PAGE and autoradiography for 1 month (**a**, upper panels) or resolved directly by SDS-PAGE and immunoblotted with antiserum ON1 (**a**, lower panels). **Figure 3.3** (**a**) shows one representative palmitoylation experiment with corresponding western blot analysis. Similar results were obtained for three separate experiments.

Autoradiographs as in the upper panels of **a** were scanned and signals quantitated (**b**) in the area of the film shown. Open circles = absence, filled circles = presence of adrenaline. Results for three separate experiments were quantified and data is shown as mean +/- S.E.M., n= 3. In order to compare levels of incorporation from separate experiments it was necessary to express the levels of incorporation for each sample as a percentage of the maximal incorporation level observed (60 min time-point of unstimulated construct).

Figure 3.3

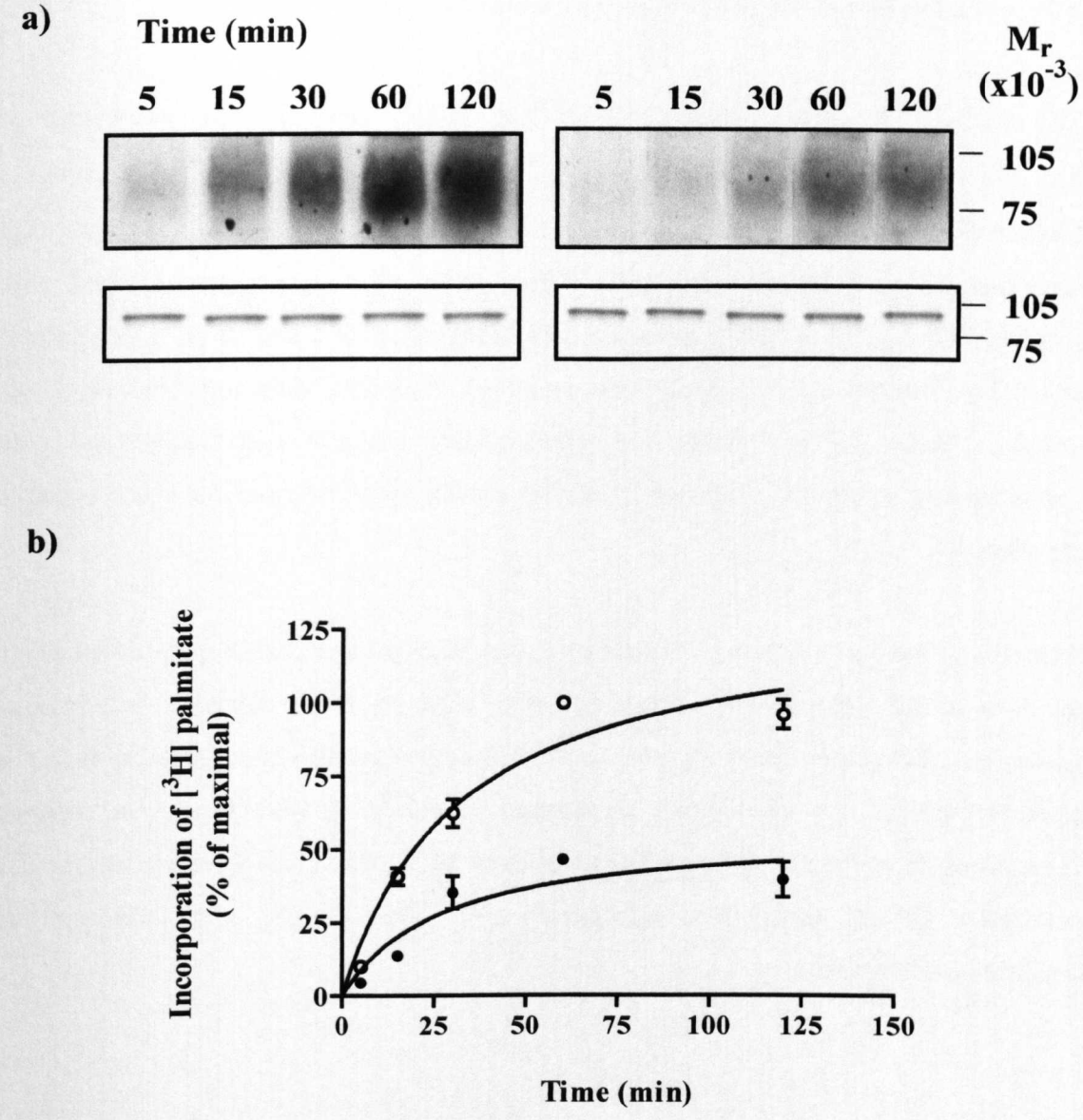

a)
Time (min)
5
15
30
60
120
5
15
30
60
120
M_r ($x10^{-3}$)
105
75
105
75
b)
Incorporation of [^{3}H] palmitate (% of maximal)
125
100
75
50
25
0
0
25
50
75
100
125
150
Time (min)

Figure 3.4

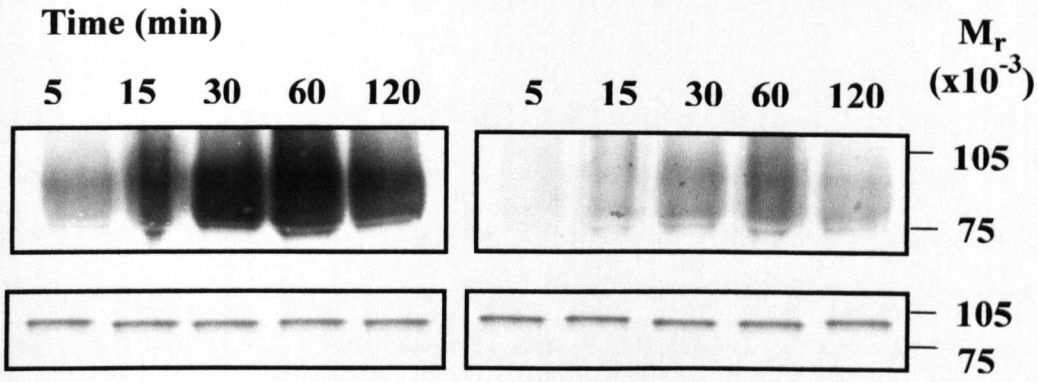
Incorporation of [^{3}H] palmitate into the α_{2A}Cys^{442}Ala-adrenoceptor-$G_{o1}\alpha$Cys^{351}Ile ($C^{442}A$) fusion protein in the presence and absence of adrenaline.

An α_{2A}Cys^{442}Ala-adrenoceptor-$G_{o1}\alpha$Cys^{351}Ile fusion protein was expressed in HEK293T cells. Cells were incubated with [^{3}H] palmitate for the indicated times in the absence (left panels) or presence (right panels) of 100μM adrenaline. Samples were harvested and cell lysates produced. These were either immunoprecipitated with antiserum ON1 prior to SDS-PAGE and autoradiography for 1 month (**a**, upper panels) or resolved directly by SDS-PAGE and immunoblotted with antiserum ON1 (**a**, lower panels). **Figure 3.4** (**a**) shows one representative palmitoylation experiment with corresponding western blot analysis. Similar results were obtained for three separate experiments.

Autoradiographs as in the upper panels of **a** were scanned and signals quantitated (**b**) in the area of the film shown. Open circles = absence, filled circles = presence of adrenaline. Results for three separate experiments were quantified and data is shown as mean +/- S.E.M., n= 3. In order to compare levels of incorporation from separate experiments it was necessary to express the levels of incorporation for each sample as a percentage of the maximal incorporation level observed (60 min time-point of unstimulated construct).

Figure 3.4

a)


Time (min)
5
15
30
60
120
5
15
30
60
120
M_r ($x10^{-3}$)
105
75
105
75

b)

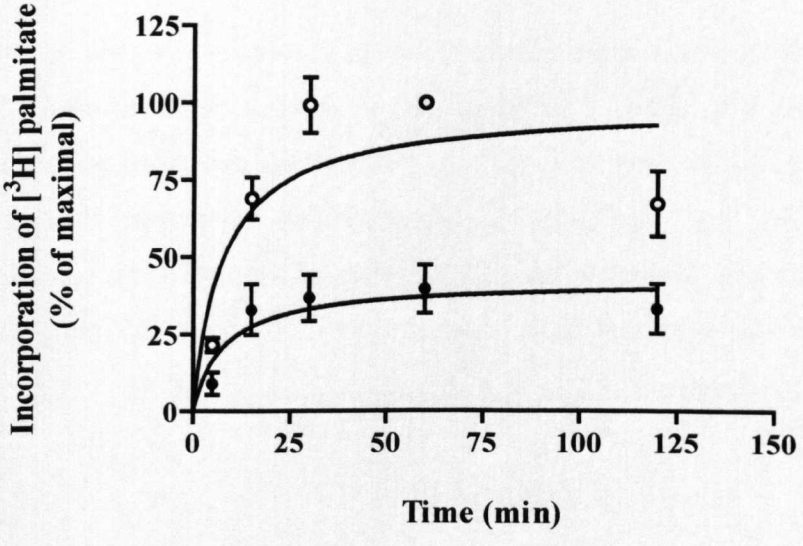

Incorporation of [^{3}H] palmitate (% of maximal)
125
100
75
50
25
0
0
25
50
75
100
125
150
Time (min)

Figure 3.5

Incorporation of [^{3}H] palmitate into the α_{2A}-adrenoceptor-$G_{o1}\alpha$ Cys^{3}Ser, Cys^{351}Ile (C^3S) fusion protein in the presence and absence of adrenaline.

An α_{2A}-adrenoceptor-$G_{o1}\alpha$ Cys^{3}Ser, Cys^{351}Ile fusion protein was expressed in HEK293T cells. Cells were incubated with [^{3}H] palmitate for the indicated times in the absence (left panels) or presence (right panels) of 100μM adrenaline. Samples were harvested and cell lysates produced. These were either immunoprecipitated with antiserum ON1 prior to SDS-PAGE and autoradiography for 1 month (**a**, upper panels) or resolved directly by SDS-PAGE and immunoblotted with antiserum ON1 (**a**, lower panels). **Figure 3.5** (**a**) shows one representative palmitoylation experiment with corresponding western blot analysis. Similar results were obtained for three separate experiments.

Autoradiographs as in the upper panels of **a** were scanned and signals quantitated (**b**) in the area of the film shown. Open circles = absence, filled circles = presence of adrenaline. Results for three separate experiments were quantified and data is shown as mean +/- S.E.M., n= 3. In order to compare levels of incorporation from separate experiments it was necessary to express the levels of incorporation for each sample as a percentage of the maximal incorporation level observed (60 min time-point of unstimulated construct).

Figure 3.5

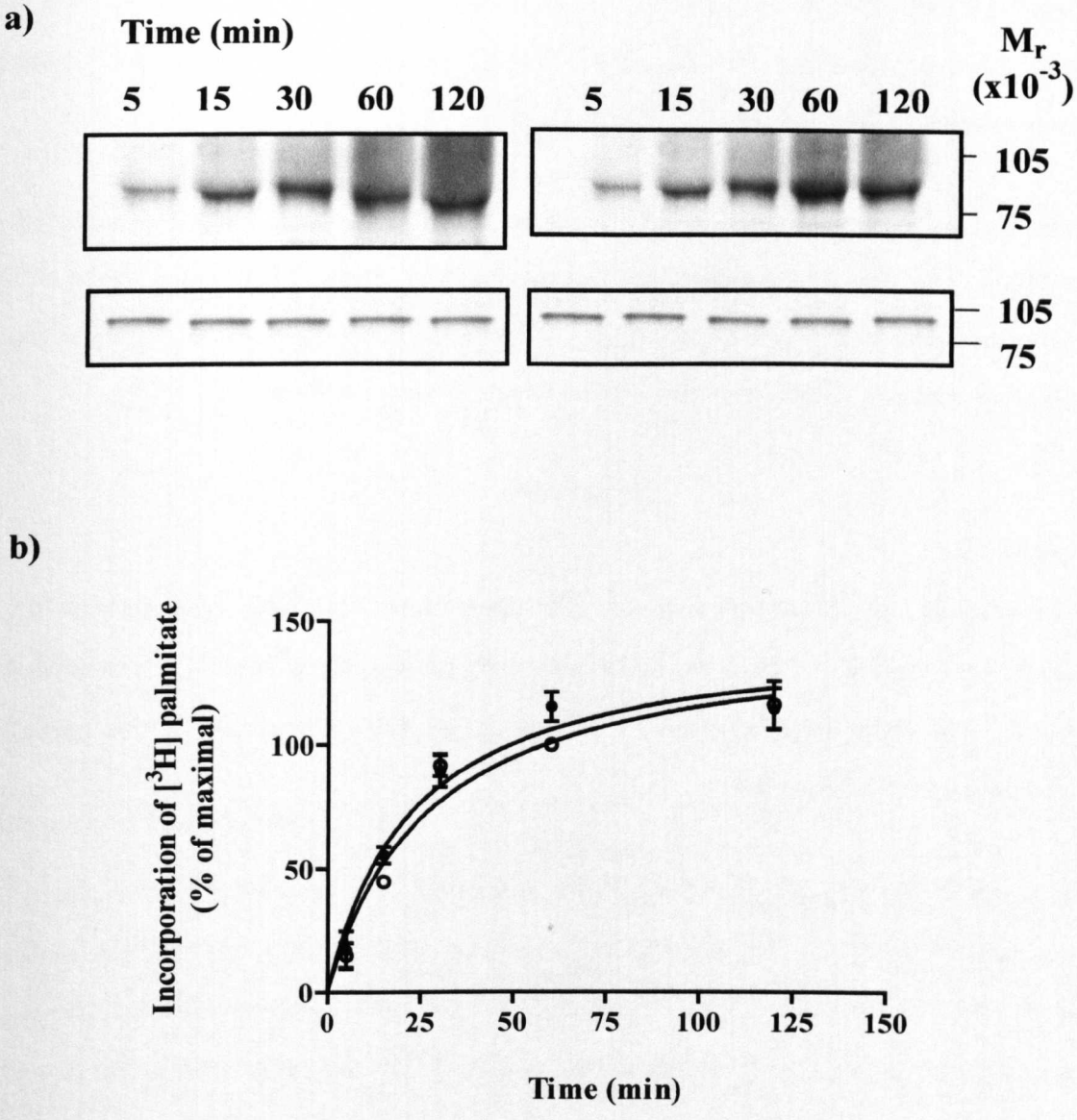

a)
Time (min)
5 15 30 60 120
5 15 30 60 120
M_r ($x10^{-3}$)
105
75
105
75
b)
Incorporation of [^{3}H] palmitate (% of maximal)
150
100
50
0
0 25 50 75 100 125 150
Time (min)

Figure 3.6

Comparison of incorporation of [^{3}H] palmitate into the α_{2A}-adrenoceptor-$G_{o1}\alpha$Cys^{351}Ile (WT), α_{2A}Cys^{442}Ala-adrenoceptor-$G_{o1}\alpha$Cys^{351}Ile (C^{442}A) and the α_{2A}-adrenoceptor-$G_{o1}\alpha$ Cys^{3}Ser, Cys^{351}Ile (C^{3}S) fusion proteins in the presence and absence of adrenaline

The autoradiographs quantitated in **Figures 3.3 b**, **3.4 b** and **3.5 b** are analysed in parallel for clarity. Open circles with dashed lines = absence of adrenaline, filled circles with solid lines = presence of adrenaline. **WT**=black, **C^{442}A**=red and **C^{3}S**=blue. Data is shown as mean +/- S.E.M., n= 3.

Table 3.2

Comparison of incorporation of [^{3}H] palmitate into the α_{2A}-adrenoceptor-$G_{o1}\alpha$Cys^{351}Ile (WT), α_{2A}Cys^{442}Ala-adrenoceptor-$G_{o1}\alpha$Cys^{351}Ile (C^{442}A) and the α_{2A}-adrenoceptor-$G_{o1}\alpha$ Cys^{3}Ser, Cys^{351}Ile (C^{3}S) fusion proteins in the presence and absence of adrenaline

The results from **Figures 3.3-3.5** were presented in a tabular form for clarity of comparison between the constructs. Non-linear regression analysis was used to determine the maximal incorporation level of [^{3}H] palmitate and the $t_{1/2}$ (min) for incorporation into each α_{2A}-adrenoceptor-$G_{o1}\alpha$Cys^{351}Ile fusion protein in the presence and absence of adrenaline.

Figure 3.6

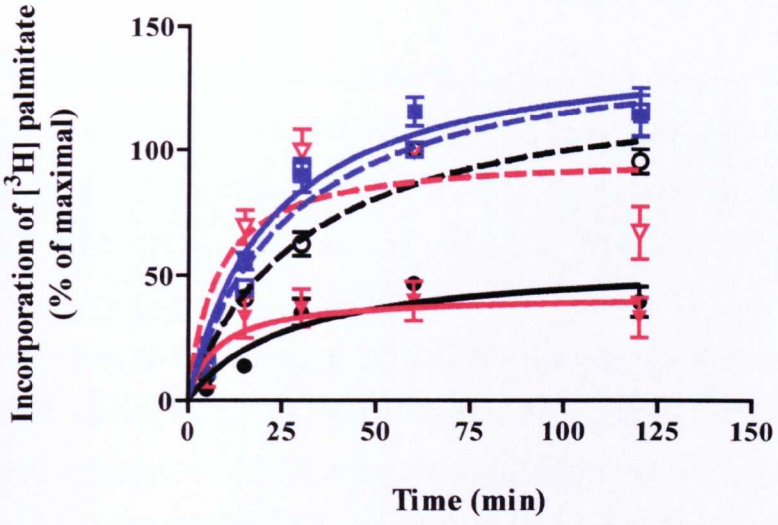


Table 3.2

Construct	Potential Site of [^{3}H] Palmitate Incorp.	Max Palmitate Incorp. -Adrenaline (%)	$t_{1/2}$ Palmitate Incorp. -Adrenaline (min)	Max Palmitate Incorp. +Adrenaline (%)	$t_{1/2}$ Palmitate Incorp. +Adrenaline (min)
WT	GPCR Cys^{442} residue and G protein Cys^3 residue	131 +/- 12	31.4 +/- 4.4	56 +/- 9	26.8 +/- 2.2
$C^{442}A$	G protein Cys^3 residue	98 +/- 12	8.2 +/- 1.3	42 +/- 7	8.3 +/- 2.0
C^3S	GPCR Cys^{442} residue	146 +/- 12	27.4 +/- 2.9	148 +/- 12	22.3 +/- 1.1

Figure 3.7

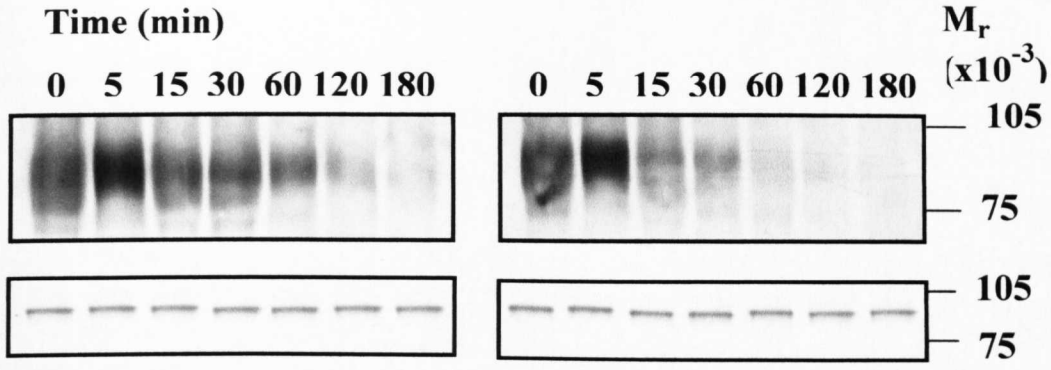
Basal and adrenaline-stimulated de-palmitoylation of an α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ (WT) fusion protein

HEK293T cells were transfected to express an α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ fusion protein. Cells were incubated with [^{3}H] palmitate for 30 min, washed and then excess non-radioactive palmitate was added in the absence (**a**, left panels) or presence (**a, right** panels) of adrenaline (100μM). Samples were harvested at varying times and cell lysates produced that were either immunoprecipitated with antiserum ON1 prior to SDS-PAGE and autoradiography for 1 month (**a**, upper panels) or resolved directly by SDS-PAGE and immunoblotted with antiserum ON1 (**a**, lower panels). **Figure 3.7 (a)** shows one representative depalmitoylation experiment with corresponding western blot analysis. Similar results were obtained for three separate experiments.

Autoradiographs as in the upper panel of **a** were scanned and signals quantitated **(b)** in the area of the film shown. Open symbols = absence of adrenaline, filled symbols = presence of adrenaline. Results for three separate experiments were quantified and data is shown as mean +/- S.E.M., n= 3. In order to compare remaining levels of [^{3}H] palmitate from separate experiments it was necessary to express the remaining levels of [^{3}H] palmitate for each sample as a percentage of the maximal level observed (0 min chase time).

Figure 3.7

a)


Time (min)
0 5 15 30 60 120 180
0 5 15 30 60 120 180
M_r ($x10^{-3}$)
105
75
105
75

b)

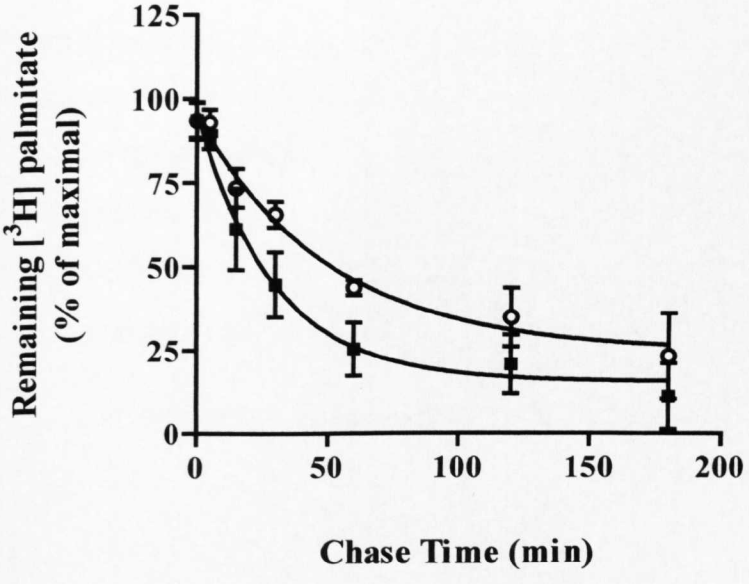

Remaining [3H] palmitate (% of maximal)
125
100
75
50
25
0
0
50
100
150
200
Chase Time (min)

Figure 3.8

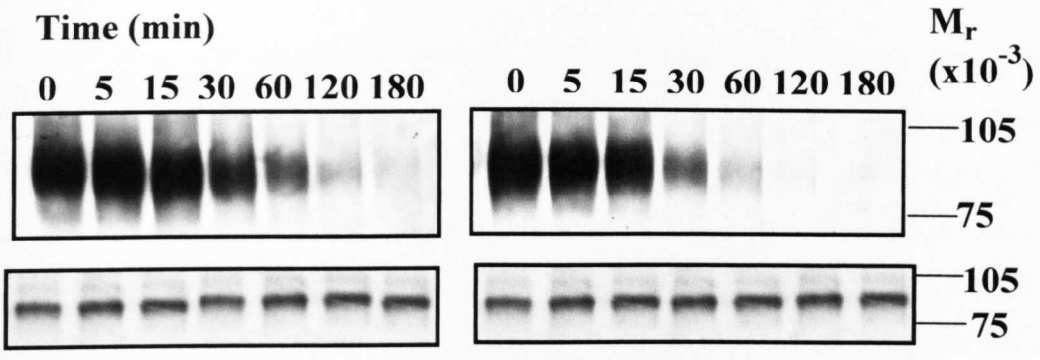
Basal and adrenaline-stimulated de-palmitoylation of an $\alpha_{2A}Cys^{442}Ala$-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ ($C^{442}A$) fusion protein

HEK293T cells were transfected to express an $\alpha_{2A}Cys^{442}Ala$-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ fusion protein. Cells were incubated with [^{3}H] palmitate for 30 min, washed and then excess non-radioactive palmitate was added in the absence (**a**, left panels) or presence (**a**, right panels) of adrenaline (100μM). Samples were harvested at varying times and cell lysates produced that were either immunoprecipitated with antiserum ON1 prior to SDS-PAGE and autoradiography for 1 month (**a**, upper panels) or resolved directly by SDS-PAGE and immunoblotted with antiserum ON1 (**a**, lower panels). **Figure 3.8** (**a**) shows one representative depalmitoylation experiment with corresponding western blot analysis. Similar results were obtained for three separate experiments.

Autoradiographs as in the upper panel of **a** were scanned and signals quantitated (**b**) in the area of the film shown. Open symbols = absence of adrenaline, filled symbols = presence of adrenaline. Results for three separate experiments were quantified and data is shown as mean +/- S.E.M., n= 3. In order to compare remaining levels of [^{3}H] palmitate from separate experiments it was necessary to express the remaining levels of [^{3}H] palmitate for each sample as a percentage of the maximal level observed (0 min chase time).

Figure 3.8

a)


Time (min)
0 5 15 30 60 120 180
0 5 15 30 60 120 180
M_r ($x10^{-3}$)
105
75
105
75

b)

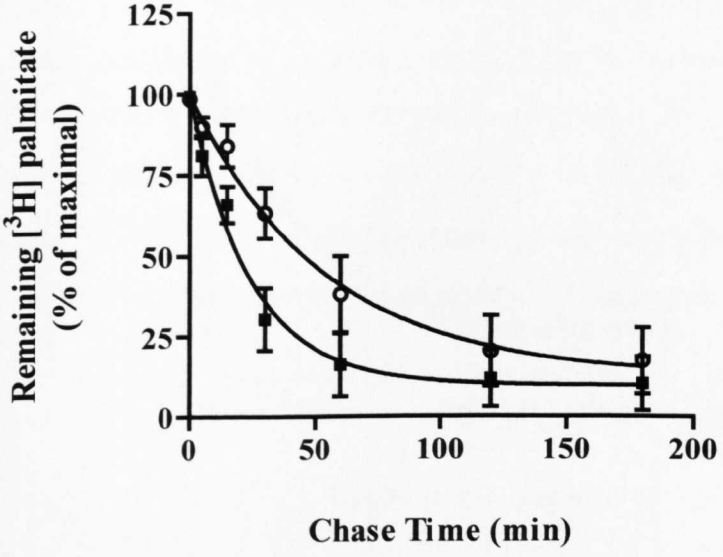

Remaining [^{3}H] palmitate (% of maximal)
125
100
75
50
25
0
0
50
100
150
200
Chase Time (min)

Figure 3.9

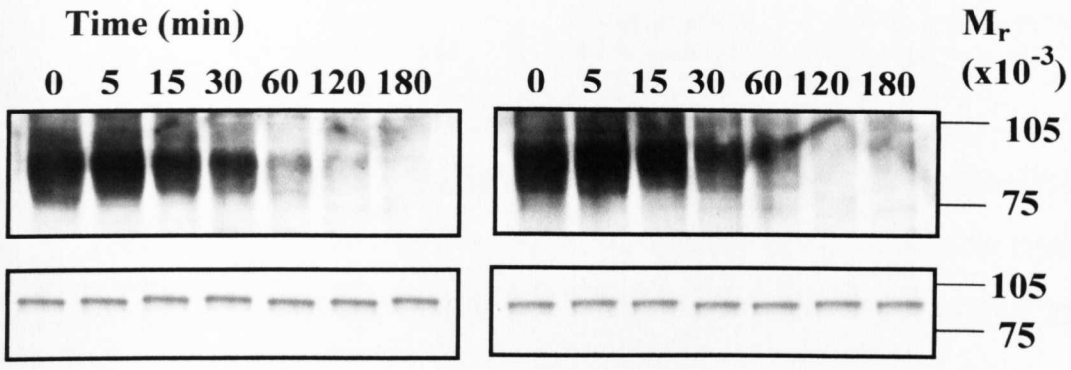
Basal and adrenaline-stimulated de-palmitoylation of an α_{2A}-adrenoceptor-$G_{o1}\alpha$ $Cys^{3}Ser$, $Cys^{351}Ile$ ($C^{3}S$) fusion protein

HEK293T cells were transfected to express an α_{2A}-adrenoceptor-$G_{o1}\alpha$ $Cys^{3}Ser$, $Cys^{351}Ile$ fusion protein. Cells were incubated with [^{3}H] palmitate for 30 min, washed and then excess non-radioactive palmitate was added in the absence (**a**, left panels) or presence (**a**, right panels) of adrenaline (100μM). Samples were harvested at varying times and cell lysates produced that were either immunoprecipitated with antiserum ON1 prior to SDS-PAGE and autoradiography for 1 month (**a**, upper panels) or resolved directly by SDS-PAGE and immunoblotted with antiserum ON1 (**a**, lower panels). **Figure 3.9** (**a**) shows one representative depalmitoylation experiment with corresponding western blot analysis. Similar results were obtained for three separate experiments.

Autoradiographs as in the upper panel of **a** were scanned and signals quantitated (**b**) in the area of the film shown. Open symbols = absence of adrenaline, filled symbols = presence of adrenaline. Results for three separate experiments were quantified and data is shown as mean +/- S.E.M., n= 3. In order to compare remaining levels of [^{3}H] palmitate from separate experiments it was necessary to express the remaining levels of [^{3}H] palmitate for each sample as a percentage of the maximal level observed (0 min chase time).

Figure 3.9

a)


Time (min)
0 5 15 30 60 120 180
0 5 15 30 60 120 180
M_r ($x10^{-3}$)
105
75
105
75

b)

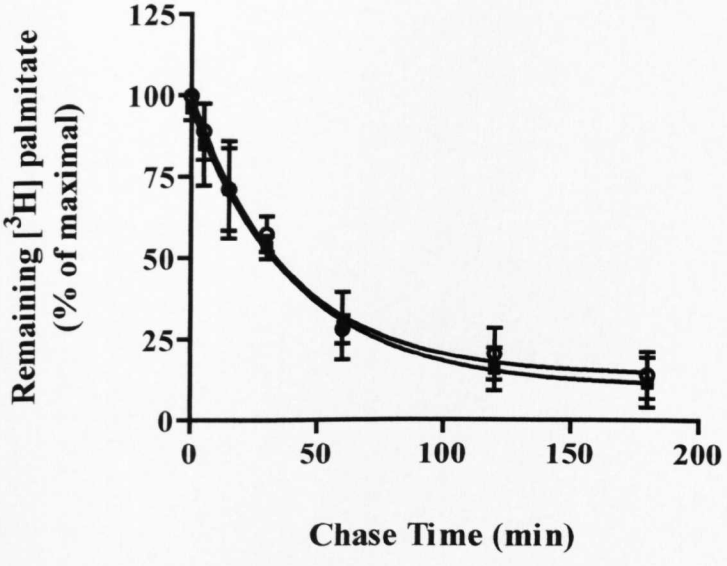

Remaining [^{3}H] palmitate (% of maximal)
125
100
75
50
25
0
0
50
100
150
200
Chase Time (min)

Table 3.3

Comparison of depalmitoylation for the three selected palmitoylation-variant α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ fusion proteins in the presence and absence of adrenaline

The results from **Figures 3.7-3.9** were presented in a tabular form for clarity of comparison between the constructs. Non-linear regression analysis was used to determine the $t_{1/2}$ (min) for depalmitoylation of α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ fusion proteins in the presence and absence of adrenaline.

Table 3.3

Construct	Potential Site of [^{3}H] Palmitate Incorporation	$t_{1/2}$ (min) Depalmitoylation (- Adrenaline)	$t_{1/2}$ (min) Depalmitoylation (+ Adrenaline)
α_{2A}-adrenoceptor-$G_{o1}\alpha C^{351}I$ (**WT**)	GPCR Cys^{442} and G protein Cys^3 residues	34.8 +/- 9.2	20.0 +/- 3.3
$\alpha_{2A}C^{442}A$ adrenoceptor-$G_{o1}\alpha C^{351}I$ (**$Cys^{442}Ala$**)	G protein Cys^3 residue	37.0 +/- 4.9	17.1 +/- 2.1
α_{2A}-adrenoceptor-$G_{o1}\alpha C^3S$, $C^{351}I$ (**Cys^3Ser**)	GPCR Cys^{442} residue	27.3 +/- 4.1	28.9 +/- 2.0

Figure 3.10

Concentration-response of adrenaline regulated incorporation of [^{3}H] palmitate into the α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ (WT) fusion protein

An α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ fusion protein was expressed in HEK293T cells. Cells were incubated with [^{3}H] palmitate for 30 min in the presence of varying concentrations of adrenaline. Samples were harvested and cell lysates produced. These were either immunoprecipitated with antiserum ON1 prior to SDS-PAGE and autoradiography for 1 month (**a**, upper panel) or resolved directly by SDS-PAGE and immunoblotted with antiserum ON1 (**a**, lower panel). **Figure 3.10** (**a**) shows one representative concentration-response palmitoylation experiment with corresponding western blot analysis. Similar results were obtained for three separate experiments.

Autoradiographs as in the upper panel of **a** were scanned and signals quantitated **(b)** in the area of the film shown. The effect of adrenaline was quantified for three separate experiments and data is shown as mean +/- S.E.M., n= 3. In order to compare levels of incorporation from separate experiments it was necessary to express the levels of incorporation for each sample as a percentage of the maximal incorporation level observed (unstimulated construct).

Figure 3.10

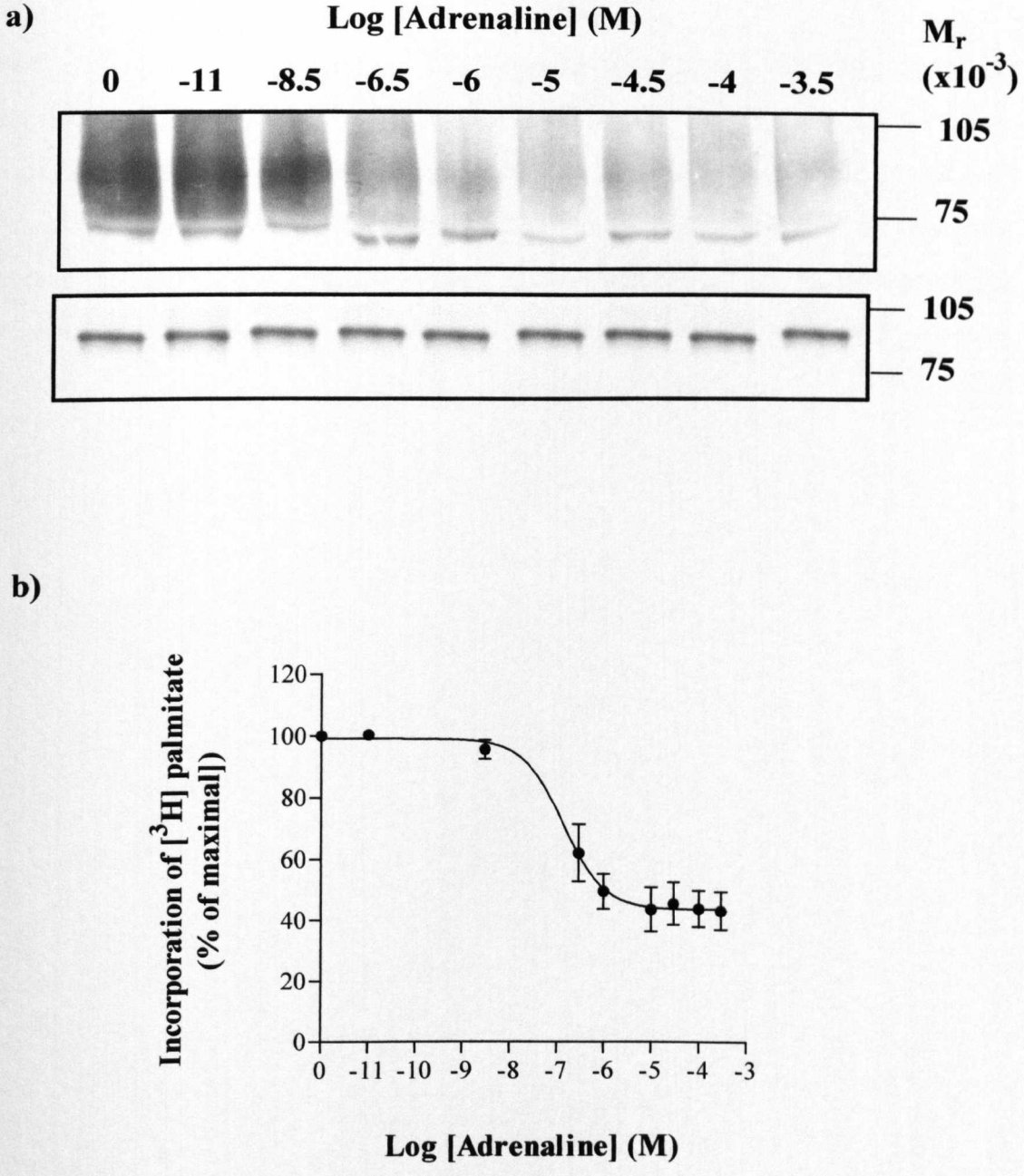

a)
Log [Adrenaline] (M)
0
-11
-8.5
-6.5
-6
-5
-4.5
-4
-3.5
M_r ($x10^{-3}$)
105
75
105
75
b)
Incorporation of [^{3}H] palmitate (% of maximal])
120
100
80
60
40
20
0
0
-11
-10
-9
-8
-7
-6
-5
-4
-3
Log [Adrenaline] (M)

Figure 3.11

Competitive inhibition of antagonist radioligand binding by the agonist adrenaline to membranes expressing the α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}$Ile (WT) fusion protein

HEK293T cells were transfected to express α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}$Ile **(WT)** fusion protein. Membranes were prepared and the ability of adrenaline to compete with [^{3}H]-RS-79948-197 for binding to the α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}$Ile fusion protein was assessed. This allowed calculation of the binding affinity of adrenaline for the α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}$Ile fusion protein. Results are from triplicate determinations. Analysis is representative of three similar experiments. Data is shown as mean +/- S.E.M., n= 3

Figure 3.11

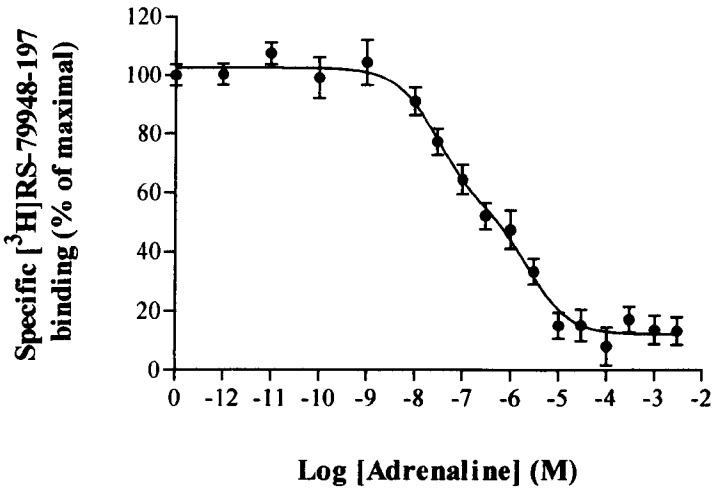

Specific [3H]RS-79948-197 binding (% of maximal)
120
100
80
60
40
20
0
0 -12 -11 -10 -9 -8 -7 -6 -5 -4 -3 -2
Log [Adrenaline] (M)

Figure 3.12

Lack of ability of α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ fusion proteins to bind [^{35}S] GTPγS upon mutation of residue Gly^{204} of the $G_{o1}\alpha$ protein.

HEK293T cells were transfected with empty vector (**pcDNA3**) or to express either α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ (**WT**), $\alpha_{2A}Cys^{442}Ala$-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ (**$Cys^{442}Ala$**), α_{2A}-adrenoceptor-$G_{o1}\alpha Gly^{204}Ala$, $Cys^{351}Ile$ (**$Gly^{204}Ala$**) or $\alpha_{2A}Cys^{442}Ala$ - adrenoceptor-$G_{o1}\alpha Gly^{204}Ala$, $Cys^{351}Ile$ (**$Cys^{442}Ala$, $Gly^{204}Ala$**) fusion proteins. Membranes were prepared from these cells and samples containing 10fmol of [^{3}H]-RS-79948-197 binding sites were used to measure basal (open bars) and 100 μM adrenaline-stimulated (filled bars) binding of [^{35}S] GTPγS to the various fusion proteins. Results are from triplicate determinations. Analysis is representative of three similar experiments. Data is shown as mean +/- S.E.M., n= 3

Figure 3.12

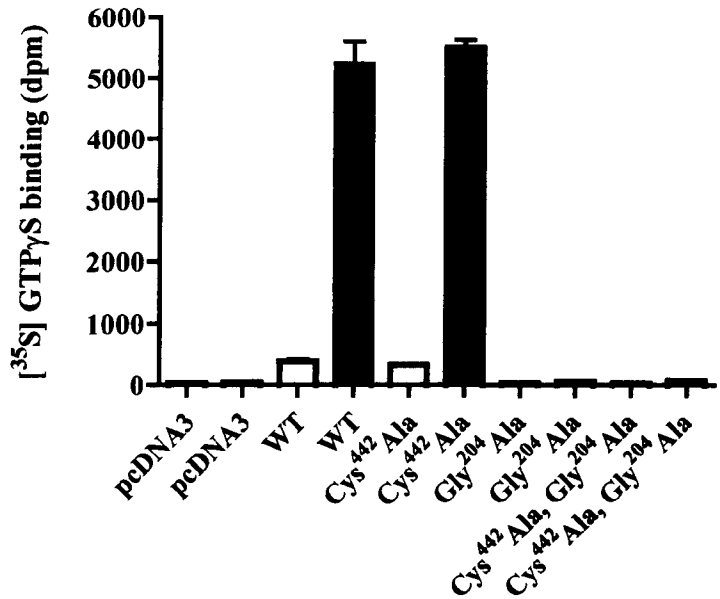

[35S] GTPγS binding (dpm)
6000
5000
4000
3000
2000
1000
0
pcDNA3
pcDNA3
WT
WT
Cys442 Ala
Cys442 Ala
Gly204 Ala
Gly204 Ala
Cys442 Ala, Gly204 Ala
Cys442 Ala, Gly204 Ala

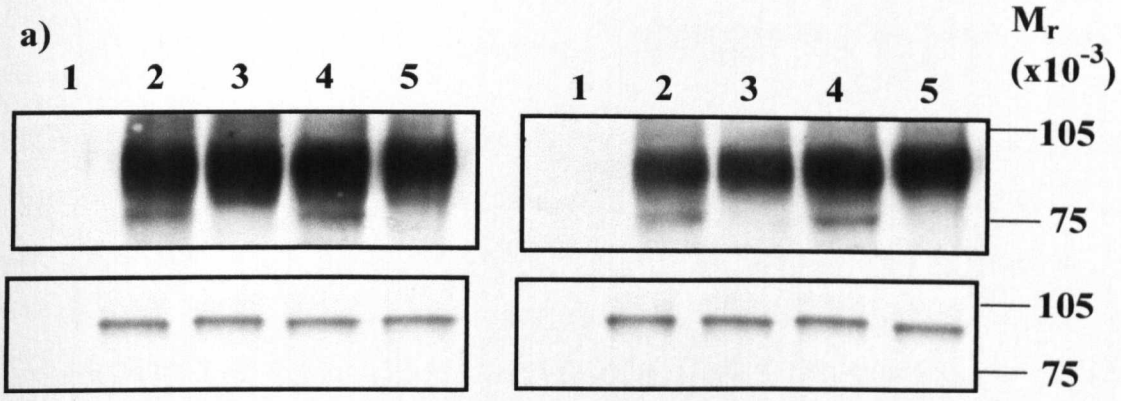
Figure 3.13

Lack of ability of adrenaline to regulate the palmitoylation of α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ fusion proteins upon mutation of residue Gly^{204} of the $G_{o1}\alpha$ protein

HEK293T cells were transfected with empty vector (**pcDNA3, 1**) or to express either α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ (**WT, 2**), $\alpha_{2A}Cys^{442}Ala$-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ (**$Cys^{442}Ala$, 3**), α_{2A}-adrenoceptor-$G_{o1}\alpha Gly^{204}Ala$, $Cys^{351}Ile$ (**$Gly^{204}Ala$, 4**) or $\alpha_{2A}Cys^{442}Ala$-adrenoceptor-$G_{o1}\alpha Gly^{204}Ala$, $Cys^{351}Ile$ (**$Cys^{442}Ala$, $Gly^{204}Ala$, 5**) fusion proteins. Cells were incubated with [^{3}H] palmitate for 30 min in the absence (left panels) or presence (right panels) of 100μM adrenaline. Samples were harvested and cell lysates produced. These were either immunoprecipitated with antiserum ON1 prior to SDS-PAGE and autoradiography for 1 month (upper panels) or resolved directly by SDS-PAGE and immunoblotted with antiserum ON1 (lower panels). **Figure 3.13 (a)** shows one representative palmitoylation experiment with corresponding western blot analysis. Similar results were obtained for three separate experiments.

Autoradiographs as in the upper panel of **a** were scanned and signals quantitated **(b)** in the area of the film shown. Open bars = absence, filled bars = presence of adrenaline. Results for three separate experiments were quantified and data is shown as mean +/- S.E.M., n= 3. In order to compare levels of incorporation from separate experiments it was necessary to express the levels of incorporation for each sample as a percentage of the incorporation observed for the unstimulated **WT** construct.

Figure 3.13


a)
1
2
3
4
5
1
2
3
4
5
M_r
($x10^{-3}$)
105
75
105
75

b)

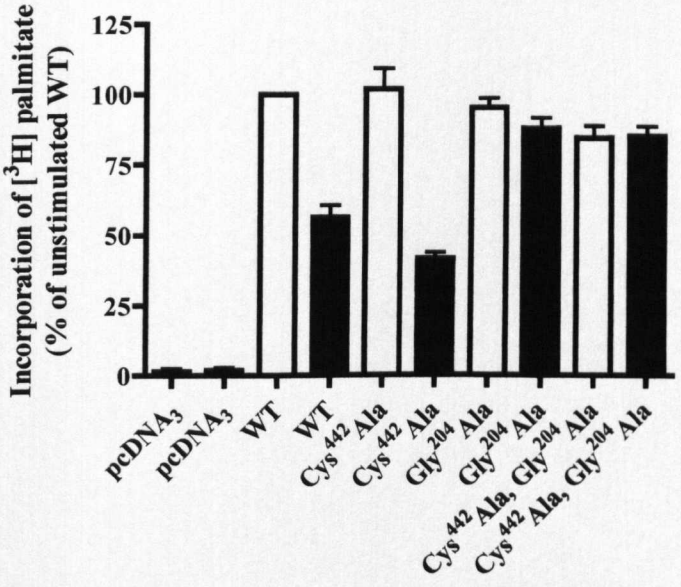

Incorporation of [3H] palmitate
(% of unstimulated WT)
125
100
75
50
25
0
pcDNA3
pcDNA3
WT
WT
Cys442 Ala
Cys442 Ala
Gly204 Ala
Gly204 Ala
Cys442 Ala, Gly204 Ala
Cys442 Ala, Gly204 Ala

Figure 3.14

Incorporation of [^{3}H] palmitate into endogenously expressed $G_{o1}\alpha$ protein in the presence and absence of adrenaline.

An α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ (**WT**) fusion protein was expressed in HEK293T cells. Cells were incubated with [^{3}H] palmitate for the indicated times in the absence (-) or presence (+) of 100μM adrenaline. Samples were harvested and cell lysates produced. These were either immunoprecipitated with antiserum ON1 prior to SDS-PAGE and autoradiography for 1 month (upper panel) or resolved directly by SDS-PAGE and immunoblotted with antiserum ON1 (lower panel). Rather than the fusion protein, labelling and expression of endogenous $G\alpha_{o1}$ is shown. **Figure 3.14** shows one representative palmitoylation experiment with corresponding western blot analysis. Similar results were obtained for three separate experiments.

Figure 3.14

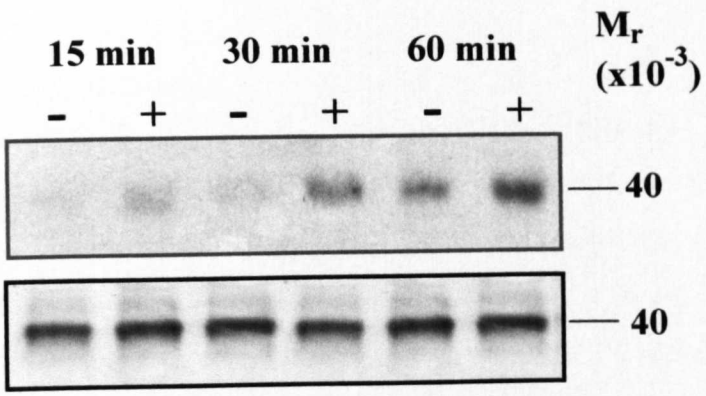


Figure 3.15

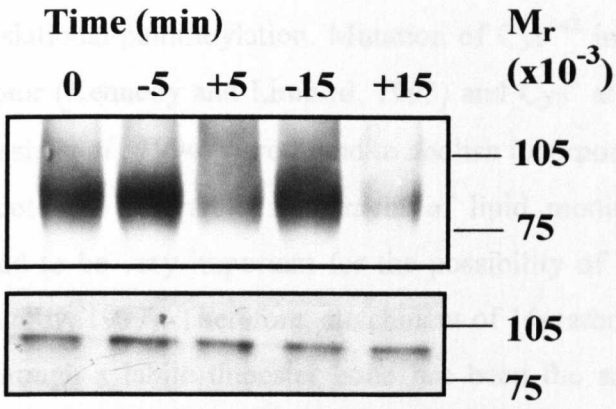
Repalmitoylation of the $\alpha_{2A}Cys^{442}$Ala-adrenoceptor-$G_{o1}\alpha Cys^{351}$Ile ($C^{442}A$) fusion protein

An $\alpha_{2A}Cys^{442}$Ala-adrenoceptor-$G_{o1}\alpha Cys^{351}$Ile fusion protein was expressed in HEK293T cells. Cells were incubated with [^{3}H] palmitate for 30 min in the absence of agonist to allow approximately steady-state levels of [^{3}H] palmitate incorporation to be reached. After the 30 min incubation, cells were then incubated with [^{3}H] palmitate for the indicated times in the absence (-) or presence (+) of 100μM adrenaline. Samples were harvested and cell lysates produced. These were either immunoprecipitated with antiserum ON1 prior to SDS-PAGE and autoradiography for 1 month (upper panel) or resolved directly by SDS-PAGE and immunoblotted with antiserum ON1 (lower panel). **Figure 3.15** (**a**) shows one representative repalmitoylation experiment with corresponding western blot analysis. Similar results were obtained for three separate experiments.

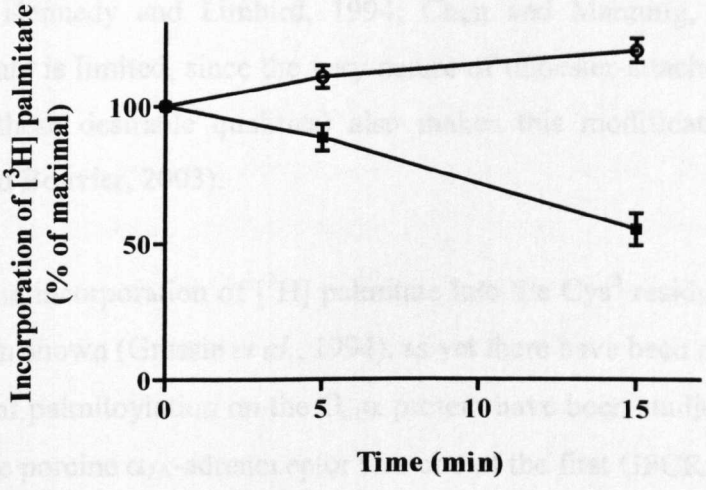
Autoradiographs as in the upper panel of **a** were scanned and signals quantitated (**b**) in the area of the film shown. Open circles = absence, filled squares = presence of adrenaline. Results for three separate experiments were quantified and data is shown as mean +/- S.E.M., n= 3. In order to compare levels of incorporated [^{3}H] palmitate from separate experiments it was necessary to express the levels of [^{3}H] palmitate for each sample as a percentage of the maximal level observed (0 min post-steady-state incubation).

Figure 3.15

a)



b)



3.3 Discussion

The α_{2A}-adrenoceptor and the $G_{o1}\alpha$ protein used in this study have both been shown to undergo post translational-palmitoylation. Mutation of Cys^{442} in the C-terminal tail of the α_{2A}-adrenoceptor (Kennedy and Limbird, 1993) and Cys^3 at the N-terminus of the $G_{o1}\alpha$ protein (Grassie *et al.*, 1994) were found to abolish incorporation of [^{3}H] palmitate into these two proteins. Reversible attachment of lipid modifications to signalling proteins is assumed to be very important for the possibility of dynamic regulation of these proteins (Mumby, 1997). Therefore, attachment of 16-carbon palmitate to GPCRs and G proteins through a labile thioester bond has been the subject of a number of investigations to date (Mouillac *et al.*, 1992; Loisel *et al.*, 1996; Ponimaskin *et al.*, 2001; Wedegaertner and Bourne, 1994; Chen and Manning, 2000; Stanislaus *et al.*, 1997; Bhamre *et al.*, 1998). For some of these investigations, focus is specifically placed on the dynamic regulation of palmitoylation of GPCRs and G proteins (Loisel *et al.*, 1996, Kennedy and Limbird, 1994; Chen and Manning, 2000). However, the available data is limited, since the very nature of thioester-attached palmitate (assumed to impart these desirable qualities) also makes this modification difficult to study (Qanbar and Bouvier, 2003).

Although the incorporation of [^{3}H] palmitate into the Cys^3 residue of $G_{o1}\alpha$ protein has already been shown (Grassie *et al.*, 1994), as yet there have been no studies in which the regulation of palmitoylation on the $G_{o1}\alpha$ protein have been studied in detail. Similarly, although the porcine α_{2A}-adrenoceptor was one of the first GPCRs demonstrated to be a target for post-translational palmitoylation (Kennedy and Limbird, 1993, 1994), it has been some 10 years since the limited studies of regulation of palmitoylation were performed for this receptor. With these facts in mind and given that in recent years there have been a number of improvements in the means to monitor regulated palmitoylation, we considered these two proteins to be suitable subjects for further investigation.

In the current study it was decided to use fusion proteins in which the N-terminus of the G_{o1} protein α subunit is linked in-frame to the C-terminal tail of the α_{2A}-adrenoceptor. A number of other groups have routinely used similar fusion proteins to explore many aspects of receptor and G protein interaction and function (Bertin *et al.*, 1994; Wise and

Milligan, 1997; Seifert *et al.*, 1998; Guo *et al.*, 2001). The GPCR-G protein fusion protein system, as a tool for studying cell-signalling mechanisms, provides the user with a number of advantages. Indeed, fusion proteins have already been shown to provide an efficient system for studying the regulation of palmitoylation on GPCRs and G proteins (Loisel *et al.*, 1999; Stevens *et al.*, 2001). The specific advantages to using a fusion protein approach for the current study were two-fold. Firstly, anti-$G_{o1}\alpha$ antisera was available (Georgoussi *et al.*, 1993) which had the capacity to quantitatively immunoprecipitate $G_{o1}\alpha$ protein (and by extention GPCR-G protein fusion proteins containing this G protein). Secondly, given that the fused G protein is in close physical proximity to the receptor, the fusion allows us to look at activated G protein (Wise and Milligan, 1997; Ugur *et al.*, 2003) in isolation. This is important in order to overcome any potential dilution of agonist effects arising from immunoprecipitation of non-activated G protein (possible in a non-fused system).

Four palmitoylation-variant fusion constructs were used in this study. Firstly, the α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ (**WT**) construct which has the ability to undergo palmitoylation on two sites, one site in the GPCR and one site in the G protein. Secondly, the $\alpha_{2A}Cys^{442}Ala$-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ (**$C^{442}A$**) construct which has the ability to undergo palmitoylation only in the G protein. Thirdly, the α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{3}Ser,Cys^{351}Ile$ (**$C^{3}S$**) construct which has the ability to undergo palmitoylation only in the GPCR. Lastly, the $\alpha_{2A}Cys^{442}Ala$-adrenoceptor-$G_{o1}\alpha Cys^{3}Ser$, $Cys^{351}Ile$ (**$C^{442}A$, $C^{3}S$**) construct which has had both potential palmitoylation sites removed. All constructs contained a $Cys^{351}Ile$ mutation in the C-terminal tail of the $G_{o1}\alpha$ protein. This mutation was desirable for our fusion proteins in order to do functional studies of our constructs (**Chapter 4**) without interference from endogenous G proteins (Jones and Reed, 1987; Lochrie and Simon, 1988; Burt *et al.*, 1998). Mutation of this Cys^{351} residue, renders the G protein resistant to ADP-ribosylation by pertussis toxin. Practically, this allows the endogenous $G_{o1}\alpha$ proteins to be inactivated by the toxin such that any $G_{o1}\alpha$ protein signalling remaining will be through the fused $G_{o1}\alpha Cys^{351}Ile$ protein only. In previous studies on the related G protein, G_{i1}, the effectiveness of activation by the α_{2A}-adrenoceptor was correlated highly with hydrophobicity of the amino acid at this site (Bahia *et al.*, 1998). As a result many

groups now use the Ile containing versions of G_i-family G proteins routinely (Jeong and Ikeda, 2000; Benians *et al.*, 2003).

Before performing a variety of experiments to assess the regulation of palmitoylation in the GPCR and G protein parts of α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ fusion proteins, it was necessary to confirm the Cys^{442} site on the GPCR and the Cys^{3} site on the G protein as the sites for incorporation of [^{3}H] palmitic acid into these fusions. When the four palmitoylation-variant α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ fusion proteins were pulse-labelled with [^{3}H] palmitate for 30 min, the fusion containing both the $Cys^{442}Ala$ and the $Cys^{3}Ser$ mutations did not incorporate [^{3}H] palmitate, confirming these sites as the only ones for dynamic post-translational acylation in these constructs. Similar experiments have been performed routinely for a number of GPCRs and G proteins (Mouillac *et al.*, 1992; Ponimaskin *et al.*, 2001; Chen and Manning, 2000). Both the $Cys^{442}Ala\alpha_2$-adrenoceptor-$G_{o1}\alpha$ and the α_2-adrenoceptor-$Cys^{3}SerG_{o1}\alpha$ fusion proteins did incorporate [^{3}H] palmitate, demonstrating that both the receptor and G protein elements are targets for dynamic acylation. Interestingly and unexpectedly, they were not equivalent. Using equal amounts of the two fusions, the α_2-adrenoceptor-$Cys^{3}SerG_{o1}\alpha$ fusion (in which only the single site in the GPCR can be modified) incorporated significantly less [^{3}H] palmitate within a 30 min period than the $Cys^{442}Ala\alpha_2$-adrenoceptor-$G_{o1}\alpha$ fusion in which only the G protein can be the target. These observations suggested that acylation of the GPCR segment is slower than that of the G protein. In order to address this hypothesis, the kinetics of GPCR and G protein palmitoylation were assessed. This was done by observation of the time courses of incorporation of [^{3}H] palmitic acid into the palmitoylation-variant α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ fusion proteins. Following expression of the α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ (**WT**) fusion protein in HEK293T cells, this construct incorporated [^{3}H] palmitate in a time-dependent manner. This incorporation was substantially lower when the α_{2A}-adrenoceptor agonist adrenaline was present during the labelling period. A similar effect of agonist was observed for palmitoylation of a β_2-adrenoceptor-$G_s\alpha$ fusion protein (Loisel *et al.*, 1999). In contrast, a distinct effect of agonist was observed for an α_{1b}-adrenoceptor-$G_{11}\alpha$ fusion protein. In the latter case, agonist enhanced the kinetics of palmitoylation (Stevens *et al.*, 2001). Given that the incorporation of [^{3}H] palmitic acid into the α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ (**WT**)

fusion protein represented a combination of incorporation into both the GPCR and G protein elements of the fusion, the α_{2A}Cys^{442}Ala-adrenoceptor-$G_{o1}\alpha$Cys^{351}Ile (**C^{442}A**) and α_{2A}-adrenoceptor-$G_{o1}\alpha$Cys^{3}Ser,Cys^{351}Ile (**C^{3}S**) fusions were used to obtain the kinetic data for palmitoylation of the GPCR and G protein. From this data, the half-time of [^{3}H] palmitate incorporation into the receptor was observed to be some 3 times slower than into the G protein. It is important to note here that experiments on the extent and dynamics of acylation must always consider the reversibility of the process (Qanbar and Bouvier, 2003). This point is especially important when pulse-labelling studies are employed. In pulse-labelling assays the levels of incorporation of [^{3}H] palmitate observed are the net result of palmitoylation and depalmitoylation reactions. Thus, variations in the observed changes in [^{3}H] palmitate incorporation may not reflect true differences in the palmitate dynamics. In fact, an agonist-promoted increase in the palmitate turnover rate could potentially give rise to either a decrease or an increase in labelled palmitate incorporation, dependent on the kinetics and experimental conditions. These type of pulse-labelling assays are the normal type of palmitoylation assay carried out for most GPCRs and G proteins (Mouillac *et al.*, 1992; Ponimaskin *et al.*, 2001; Chen and Manning, 2000), however, data from pulse-chase format palmitoylation experiments generally proves far more valuable.

De-palmitoylation studies have been carried out for a number of GPCRs, including the β_2-adrenoceptor (Loisel *et al.*, 1996, 1999) and the α_{2A}-adrenoceptor (Kennedy and Limbird, 1994). In the work of Kennedy and Limbird (1994) it was reported that the half-life of [^{3}H] palmitate on the GPCR was in the region of 10 hours and was similar to the half-life of the protein. In addition, these authors reported only a very slight agonist stimulation of de-palmitoylation rate for the α_{2A}-adrenoceptor. These results suggest the role of palmitoylation for the α_{2A}-adrenoceptor may be distinct from many other protein targets, including other GPCRs, for which the fatty acid is turned over rapidly, allowing proteins to undergo many cycles of acylation and de-acylation during their lifetime (Loisel *et al.*, 1996; Qanbar and Bouvier, 2003; Bijlmakers and Marsh, 2003). In the present de-palmitoylation studies, adrenaline accelerated removal of palmitate from the G protein but not from the receptor. This indicated that the acylation cycle of the G protein is regulated by agonist whereas that of the GPCR is not. As previously mentioned, a very slight agonist effect was observed for the acylation cycle of the non-

fused α_{2A}-adrenoceptor (Kennedy and Limbird, 1993, 1994), results in contrast to the current study with the fusion protein. The current results are also in contrast to studies with an α_{1b}-adrenoceptor-$G_{i1}\alpha$ fusion protein where agonist enhanced labelling of both the GPCR and G protein elements (Stevens *et al.*, 2001). The other major difference in the results of the current study and the previously published work for the α_{2A}-adrenoceptor (Kennedy and Limbird, 1994) is in the half-life of palmitate attached to this receptor. Although the previous study decribed a half-life in the region of 10 hours for palmitate attached to the α_{2A}-adrenoceptor, in our hands (and via the use of a α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{3}Ser,Cys^{351}Ile$ (**$C^{3}S$**) fusion protein) this half-life was found to be in the region of 30 min. This much shorter, more dynamic turnover of palmitate attached to the α_{2A}-adrenoceptor (observed in the current studies) would seem to be much more in keeping with the suggested role of palmitoylation on other signalling proteins, including GPCRs (Loisel *et al.*, 1996) and G proteins (Chen and Manning, 2000).

Activation of G proteins is often associated with alterations in palmitoylation (Chen and Manning, 2001; Wedegaertner, 1998). In order to test this directly we made use of fusion proteins incorporating a form of $G_{o1}\alpha Cys^{351}Ile$ that is unable to exchange GDP for GTP (**$Gly^{204}Ala$** mutant). These **$Gly^{204}Ala$** mutant forms are unable to attain the activated state. Although dynamic in that this form of the G protein did incorporate [^{3}H] palmitate, acylation of this form of the G protein was not regulated by agonist. This allowed us to conclude that activation of the G protein was required for the agonist-stimulated regulation of palmitoylation of the α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ fusion proteins.

Given that dynamic regulation of G protein palmitoylation in response to agonist had already been observed for G_{i1} protein following agonist-stimulation of the 5-HT_{1A} receptor (Chen and Manning, 2000), it was deemed important to assess whether the adrenaline-promoted regulation of G protein palmitoylation in α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ fusion proteins was concentration-dependent. Like for the G_{i1} protein, the agonist-regulation of palmitate on the $G_{o1}\alpha Cys^{351}Ile$ element of the fusion occurred in a concentration-dependent manner. In addition, this occurred at a concentration

correlating with agonist occupancy of the receptor for the α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ fusion protein.

A number of other studies have suggested that activation of a G protein can regulate palmitoylation. Loisel *et al.*, (1999) also demonstrated agonist block of incorporation of [^{3}H] palmitate into a fusion protein, in this case between the β_2-adrenoceptor and $G_s\alpha$ (although they raised the prospect of this reflecting decreased re-palmitoylation following de-palmitoylation). These authors had also noted that in pulse-chase experiments the agonist caused more rapid removal of the [^{3}H] palmitate. This combination of events was taken to reflect an inability of the β_2-adrenoceptor-$G_s\alpha$ fusion protein to be re-palmitoylated in the presence of agonist. Loisel *et al.*, (1999) did, however, observe re-palmitoylation when the studies were performed with the isolated β_2-adrenoceptor and concluded that desensitization or other turn-off processes might be required for re-palmitoylation. Similar results were obtained in the current study, whereby the pattern of agonist-stimulated incorporation of [^{3}H] palmitate into the small amount of $G_{o1}\alpha$ that is expressed endogenously in HEK293 cells appeared to be opposite to that obtained for the G protein element of the fusion protein. When the ability of the $\alpha_{2A}Cys^{442}Ala$-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ (**$C^{442}A$**) fusion protein to be repalmitoylated was assessed, it was found to show the same characteristics as the β_2-adrenoceptor-$G_s\alpha$ fusion protein (Loisel *et al.*, 1999), thus supporting the hypothesis that some limitation of re-palmitoylation of fused proteins may occur.

The differences in results for the regulation of GPCR and G protein palmitoylation in the α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ fusion proteins compared with the non-fused α_{2A}-adrenoceptor and the $G_{o1}\alpha$ protein are, for some aspects, difficult to ascertain. In the case of the $G_{o1}\alpha$ protein, the apparently opposite pattern of [^{3}H] palmitate incorporation into the non-fused protein compared with the $G_{o1}\alpha$ protein constrained within the α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ fusion, is the perfect example of how results obtained from pulse-labelling assays can vary dependent on the experimental conditions. We have been able to show that this difference may be due to the inability of the G protein (as assessed by use of the $\alpha_{2A}Cys^{442}Ala$-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ (**$Cys^{442}Ala$**) construct) to undergo agonist-stimulated repalmitoylation (a phenomena previously observed for a

β_2-adrenoceptor-$G_s\alpha$ fusion protein (Loisel *et al.*, 1999)). However, the fact that the G protein part of the fusion protein is exhibiting the opposite pattern of [^{3}H] palmitate incorporation to the non-fused G protein in the pulse-labelling study may not necessarily indicate that the fusion system is unsuitable for palmitoylation studies. As already discussed, the results from pulse-labelling studies do not give us definitive descriptions of changes in palmitoylation, since it represents both the palmitoylation and depalmitoylation reactions. As a result, the only real conclusion which can be drawn from the pulse-labelling of the endogenous $G_{o1}\alpha$, is that the [^{3}H] palmitate incorporation in the protein can be regulated by agonist. It is the very same conclusion that can be drawn from studies of the [^{3}H] palmitate incorporation into the G protein part of the fusion protein. The most useful information concerning palmitoylation studies comes from experiments in pulse-chase format, because these assays represent only the rate of de-palmitoylation of the target protein. We assume, by inference from previous studies (Loisel *et al.*, 1999), that the nature of the target protein (fused or non-fused) will not affect the rate of depalmitoylation observed, although this has not been directly tested herein. For the same reasons, the difference in depalmitoylation rate of the fused α_{2A}-adrenoceptor and the non-fused protein are difficult to comprehend. In order to address this difference we attempted to perform palmitoylation studies for the non-fused α_{2A}-adrenoceptor. In these assays we made use of a HA-tag, present on the N-terminus of the protein, for immunoprecipitation. Unfortunately, the extent of immunoprecipitation achieved using anti-HA antibodies was relatively poor, resulting in no detectable signal for incorporation of [^{3}H] palmitate with exposure times of up to one month. As a result the differences could not be addressed in the present study.

Concerning the study of endogenous $G_{o1}\alpha$ protein palmitoylation, there is one very important point to note in relation to the very reasons we decided to use a fusion-based approach for the current study. The very fact that there will not be a 1:1 stoichiometry of GPCR to G protein and that not all G protein will become activated by GPCR, means that no real kinetic analyses can be performed for these experiments. The results observed will always reflect some "interference" from non-activated G protein. This fact is illustrated in the pulse-labelling study of [^{3}H] palmitate incorporation into the endogenous $G_{o1}\alpha$ protein (**Figure 3.14**), in which the incorporation levels do not display saturation over the time-course. In conclusion, like with any experimental

system, there are advantages and disadvantages. In terms of studying palmitoylation, I believe the advantages of the fusion system outweigh the disadvantages. So long as the results are taken in context and some effort is made to address how the results might relate to the situation in non-fused proteins, then the data is very useful. With this in mind it is important to reflect that fusion-proteins are already an accepted model-system for the study of many features of GPCR-G protein signalling. In support of this, the intrinsic GTPase activity of the G protein functions to deactivate this construct are as expected (Wise and Milligan, 1997) and Regulators of G protein Signalling are effective GTPase activating proteins for this construct (Cavalli *et al.*, 2000). Furthermore, the G protein βγ-complex interacts effectively and can be co-immunoprecipitated with α_{2A}-adrenoceptor-$G_{o1}\alpha$ fusion proteins (Bertaso *et al.*, 2003). Therefore, basic features and regulation of interactions between the α_{2A}-adrenoceptor, the G protein α-subunit and its interacting proteins are preserved in the fusion proteins.

In summary, the current data provides rather different conclusions on the kinetics of acylation and its regulation by agonist than previous work on the α_{2A}-adrenoceptor. It demonstrates that agonist occupancy and activation of an α_{2A}-adrenoceptor-$G_{o1}\alpha$ fusion protein regulates the palmitoylation status of the G protein but not the receptor. This effect is produced, at least partially, via agonist-induced enhancement of G protein de-palmitoylation. Given the differences in results obtained with related experiments for both the β_2- and α_{1b}-adrenoceptors, simple and universal rules on the regulation of thio-acylation may be difficult to define, even for closely related receptors. This implies that in future studies regulation of GPCR palmitoylation will probably have to be analysed on a case-by-case basis.

In order to interpret the results of regulation of GPCR and G protein palmitoylation from the current study in terms of functional consequences, the palmitoylation-variant fusions were subsequently used in a variety of functional assays (detailed in **Chapter 4**).

Chapter 4

Functional consequences of palmitoylation in α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ fusion proteins

4.1 Introduction

Reversible attachment of palmitate to some GPCRs and G proteins is one example of a dynamic modification thought to modulate cell signalling. Agonist-dependent regulation of palmitoylation status has been shown to occur for both GPCRs (Ponimaskin *et al.*, 2001; Ng *et al.*, 1994; Mouillac *et al.*, 1992) and G proteins (Wedegartner and Bourne, 1994; Chen and Manning, 2000). The observation of agonist-regulation of palmitoylation is suggestive of some important functional role for this modification. Consequently a number of studies of GPCR and G protein palmitoylation have been performed (Ponimaskin *et al.*, 2001; Chen and Manning, 2000; Papoucheva *et al.*, 2004) and a wide variety of functional consequences have been reported (Qanbar and Bouvier, 2003; Hawtin *et al.*, 2001: Ponimaskin *et al.*, 2002; Miggin *et al.*, 2003).

For G protein α-subunits two main roles of palmitoylation have been suggested. The first is the regulation of the efficiency of interactions between RGS proteins and the Gα protein. Gα subunit palmitoylation has been reported to cause decreased efficiency of the RGS protein to stimulate GAP activity (Tu *et al.*, 1997). This has been shown for a number of Gα and RGS protein pairs and the extent of inhibition has been observed to correlate with how well the two proteins interact (Ross and Wilkie, 2000). The second role of palmitoylation is thought to be in targeting and anchoring the soluble Gα polypeptides to the membrane (Wedegaertner, 1998; Dunphy and Linder, 1998) and specialised sub-domains of the membrane such as lipid rafts (Mumby, 1997; Song *et al.*, 1997). Mutation of palmitoylation sites of some G protein α-subunits has also been reported to decrease the efficiency of signalling. This has been shown for $G_s\alpha$ (Wedegaertner *et al.*, 1993) and $G_q\alpha$ (Edgerton *et al.*, 1994). However, the apparent alterations in efficiency of signalling may be attributable to absence of membrane localisation. This was shown to be the case for the G_s protein α-subunit (Ugur *et al.*, 2003), whereby normal signalling efficiency was recovered upon fusion of the Gα subunit to the β_2-adrenoceptor.

It is assumed that the reason for attachment of palmitate to many rhodopsin-like GPCRs must be for something other than membrane association, given that they are integral

membrane proteins spanning the membrane seven times. It has therefore been suggested that palmitoylation may play an important role in the functioning of GPCRs. For most but not all (Chen *et al.*, 1998, Hawtin *et al.*, 2001) rhodopsin-like GPCRs, attachment of palmitate occurs at one or more cysteine residues within the C-terminal tail. For rhodopsin it was shown that this palmitate was able to insert into the bilayer and thereby create a 'fourth intracellular loop' (Ganter *et al.*, 1992; Moench *et al.*, 1994). From the high-resolution three-dimensional structural information obtained by X-ray crystallography of inactive bovine rhodopsin (Palczewski *et al.*, 2000), the existence of an eighth helix was reported. This helix is thought to have arisen by palmitate insertion into the bilayer and several studies have suggested a role for this region in G protein activation (Altenbach *et al.*, 2001; Krishna *et al.*, 2002; Okuno *et al.*, 2003). The conservation of sequence and structure across the family of rhodopsin-like GPCRs suggests this will likely be the case for other family members. The downstream consequences of GPCR palmitoylation have been studied for a significant number of GPCRs and a number of very different responses have been observed. In some cases mutations of the sites of palmitoylation have been shown to alter downstream signalling or the regulation of receptors (O'Dowd *et al.*, 1989, Kennedy and Limbird, 1993, 1994, Loisel *et al.*, 1996, Chen *et al.*, 1998, Hawtin *et al.*, 2001, Ponimaskin *et al.*, 2002, Miggin *et al.*, 2003). In contrast, there are a number of other cases where mutations of the sites of palmitoylation have been shown not to have any effect on the downstream signalling (Eason *et al.*, 1994) or the regulation of the receptor (Jin *et al.*, 1997).

In this study the functional consequences of palmitoylation of the α_{2A}-adrenoceptor and the $G_{o1}\alpha$ protein are studied for the same palmitoylation-variant α_{2A}-adrenoceptor-$G_{o1}\alpha$ fusion proteins used in **Chapter 3**. At present the data is limited concerning the functional consequences of $G_{o1}\alpha$ protein (Grassie *et al.*, 1994) and α_{2A}-adrenoceptor (Kennedy and Limbird, 1993) palmitoylation. The ability of the α_{2A}-adrenoceptor to be palmitoylated was shown to have no apparent affect on functional properties such as coupling to G_i/G_o proteins or receptor phosphorylation (Kennedy and Limbird, 1993; 1994). However in one study the removal of the palmitoylation site (by removal of a portion of the C-terminal tail) of the α_{2A}-adrenoceptor resulted in altered desensitisation properties for this receptor (Eason *et al.*, 1994). Given this limited breadth of knowledge for the α_{2A}-adrenoceptor and the $G_{o1}\alpha$ protein, it was decided to use the

palmitoylation-variant α_{2A}-adrenoceptor-$G_{o1}\alpha$ fusion proteins in a range of functional assays such as radioligand binding assays, GTPγS binding assays, GTPase assays and receptor internalisation assays in order to assess the importance of protein palmitoylation for this selection of functional properties.

4.2 Results

Expression of α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ fusion proteins and determination of their affinity for the agonist adrenaline and the α_2-adrenoceptor antagonist [^{3}H]-RS-79948-197

The palmitoylation variant α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ fusion proteins already created and studied in terms of regulation of palmitoylation (**Chapter 3**) were transiently transfected into HEK293T cells. After transfection, cells were harvested and cell membranes were prepared for analysis. α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ fusion protein expression level was initially investigated by Western blot analysis. Immuno detection of the $G_{o1}\alpha$ protein N terminus (via ON1 antiserum) or the C terminus (via OC2 antiserum) confirmed expression of the fusion proteins post transfection (**Figure 4.1**). Immunoblotting of transiently transfected membranes with both the ON1 and OC2 antisera detected immuno-reactive bands of molecular mass ~75-100kDa (predicted molecular weight 89kDa) which were not present in mock transfected membranes. In addition, the immunodetection levels of the α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{3}Ser$, $Cys^{351}Ile$ (**C^3S**) and α_{2A}-adrenoceptor $Cys^{442}Ala$-$G_{o1}\alpha Cys^{3}Ser$, $Cys^{351}Ile$ (**$C^{442}A$, C^3S**) fusion proteins were similar to the levels of the other constructs when immunoblotting was carried out with either the ON1 or the OC2 antisera. This confirmed that the presence of the Cys^3Ser mutation on the $G_{o1}\alpha$ protein did not affect the ability of the ON1 antiserum to recognise this region of the $G_{o1}\alpha$ protein.

The expression of the α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ fusion proteins was further assessed by the binding of a near saturating concentration (~5nM) of the tritiated antagonist [^{3}H]-RS-79948-197 (Milligan *et al.*, 1997). From these experiments the transient membrane expression of each construct was estimated to be in the region of 6.5 pmol/mg (**Figure 4.2, Table 4.1**).

In order to obtain a more accurate measurement of each α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ fusion protein expression level, saturation binding assays were performed using various concentrations of tritiated antagonist [^{3}H]-RS-79948-197 (ranging from

0.05 to 5nM). From these experiments the expression level (B_{max}) and the affinity for the antagonist (K_d) for each construct was obtained (**Figure 4.3, Table 4.2**). Again, the expression levels of all α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ fusion protein constructs were in the region of 6.5 pmol/mg. The affinity for [^{3}H]-RS-79948-197 for all four palmitoylation variant fusion proteins was approximately 0.32nM. Neither the values for B_{max} nor K_d were significantly different ($p>0.05$) for each of the α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ fusion protein constructs.

Next the affinity for the natural ligand adrenaline was calculated by assessing its ability to compete with [^{3}H]-RS-79948-197 for binding to the α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ fusion protein constructs (**Figure 4.4, Table 4.3**). From these experiments is was apparent that the presence of adrenaline caused a competitive biphasic inhibition of [^{3}H]-RS-79948-197 binding to the α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ fusion protein constructs with IC_{50} values in the region of 20nM and 2.2μM (corresponding to high and low affinity binding sites) for all four palmitoylation variant constructs. From these IC_{50} values the K_i for adrenaline was calculated (see **Table 4.3**) for each construct and were not found to be statistically different ($p>0.05$).

Assessment of the ability of the α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ fusion proteins to activate signalling

The ability of the α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ fusion proteins to activate signalling was assessed in terms of two properties, the ability to bind GTP (assessed via a GTPγS binding assay) and the ability to hydrolyse GTP (assessed via a high affinity GTPase assay).

In order to compare the GTPγS binding of all palmitoylation variant α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ fusion proteins a number of control experiments had to be performed in order to establish appropriate experimental conditions. These control experiments were not performed on all four palmitoylation variant constructs but instead were carried out for the α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ **(WT)** and the α_{2A}-adrenoceptor $Cys^{442}Ala$-$G_{o1}\alpha Cys^{3}Ser$, $Cys^{351}Ile$ **($C^{442}A$, $C^{3}S$)** fusion proteins only. The effects of increasing the

amount of fusion protein used in the assay and increasing incubation times were assessed in **Figure 4.5** and **Figure 4.6** respectively. From **Figure 4.5** increasing [^{35}S] GTPγS binding was observed with increasing quantities of fusion protein. [^{35}S] GTPγS binding was saturated with quantities of fusion protein of 50 fmol and above. For this reason it was decided to use 10fmol fusion protein/tube in subsequent assays.

From **Figure 4.6** increasing [^{35}S] GTPγS binding was observed with increasing lengths of incubation. Saturation of [^{35}S] GTPγS binding was observed after ~5 min. For this reason it was decided to use a 2.5 min incubation length in subsequent assays. Therefore in all subsequent assays an incubation time of 2.5 min was used to assess GTPγS binding to 10 fmol/tube of each α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}$Ile fusion protein.

Next a concentration-response experiment was performed in order to establish an appropriate concentration of adrenaline to be used in subsequent assays (**Figure 4.7, Table 4.4**). **Figure 4.7** showed a concentration dependent increase in [^{35}S] GTPγS binding for both fusion proteins. The concentration-response curves and resultant EC_{50} determination for GTPγS binding to the two α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}$Ile fusion proteins was found not to be statistically different ($p>0.05$) with the EC_{50} value for both in the region of 10nM.

Once the appropriate experimental conditions had been established, GTPγS binding for all constructs were compared within one assay (**Figure 4.8, Table 4.5**). In the presence of the maximally effective concentration of adrenaline (10^{-4}M) GTPγS binding was significantly stimulated over basal levels ($p<0.05$) for all α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}$Ile fusion constructs. However upon comparison of the levels of adrenaline stimulated GTPγS binding for each α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}$Ile fusion construct they were found not to be statistically different ($p>0.05$).

In order to assess the ability of the four α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}$Ile fusion proteins to hydrolyse GTP, it was necessary to perform a number of control experiments to establish appropriate experimental conditions for subsequent assays. The effects of different incubation lengths and amounts of fusion protein used in the assay were assessed in **Figure 4.9** (for **WT**) and **Figure 4.10** (for **$C^{442}A$, $C^{3}S$**). From these

experiments it was concluded that no significant difference in either basal or 100μM adrenaline-stimulated GTPase activity was found for the incubation periods of 10, 20, 30 or 40 min or at four different protein amounts (0.5μg, 1.5μg, 3.0μg and 6.0μg) for either fusion protein. It was therefore decided that an incubation time of 40 min (as used in Hoffman *et al.*, 2001) would be used to assess high affinity GTPase activity of 1.5μg/tube of membranes expressing each α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ fusion protein.

Next a concentration-response experiment was performed in order to establish an appropriate concentration of adrenaline to be used in subsequent assays (**Figure 4.11, Table 4.6**). **Figure 4.11** showed a concentration dependent increase in high affinity GTPase activity for both fusion proteins. The concentration-response curves and resultant EC_{50} determination for high affinity GTPase activity for the two α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ fusion proteins (**WT** and **$C^{442}A$, $C^{3}S$**) was found not to be statistically different ($p>0.05$) with the EC_{50} value for each in region of 65nM.

Once the appropriate experimental conditions had been established, high affinity GTPase activities for all constructs were compared within one assay (**Figure 4.12, Table 4.7**). In the presence of the maximally effective concentration of adrenaline (10^{-4}M), high affinity GTPase activity was significantly stimulated ($p<0.05$) ~3-4-fold compared with basal levels for all α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ fusion constructs. However upon comparison of the levels of adrenaline stimulated high affinity GTPase activity for each α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ fusion construct they were found not to be statistically different ($p>0.05$).

High affinity GTPase activities for all constructs were also compared in the presence of GST purified (**Figure 4.13**) RGS16 protein (both in the presence and absence of the maximally effective concentration of adrenaline (10^{-4}M). In this experiment, high affinity GTPase activity was significantly stimulated ~3-fold compared with basal levels ($p<0.05$) for all α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ fusion constructs in the presence of adrenaline and was significantly stimulated ~11-fold compared with basal levels ($p<0.05$) for all α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ fusion constructs in the presence of adrenaline plus RGS16 protein (**Figure 4.14, Table 4.8**). Interestingly and

unexpectedly, there was also a very small but nevertheless significant ($p=0.042$) stimulation of RGS16-stimulated high GTPase activity of ~1.3-fold compared with basal levels. This is suggestive of a modest effect of RGS16 on rates of basal GTPase activity. Once again, upon comparison of the levels of adrenaline stimulated high affinity GTPase activity ($p>0.05$), adrenaline plus RGS16 stimulated high affinity GTPase activity ($p>0.05$) or RGS16-stimulated high GTPase activity ($p>0.05$) for each individual α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ fusion construct they were found not to be statistically different.

In order to obtain a more accurate comparison of the high affinity GTPase activity of all four α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ fusion constructs it was necessary to perform high affinity GTPase assays in the presence of various concentrations of GTP (substrate) (**Figures 4.15a-4.18a**). This allowed conversion of the generated data to Eadie-Hofstee plots for ease of visualisation of the effects on V_{max} and on K_m for GTP hydrolysis (**Figures 4.15b-4.18b, Tables 4.9** and **4.10**).

In these experiments the abilities of the four α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ fusion constructs to hydrolyse GTP in the basal state, in the presence of adrenaline, as well as in the presence of adrenaline plus RGS16 protein were assessed. The basal high affinity GTPase activity for each was found to be in the region of 22 pmol/mg/min, whereas the adrenaline-stimulated high affinity GTPase activity for each was found to be in the region of 44 pmol/mg/min, a ~2-fold stimulation compared with basal ($p<0.05$). The high affinity GTPase activity in the presence of RGS16 and adrenaline for each was found to be in the region of 340 pmol/mg/min, a ~15-fold stimulation compared with basal ($p<0.05$).

Given the slight differences in expression levels for each construct the data was recalculated to give turnover numbers for GTPase activity (**Table 4.11**). The turnover numbers have been calculated for basal (~3.6 min^{-1}), adrenaline-stimulated (~6.7 min^{-1}) and RGS16 (~60 min^{-1}) in the presence of adrenaline-stimulated GTPase activity. Once again, the results from the GTPase experiments show that there was no difference in the basal ($p>0.05$), adrenaline-stimulated ($p>0.05$) or RGS16 in the presence of adrenaline-

stimulated ($p>0.05$) turnover of GTP when each condition is compared for the four palmitoylation-variant fusion proteins.

Analysis of the ability of the α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ fusion proteins to internalise in response to adrenaline

After following the procedure of cell labelling, immunoprecipitation, SDS PAGE, western transfer and immunoblotting as detailed in section **2.7.7** all four palmitoylation-variant α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ fusion proteins were found to be biotin labelled (**Figure 4.19**). Immunoblotting detected immuno-reactive bands of molecular mass ~100kDa (predicted molecular weight for unglycosylated fusion protein is 89kDa). In addition, bands of molecular mass ~200-250kDa and relatively faint bands of ~40-50kDa were detected. The higher molecular weight bands may correspond to dimers of the fusion protein and the lower molecular weight bands may correspond to the α_{2A}-adrenoceptor (as a breakdown product of the fusion protein). Next, a time-course format receptor internalisation assay was performed for the α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ (**WT**) and α_{2A}-adrenoceptor $Cys^{442}Ala$-$G_{o1}\alpha Cys^{3}Ser$, $Cys^{351}Ile$ (**$C^{442}A$, $C^{3}S$**) fusion proteins (**Figure 4.20**). Cells were treated with vehicle or 100μM adrenaline for times between 5-90 min prior to the rest of the protocol as detailed in section **2.7.7**. Immunoblotting once again detected immuno-reactive bands of molecular mass ~100kDa (corresponding to the fusion protein) and ~200-250kDa (thought to be fusion protein dimers). This time, the relatively faint bands of ~40-50kDa were not detected, supporting the hypothesis that these bands may have arisen by fusion protein breakdown. Upon stimulation by 100μM adrenaline neither the α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ (**WT**) nor the α_{2A}-adrenoceptor $Cys^{442}Ala$-$G_{o1}\alpha Cys^{3}Ser$, $Cys^{351}Ile$ (**$C^{442}A$, $C^{3}S$**) fusion proteins were found to internalise after 90 min stimulation. As a control, it was decided to assess the extent of internalisation of the α_{2A}-adrenoceptor for comparison with the α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ fusion protein. In **Figure 4.21**, cells expressing the α_{2A}-adrenoceptor alone (**a**) or the α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ (**WT**) fusion protein (**b**) were treated with vehicle or 100μM adrenaline for either 15 or 45 min prior to the rest of the protocol as detailed in section **2.7.7**. Immunoblotting of α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ (**WT**) revealed the same pattern as already observed in **Figure 4.20** (**a**). When immunoblotting of α_{2A}-adrenoceptor was performed, immuno-

reactive bands of molecular mass ~50kDa corresponding to the α_{2A}-adrenoceptor were observed (the predicted molecular weight for the unglycosylated receptor is 40kDa). In addition, bands of molecular mass between 105 and 250kDa were detected. These high molecular weight bands may correspond to multimers of the α_{2A}-adrenoceptor. In support of this hypothesis, agonist-stimulated internalisation is observed for both the 50kDa α_{2A}-adrenoceptor band and for the high molecular weight band. Taken together these results demonstrate that the α_{2A}-adrenoceptor can undergo adrenaline-stimulated internalisation but the α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ fusion proteins cannot.

Figure 4.1

Western blot analysis of membranes transiently transfected with the α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ fusion proteins

3μg of membrane preparations from HEK293T cells transiently transfected with the empty vector (**pcDNA3**, **1**), α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ (**WT**, **2**), $\alpha_{2A}Cys^{442}Ala$-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ (**$C^{442}A$**, **3**), α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{3}Ser$, $Cys^{351}Ile$ (**$C^{3}S$**, **4**) or $\alpha_{2A}Cys^{442}Ala$-adrenoceptor-$G_{o1}\alpha Cys^{3}Ser$, $Cys^{351}Ile$ (**$C^{442}A$**, **$C^{3}S$**, **5**) fusion proteins were resolved on SDS-PAGE gels then transferred onto nitrocellulose membranes. Immunoblotting was carried out with a) ON1 and b) OC2 antisera. Two further experiments produced similar results.

Figure 4.1

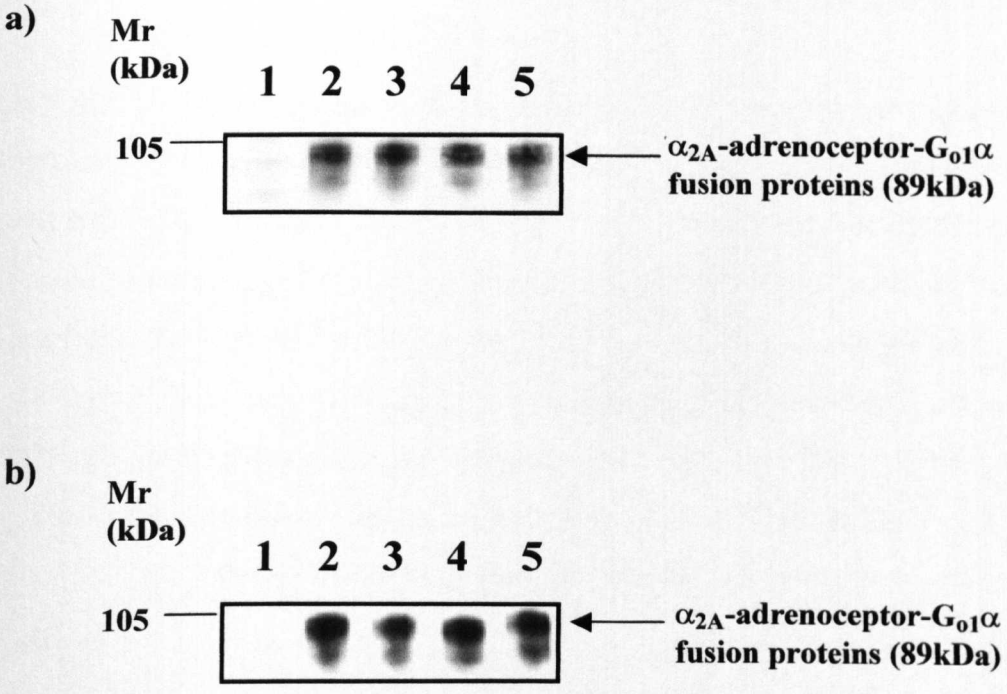

a)
Mr
(kDa)
1 2 3 4 5
105
α_{2A}-adrenoceptor-$G_{o1}\alpha$
fusion proteins (89kDa)
b)
Mr
(kDa)
1 2 3 4 5
105
α_{2A}-adrenoceptor-$G_{o1}\alpha$
fusion proteins (89kDa)

Figure 4.2

Analysis of expression levels of α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}$Ile fusion proteins: determined from binding of a 5nM concentration of [^{3}H]-RS-79948-197 to membranes expressing the fusion proteins

HEK293T cells were transfected to express the α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}$Ile fusion proteins. Membranes expressing the fusion proteins were prepared and binding of 5nM of [^{3}H]-RS-79948-197 to 0.5μg of each membrane sample was assessed. In this figure the bar corresponding to α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}$Ile (**WT**) is shown in blue, the bar corresponding to α_{2A}-adrenoceptor Cys^{442}Ala-$G_{o1}\alpha Cys^{351}$Ile (**$C^{442}A$**) is shown in green, the bar corresponding to α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{3}$Ser, Cys^{351}Ile (**$C^{3}S$**) is shown in purple and the bar corresponding to α_{2A}-adrenoceptor Cys^{442}Ala-$G_{o1}\alpha Cys^{3}$Ser, Cys^{351}Ile (**$C^{442}A$, $C^{3}S$**) is shown in brown. Results are from triplicate determinations. Analysis is representative of three similar experiments.

Table 4.1

Comparison of expression levels of α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}$Ile fusion proteins determined from binding of a 5nM concentration of [^{3}H]-RS-79948-197 to membranes expressing the fusion proteins

The results from **Figure 4.2** were presented in a tabular form for clarity of comparison between the constructs.

Figure 4.2

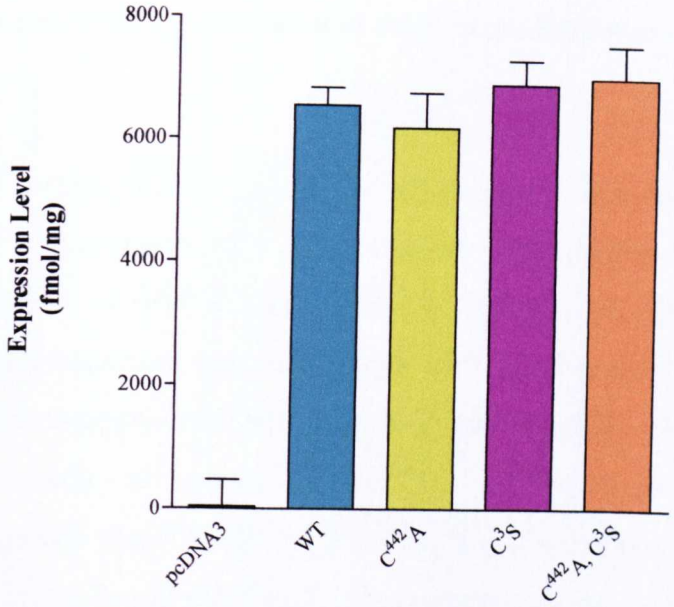


Table 4.1

Construct	Expression Level (fmol/mg)
α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ (**WT**)	6515 +/- 325
$\alpha_{2A}Cys^{442}Ala$-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ (**$C^{442}A$**)	6203 +/- 641
α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{3}Ser,Cys^{351}Ile$ (**$C^{3}S$**)	6875 +/- 411
$\alpha_{2A}Cys^{442}Ala$-adrenoceptor-$G_{o1}\alpha Cys^{3}Ser$, $Cys^{351}Ile$ (**$C^{442}A$, $C^{3}S$**)	7015 +/- 495

Figure 4.3

Analysis of expression levels of α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}$Ile fusion proteins by [^{3}H]-RS-79948-197 saturation binding analysis to membranes expressing the fusion proteins

HEK293T cells were transfected to express the α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}$Ile fusion proteins. Membranes expressing the fusion proteins were prepared and saturation binding of [^{3}H]-RS-79948-197 (using 0.05–5nM radioligand) to 0.5μg of each membrane sample was assessed. In this figure the line corresponding to α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}$Ile (**WT**) is shown in blue, the line corresponding to α_{2A}-adrenoceptor Cys^{442}Ala-$G_{o1}\alpha Cys^{351}$Ile (**$C^{442}A$**) is shown in green, the line corresponding to α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{3}$Ser, Cys^{351}Ile (**$C^{3}S$**) is shown in purple and the line corresponding to α_{2A}-adrenoceptor Cys^{442}Ala-$G_{o1}\alpha Cys^{3}$Ser, Cys^{351}Ile (**$C^{442}A$, $C^{3}S$**) is shown in brown. Results are from triplicate determinations. Analysis is representative of three similar experiments.

Table 4.2

Comparison of α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}$Ile fusion protein expression levels and K_d for [^{3}H]-RS-79948-197 binding to α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}$Ile fusion proteins: determined by saturation binding analysis to membranes expressing the fusion proteins

The results from **Figure 4.3** were presented in a tabular form for clarity of comparison between the constructs. Non-linear regression analysis was used to determine the maximal expression level (pmol/mg) and the equilibrium dissociation constant, K_d (nM) for radioligand binding to each α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}$Ile fusion protein.

Figure 4.3

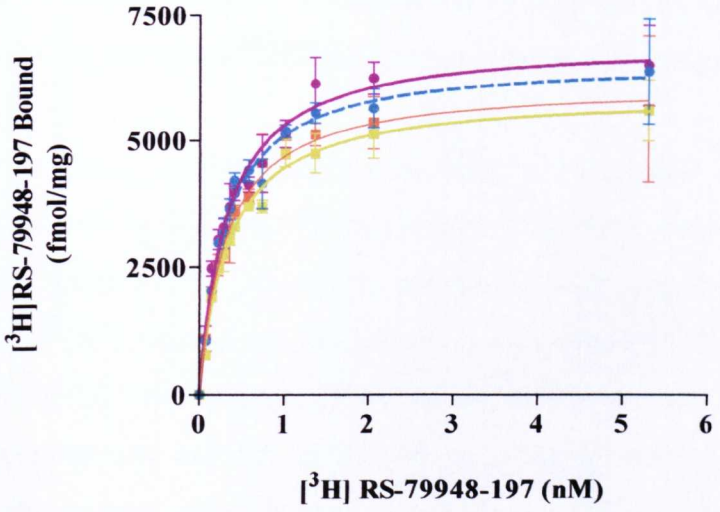


Table 4.2

Construct	Expression Level (fmol/mg)	K_d (nM) for [^{3}H] RS-79948-197
α_{2A}-adrenoceptor-$G_{o1}\alpha$Cys^{351}Ile (**WT**)	6715 +/- 199	0.30 +/- 0.03
α_{2A}Cys^{442}Ala-adrenoceptor-$G_{o1}\alpha$Cys^{351}Ile (**C^{442}A**)	5998 +/- 214	0.34 +/- 0.03
α_{2A}-adrenoceptor-$G_{o1}\alpha$Cys^{3}Ser,Cys^{351}Ile (**C^{3}S**)	6950 +/- 220	0.32 +/- 0.03
α_{2A}Cys^{442}Ala-adrenoceptor-$G_{o1}\alpha$Cys^{3}Ser, Cys^{351}Ile (**C^{442}A, C^{3}S**)	6175 +/- 131	0.32 +/- 0.02

Figure 4.4

Competitive inhibition by the agonist adrenaline of [^{3}H]-RS-79948-197 binding to membranes expressing the α_{2A}-adrenoceptor-$G_{o1}\alpha$Cys^{351}Ile fusion proteins

HEK293T cells were transfected to express α_{2A}-adrenoceptor-$G_{o1}\alpha$Cys^{351}Ile fusion proteins. Membranes were prepared and the ability of adrenaline to compete with [^{3}H]-RS-79948-197 for binding to the α_{2A}-adrenoceptor-$G_{o1}\alpha$Cys^{351}Ile fusion proteins was assessed. All results are presented as a percentage of the specific [^{3}H]-RS-79948-197 binding (in the absence of adrenaline: set as 100%). Results were fitted to a two-site curve using Graphpad Prism program. In this figure the line corresponding to α_{2A}-adrenoceptor-$G_{o1}\alpha$Cys^{351}Ile (**WT**) is shown in blue, the line corresponding to α_{2A}-adrenoceptor Cys^{442}Ala-$G_{o1}\alpha$Cys^{351}Ile (**C^{442}A**) is shown in green, the line corresponding to α_{2A}-adrenoceptor-$G_{o1}\alpha$Cys^{3}Ser, Cys^{351}Ile (**C^{3}S**) is shown in purple and the line corresponding to α_{2A}-adrenoceptor Cys^{442}Ala-$G_{o1}\alpha$Cys^{3}Ser, Cys^{351}Ile (**C^{442}A, C^{3}S**) is shown in brown. Results are from triplicate determinations. Analysis is representative of three similar experiments.

Table 4.3

Comparison of competitive inhibition by the agonist adrenaline of [^{3}H]-RS-79948-197 binding to membranes expressing the α_{2A}-adrenoceptor-$G_{o1}\alpha$Cys^{351}Ile fusion proteins

Non-linear regression analysis on the results from **Figure 4.4** determined the IC_{50} values (mean +/- SEM) for adrenaline for each α_{2A}-adrenoceptor-$G_{o1}\alpha$Cys^{351}Ile fusion protein. Subsequently, from these IC_{50} values the K_i for adrenaline was calculated. The results are presented in a tabular form for clarity of comparison between the constructs. Note that the two IC_{50} and K_i values correspond to the low and high affinity binding sites.

Figure 4.4

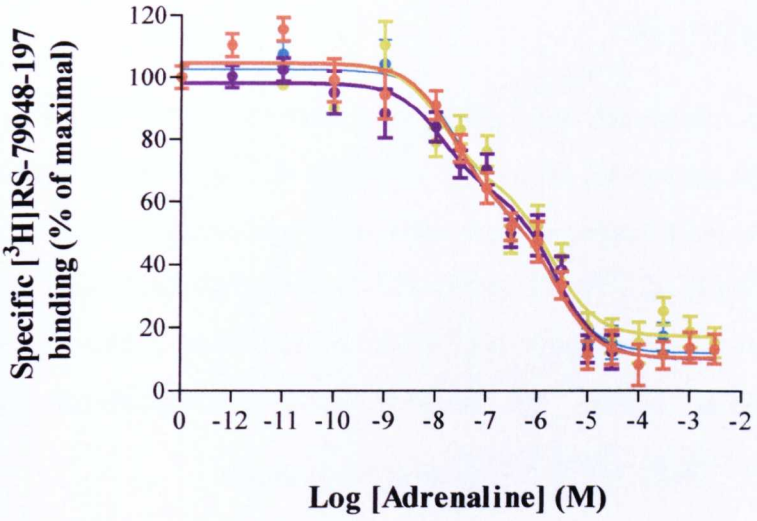


Table 4.3

Construct	IC_{50} 1 (nM) for adrenaline	IC_{50} 2 (μM) for adrenaline	K_i 1 (nM) for adrenaline	K_i 2 (μM) for adrenaline
(WT)	28.7 +/- 13.1	2.4 +/- 1.1	3.6 +/- 1.6	0.29 +/- 0.14
($C^{442}A$)	14.3 +/- 24.3	2.0 +/- 2.1	1.8 +/- 3.0	0.25 +/- 0.26
($C^{3}S$)	13.4 +/- 11.3	2.3 +/- 1.1	1.7 +/- 1.4	0.28 +/- 0.14
($C^{442}A$, $C^{3}S$)	21.0 +/- 15.4	2.1 +/- 1.5	2.6 +/- 1.9	0.26 +/- 0.19

Figure 4.5

Analysing levels of [^{35}S] GTPγS binding to various quantities of α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}$Ile fusion proteins

HEK293T cells were transfected with α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}$Ile (**WT,** blue line) or $\alpha_{2A}Cys^{442}$Ala-adrenoceptor-$G_{o1}\alpha Cys^{3}$Ser, Cys^{351}Ile (**$C^{442}A$, $C^{3}S$,** brown line) fusion proteins. Membranes were prepared from these cells and samples containing various quantities (10-100 fmol) of [^{3}H]-RS-79948-197 binding sites were used to measure 100μM adrenaline-stimulated binding of [^{35}S] GTPγS to the two fusion proteins during a 2.5 min incubation. Results are from triplicate determinations. Analysis is representative of three similar experiments.

Figure 4.5

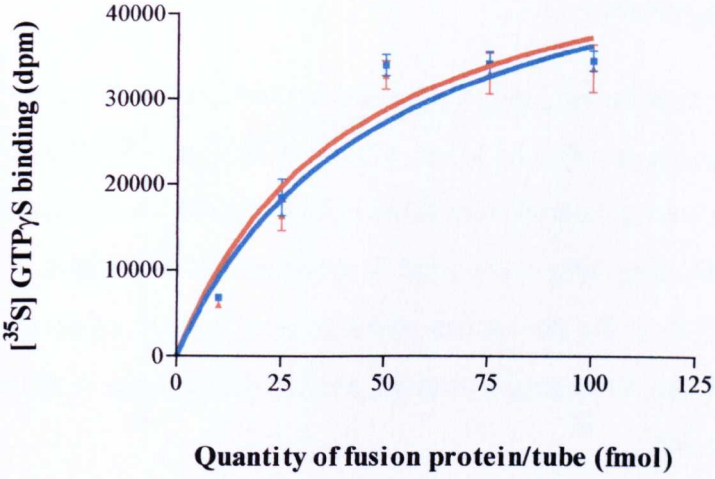

40000
30000
20000
10000
0
[35S] GTPγS binding (dpm)
0
25
50
75
100
125
Quantity of fusion protein/tube (fmol)

Figure 4.6

Analysing the time course of [^{35}S] GTPγS binding to α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}$Ile fusion proteins

HEK293T cells were transfected with α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}$Ile (**WT**, blue line) or $\alpha_{2A}Cys^{442}$Ala-adrenoceptor-$G_{o1}\alpha Cys^{3}$Ser, Cys^{351}Ile (**$C^{442}A$, $C^{3}S$**, brown line) fusion proteins. Membranes were prepared from these cells and samples containing 10 fmol of [^{3}H]-RS-79948-197 binding sites were used to measure 100μM adrenaline-stimulated binding of [^{35}S] GTPγS to the two fusion proteins over a range of incubation lengths (0.5-20 min). Results are from triplicate determinations. Analysis is representative of three similar experiments.

Figure 4.6

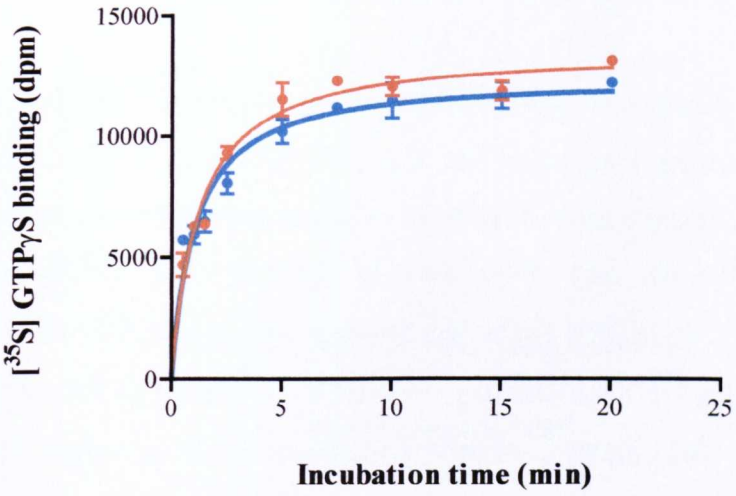

15000
10000
5000
0
[35S] GTPγS binding (dpm)
0
5
10
15
20
25
Incubation time (min)

Figure 4.7

Analysing levels of [^{35}S] GTPγS binding to α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}$Ile fusion proteins in response to various concentrations of adrenaline

HEK293T cells were transfected with α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}$Ile (**WT**, blue line) or $\alpha_{2A}Cys^{442}$Ala-adrenoceptor-$G_{o1}\alpha Cys^{3}$Ser, Cys^{351}Ile (**$C^{442}A$, $C^{3}S$**, brown line) fusion proteins. Membranes were prepared from these cells and samples containing 10 fmol of [^{3}H]-RS-79948-197 binding sites were used to measure (10^{-11}-10^{-3}M) adrenaline-stimulated binding of [^{35}S] GTPγS to the two fusion proteins. [^{35}S] GTPγS binding was expressed as a percentage of the maximal [^{35}S] GTPγS binding levels for each construct (~ 3000-4000 dpm). This allowed accurate comparison of EC_{50} values for the two constructs. Results are from triplicate determinations. Analysis is representative of three similar experiments.

Table 4.4

Comparison of levels of [^{35}S] GTPγS binding to α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}$Ile fusion proteins in response to various concentrations of adrenaline

Non-linear regression analysis on the results from **Figure 4.7** allowed calculation of the EC_{50} values (mean +/- SEM) for adrenaline-stimulated [^{35}S] GTPγS binding to each α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}$Ile fusion protein. These results are presented in a tabular form for clarity of comparison between the constructs.

Figure 4.7

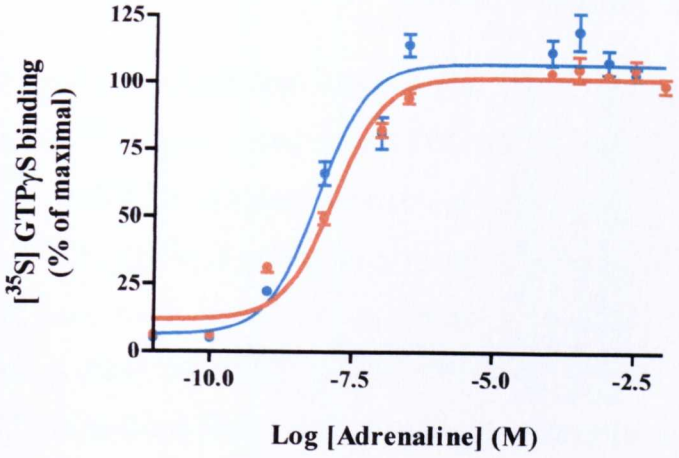


Table 4.4

Construct	EC_{50} (nM) for adrenaline stimulation of GTPγS binding
α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}$Ile (**WT**)	7.7 +/- 4.1
$\alpha_{2A}Cys^{442}$Ala-adrenoceptor-$G_{o1}\alpha Cys^{3}$Ser, Cys^{351}Ile (**$C^{442}A$, $C^{3}S$**)	15.1 +/- 6.2

Figure 4.8

Analysis of the [^{35}S] GTPγS binding of all four palmitoylation-variant α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ fusion proteins

HEK293T cells were transfected with empty vector (**pcDNA3**, black bars) or to express either α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ (**WT**, blue bars), $\alpha_{2A}Cys^{442}Ala$-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ (**$C^{442}A$**, green bars), α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{3}Ser$, $Cys^{351}Ile$ (**$C^{3}S$**, purple bars) or $\alpha_{2A}Cys^{442}Ala$-adrenoceptor-$G_{o1}\alpha Cys^{3}Ser$, $Cys^{351}Ile$ (**$C^{442}A$, $C^{3}S$**, brown bars) fusion proteins. Membranes were prepared from these cells and samples containing 10fmol of [^{3}H]-RS-79948-197 binding sites were used to measure basal (open bars) and 100 μM adrenaline-stimulated (filled bars) binding of [^{35}S] GTPγS to the various fusion proteins during a 2.5 min incubation. Results are from triplicate determinations. Analysis is representative of three similar experiments.

Table 4.5

Comparison of basal and adrenaline-stimulated [^{35}S] GTPγS binding of all four palmitoylation-variant α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ fusion proteins

The results from **Figure 4.8** were presented in a tabular form for clarity of comparison between the constructs. Results are presented as a mean +/- SEM for basal and adrenaline-stimulated [^{35}S] GTPγS binding to each α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ fusion protein.

Figure 4.8

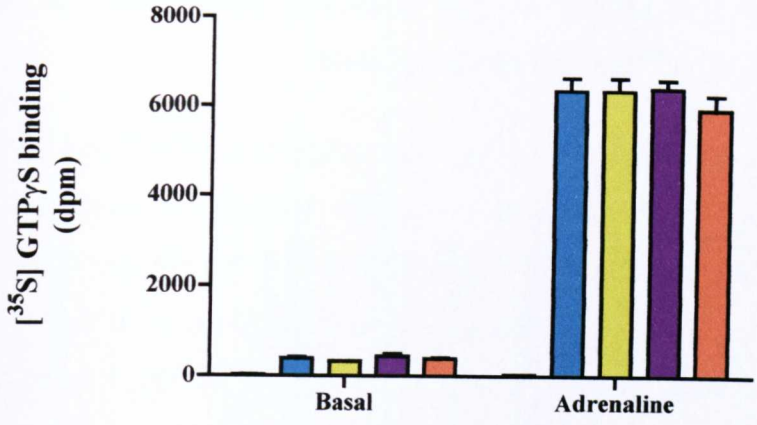


Table 4.5

Construct	**Basal [^{35}S] GTPγS binding (dpm)**	**Adrenaline-stimulated [^{35}S] GTPγS binding (dpm)**
pcDNA3	32 +/- 2	41 +/- 5
α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ **(WT)**	390 +/- 31	6378 +/- 290
$\alpha_{2A}Cys^{442}Ala$-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ **($C^{442}A$)**	333 +/- 51	6412 +/- 300
α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{3}Ser,Cys^{351}Ile$ **($C^{3}S$)**	445 +/- 52	6446 +/- 188
$\alpha_{2A}Cys^{442}Ala$-adrenoceptor-$G_{o1}\alpha Cys^{3}Ser$, $Cys^{351}Ile$ **($C^{442}A$, $C^{3}S$)**	392 +/- 23	5971 +/- 313

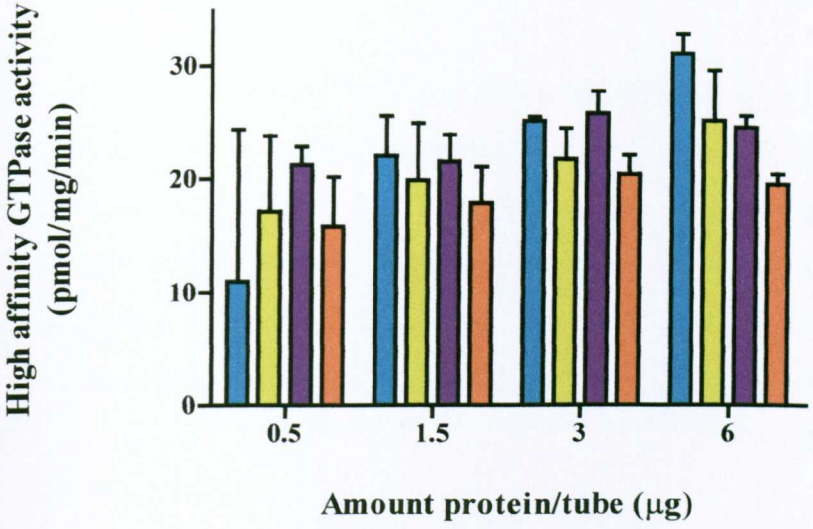
Figure 4.9

Testing various membrane amounts and incubation lengths for basal and adrenaline-stimulated high affinity GTPase activity in membranes expressing the α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ (WT) fusion protein.

HEK293T cells were transfected with α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ (**WT**) fusion protein. Membranes were prepared from these cells and samples containing various quantities (0.5μg, 1.5μg, 3μg, 6μg) of membranes for 10 min (blue), 20min (green), 30min (purple) or 40min (brown) were used to measure basal (a) or 100μM adrenaline-stimulated (b) high affinity GTPase activity to the fusion protein. Results are from triplicate determinations. Analysis is representative of three similar experiments.

Figure 4.9

a)


High affinity GTPase activity
(pmol/mg/min)
30
20
10
0
0.5
1.5
3
6
Amount protein/tube (μg)

b)

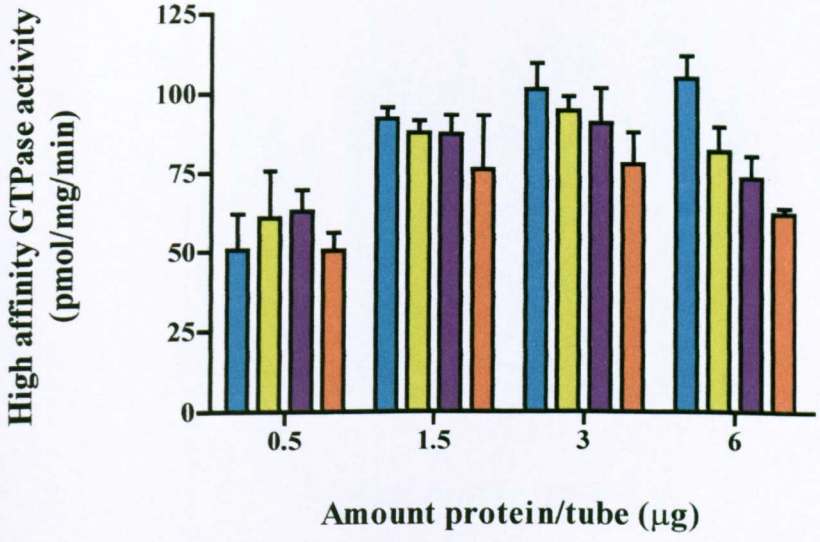

High affinity GTPase activity
(pmol/mg/min)
125
100
75
50
25
0
0.5
1.5
3
6
Amount protein/tube (μg)

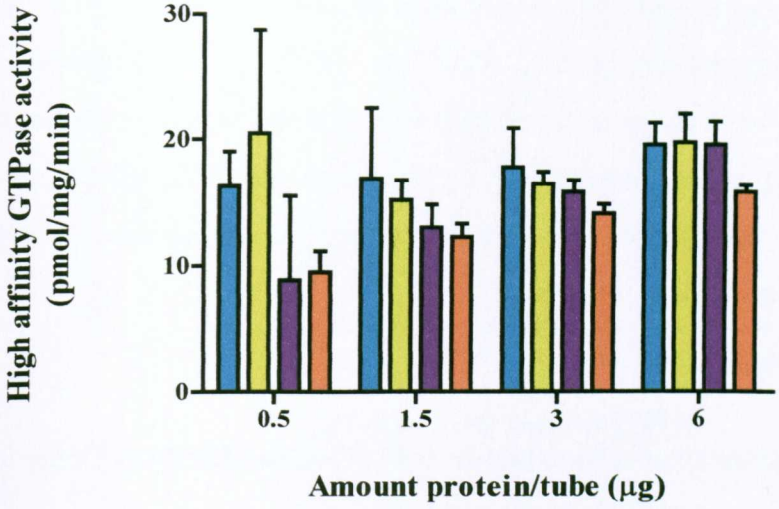
Figure 4.10

Testing various membrane amounts and incubation lengths for basal and adrenaline-stimulated high affinity GTPase activity in membranes expressing the α_{2A}Cys^{442}Ala-adrenoceptor-$G_{o1}\alpha$Cys^{3}Ser, Cys^{351}Ile (C^{442}A, C^{3}S) fusion protein.

HEK293T cells were transfected with α_{2A}Cys^{442}Ala-adrenoceptor-$G_{o1}\alpha$Cys^{3}Ser, Cys^{351}Ile (**C^{442}A, C^{3}S**) fusion protein. Membranes were prepared from these cells and samples containing various quantities (0.5μg, 1.5μg, 3μg, 6μg) of membranes for 10 min (blue), 20min (green), 30min (purple) or 40min (brown) were used to measure basal (a) or 100μM adrenaline-stimulated (b) high affinity GTPase activity to the fusion protein. Results are from triplicate determinations. Analysis is representative of three similar experiments.

Figure 4.10

a)



b)

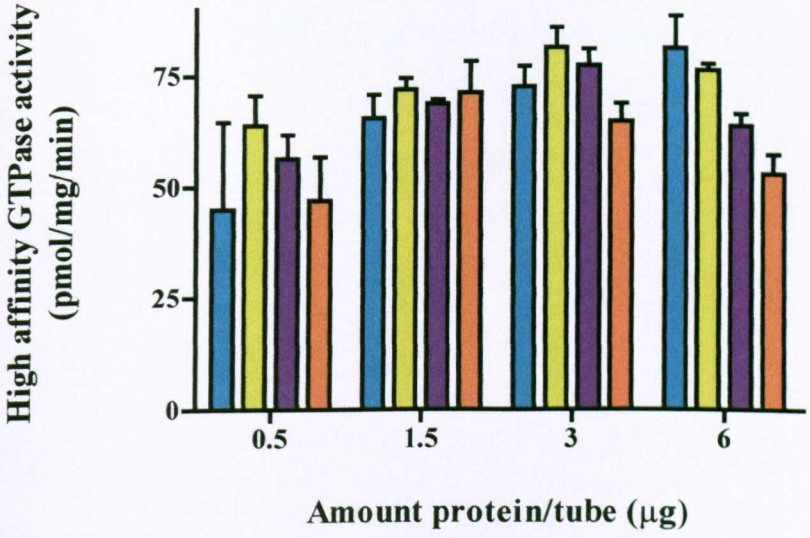


Figure 4.11

Adrenaline concentration-response curves for activation of high affinity GTPase activity in α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}$Ile fusion proteins.

HEK293T cells were transfected with α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}$Ile (**WT**, blue) or $\alpha_{2A}Cys^{442}$Ala-adrenoceptor-$G_{o1}\alpha Cys^{3}$Ser, Cys^{351}Ile (**$C^{442}A$, $C^{3}S$**, brown) fusion proteins. Membranes were prepared from these cells and the potency of adrenaline to stimulate high affinity GTPase activity in the α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}$Ile fusion proteins was examined. Results are from triplicate determinations. Analysis is representative of three similar experiments.

Table 4.6

Comparison of the potency of adrenaline to activate high affinity GTPase activity in α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}$Ile fusion proteins

Non-linear regression analysis on the results from **Figure 4.11** allowed calculation of the EC_{50} values (mean +/- SEM) for adrenaline-stimulated high affinity GTPase activity for each α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}$Ile fusion protein. These results are presented in a tabular form for clarity of comparison between the constructs.

Figure 4.11

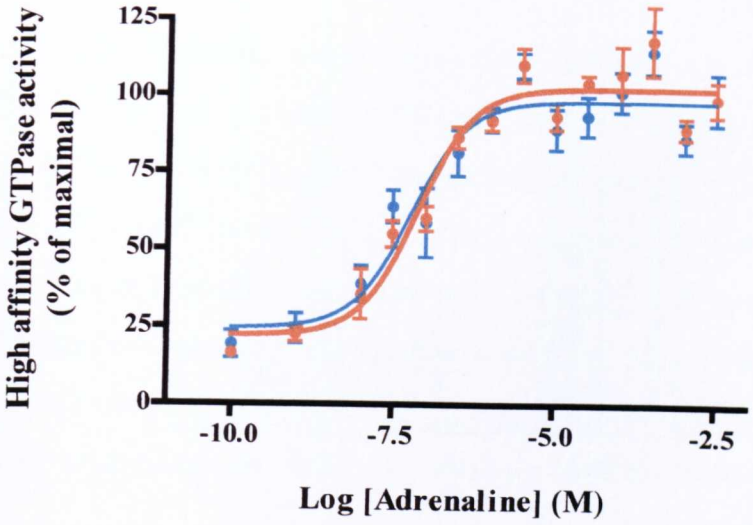


Table 4.6

Construct	EC_{50} (nM) for adrenaline stimulation of high affinity GTPase activity
α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ **(WT)**	58.4 +/- 38.6
$\alpha_{2A}Cys^{442}Ala$-adrenoceptor-$G_{o1}\alpha Cys^{3}Ser$, $Cys^{351}Ile$ **($C^{442}A$, $C^{3}S$)**	74.5 +/- 37.5

Figure 4.12

Analysis of the basal and adrenaline-stimulated high affinity GTPase activity of all four palmitoylation-variant α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ fusion proteins

HEK293T cells were transfected with empty vector (**pcDNA3**, black) or to express either α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ (**WT**, blue), $\alpha_{2A}Cys^{442}Ala$-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ (**$C^{442}A$**, green), α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{3}Ser$, $Cys^{351}Ile$ (**$C^{3}S$**, purple) or $\alpha_{2A}Cys^{442}Ala$-adrenoceptor-$G_{o1}\alpha Cys^{3}Ser$, $Cys^{351}Ile$ (**$C^{442}A$, $C^{3}S$**, brown) fusion proteins. Membranes were prepared from these cells and samples containing 1.5μg of membranes were used to measure basal and 100 μM adrenaline-stimulated high affinity GTPase activity of all four palmitoylation-variant α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ fusion proteins. Results are from triplicate determinations. Analysis is representative of three similar experiments.

Table 4.7

Comparison of basal and adrenaline-stimulated high affinity GTPase activity of all four palmitoylation-variant α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ fusion proteins

The results from **Figure 4.12** were presented in a tabular form for clarity of comparison between the constructs. Results are presented as a mean +/- SEM for adrenaline-stimulated high affinity GTPase activity of all four palmitoylation-variant α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ fusion proteins.

Figure 4.12

a)

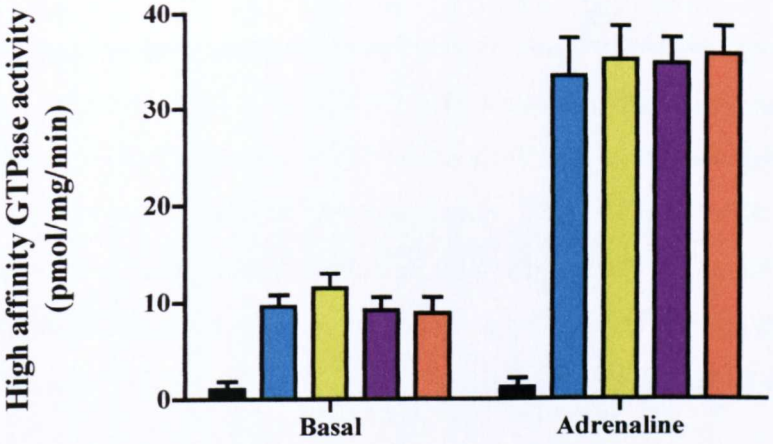


Table 4.7

Construct	**Basal high affinity GTPase activity (pmol/mg/min)**	**Adrenaline-stimulated high affinity GTPase activity (pmol/mg/min)**
pcDNA3	1.0 +/- 0.9	1.2 +/- 1.0
α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ (**WT**)	9.6 +/- 1.2	33.5 +/- 4.0
$\alpha_{2A}Cys^{442}Ala$-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ (**$C^{442}A$**)	11.52 +/- 1.5	35.3 +/- 3.5
α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{3}Ser,Cys^{351}Ile$ (**$C^{3}S$**)	9.2 +/- 1.4	34.9 +/- 2.7
$\alpha_{2A}Cys^{442}Ala$-adrenoceptor-$G_{o1}\alpha Cys^{3}Ser, Cys^{351}Ile$ (**$C^{442}A$, $C^{3}S$**)	8.9 +/- 1.7	35.9 +/- 2.9

Figure 4.13

Coommassie Blue staining for purified RGS16-GST following protein purification using Glutathione Sepharose 4B gel

Protein was stained for with Coommassie Blue following electrophoresis of purified protein and crude bacterial extracts using NuPAGE 4-12% Bis-Tris pre-cast gels. Protein of an apparent Mr of approximately 50kDa was detected, consistent with the calculated mass of the RGS16-GST fusion protein. Rainbow markers are marked M, lane 1 contains bacterial sample prior to IPTG induction, lane 2 contains bacterial sample at 4 hours post IPTG induction, lanes 3-9 contain samples of purified RGS16-GST protein. The protein at approximately 24-26kDa corresponds to degraded RGS16-GST.

Figure 4.13

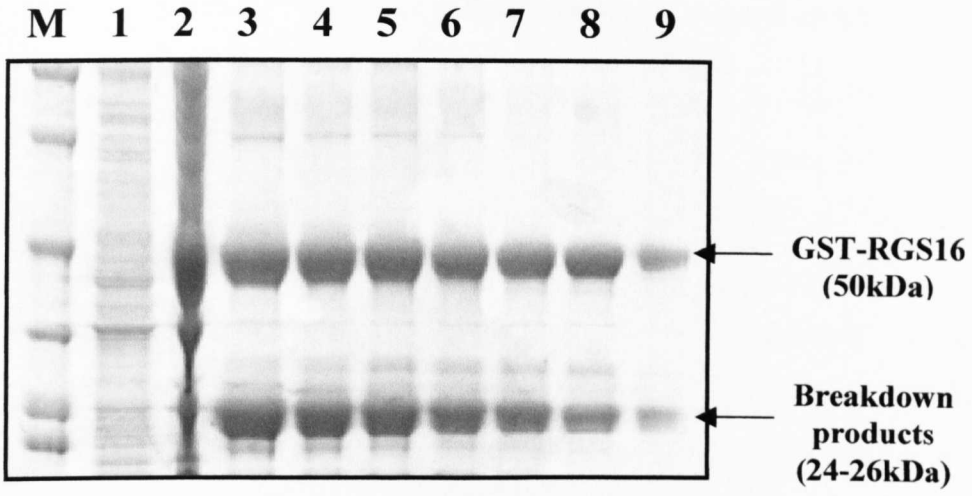


Figure 4.14

Analysis of the basal, adrenaline-stimulated, RGS16-stimulated and RGS16 in the presence of adrenaline-stimulated high affinity GTPase activity of all four palmitoylation-variant α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ fusion proteins

HEK293T cells were transfected with empty vector (**pcDNA3**, black) or to express either α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ (**WT**, blue), $\alpha_{2A}Cys^{442}Ala$-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ (**$C^{442}A$**, green), α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{3}Ser$, $Cys^{351}Ile$ (**$C^{3}S$**, purple) or $\alpha_{2A}Cys^{442}Ala$-adrenoceptor-$G_{o1}\alpha Cys^{3}Ser$, $Cys^{351}Ile$ (**$C^{442}A$, $C^{3}S$**, brown) fusion proteins. Membranes were prepared from these cells and samples containing 1.5μg of membranes were used to measure basal, 100μM adrenaline-stimulated, 1μM RGS16-stimulated as well as 1μM RGS16 in the presence of 100μM adrenaline-stimulated high affinity GTPase activity. This was done for all four palmitoylation-variant α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ fusion proteins. Results are from triplicate determinations. Analysis is representative of three similar experiments.

Table 4.8

Comparison of basal, adrenaline-stimulated, RGS16-stimulated and RGS16 in the presence of adrenaline-stimulated high affinity GTPase activity of all four palmitoylation-variant α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ fusion proteins

The results from **Figure 4.14** were presented in a tabular form for clarity of comparison between the constructs. Results are presented as a mean +/- SEM for basal, adrenaline-stimulated, RGS16-stimulated and RGS16 in the presence of adrenaline-stimulated high affinity GTPase activity of all four palmitoylation-variant α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ fusion proteins.

Figure 4.14

a)

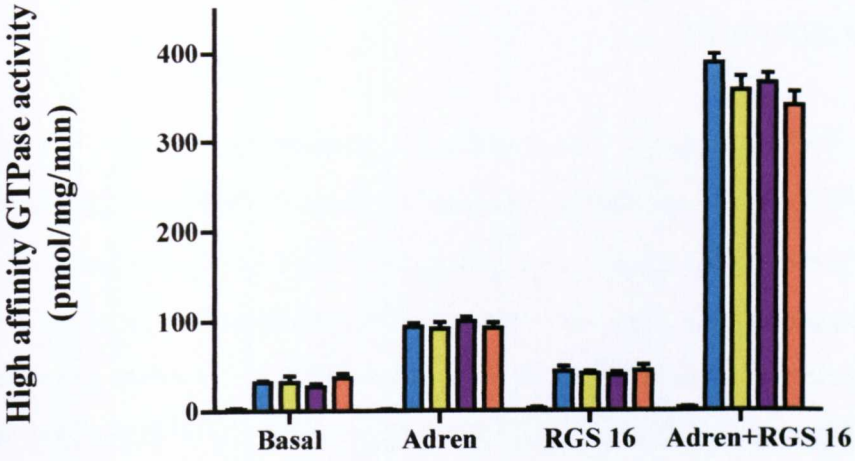


Table 4.8

Construct	**Basal high affinity GTPase activity (pmol/mg/min)**	**Adrenaline-stimulated high affinity GTPase activity (pmol/mg/min)**	**RGS16 high affinity GTPase activity (pmol/mg/min)**	**RGS16+ Adrenaline-stimulated high affinity GTPase activity (pmol/mg/min)**
pcDNA3	2.1 +/- 0.9	1.1 +/- 0.6	2.6 +/- 1.0	3.1 +/- 0.8
α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ **(WT)**	32.1 +/- 1.7	93.7 +/- 4.0	44.1 +/- 4.0	389.8 +/- 10.3
$\alpha_{2A}Cys^{442}Ala$-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ **($C^{442}A$)**	32.2 +/- 6.9	91.8 +/- 7.1	41.3 +/- 2.6	359.6 +/- 15.1
α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{3}Ser$, $Cys^{351}Ile$ **($C^{3}S$)**	27.1 +/- 3.4	99.8 +/- 4.6	40.0 +/- 3.2	368.2 +/- 9.9
$\alpha_{2A}Cys^{442}Ala$-adrenoceptor-$G_{o1}\alpha Cys^{3}Ser$, $Cys^{351}Ile$ **($C^{442}A$, $C^{3}S$)**	36.8 +/- 5.2	92.5 +/- 5.8	44.3 +/- 7.0	341.7 +/- 16.1

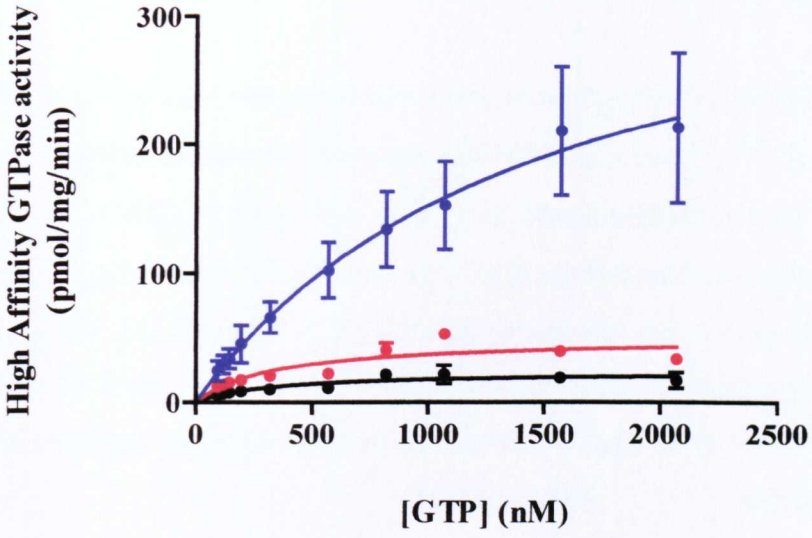
Figure 4.15

Basal and adrenaline-stimulated high affinity GTPase activity of membranes expressing the α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ (WT) fusion protein in the presence and absence of RGS16-GST

High affinity GTPase activity of membranes expressing the α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ (WT) fusion protein was assessed for three conditions: basal (black line), 100μM adrenaline-stimulated (red line) and 1μM RGS16 in the presence of 100μM adrenaline-stimulated (blue line). The rate of GTP hydrolysis, V (pmol/mg/min) was assessed for increasing concentrations of GTP as substrate (a). The data generated were converted to Eadie-Hofstee plots to analyse effects on K_m for GTP hydrolysis and V_{max} (b). Results are from triplicate determinations. Analysis is representative of three similar experiments.

Figure 4.15

a)



b)

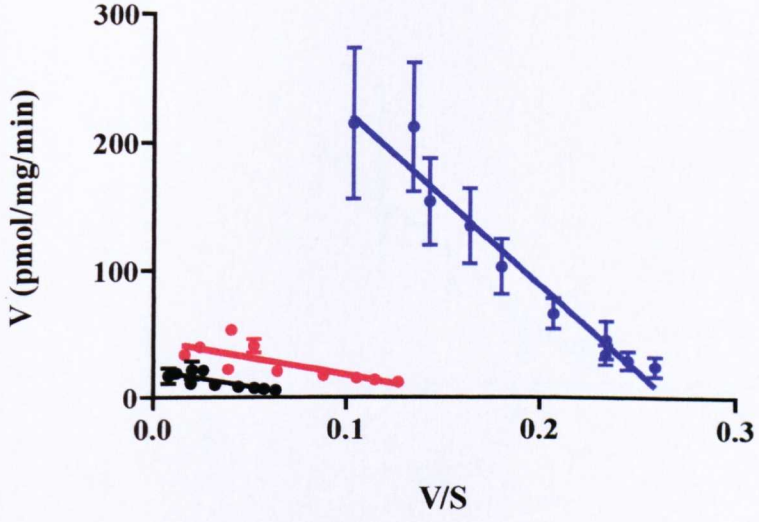


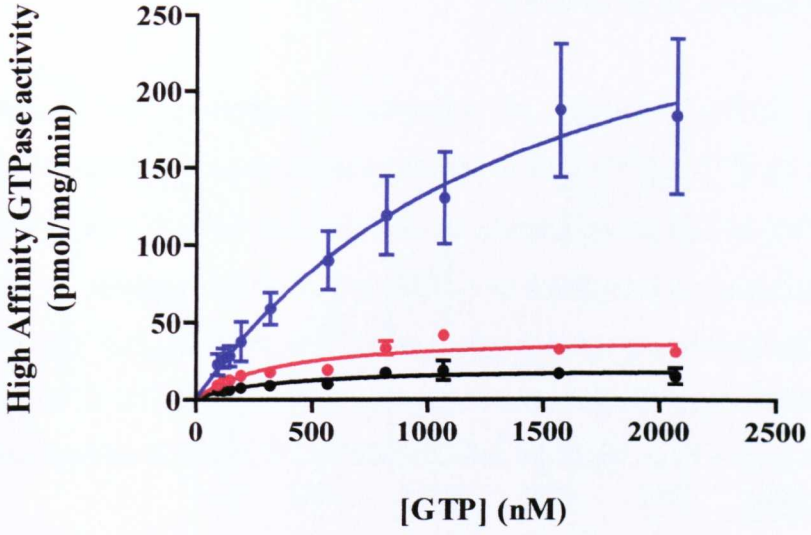
Figure 4.16

Basal and adrenaline-stimulated high affinity GTPase activity of membranes expressing the α_{2A}Cys^{442}Ala-adrenoceptor-$G_{o1}\alpha$Cys^{351}Ile (C^{442}A) fusion protein in the presence and absence of RGS16-GST

High affinity GTPase activity of membranes expressing the α_{2A}Cys^{442}Ala-adrenoceptor-$G_{o1}\alpha$Cys^{351}Ile (**C^{442}A**) fusion protein was assessed for three conditions: basal (black line), 100μM adrenaline-stimulated (red line) and 1μM RGS16 in the presence of 100μM adrenaline-stimulated (blue line). The rate of GTP hydrolysis, V (pmol/mg/min) was assessed for increasing concentrations of GTP as substrate (a). The data generated were converted to Eadie-Hofstee plots to analyse effects on K_m for GTP hydrolysis and V_{max} (b). Results are from triplicate determinations. Analysis is representative of three similar experiments.

Figure 4.16

a)



b)

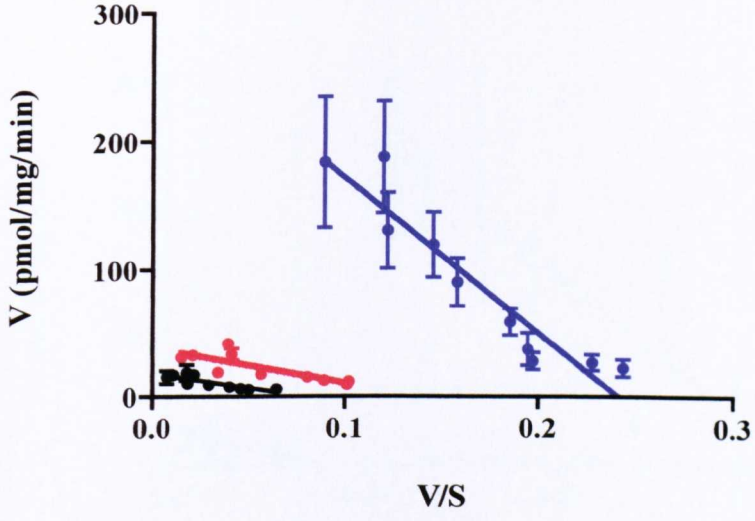


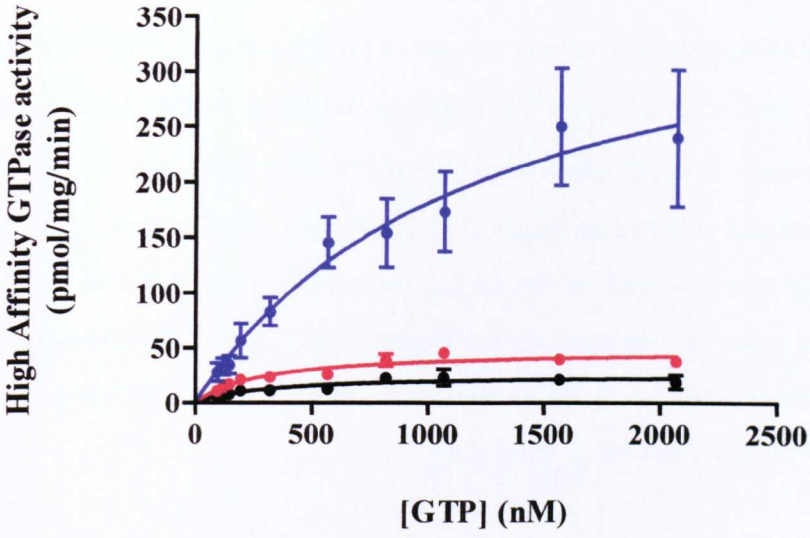
Figure 4.17

Basal and adrenaline-stimulated high affinity GTPase activity of membranes expressing the α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{3}Ser$, $Cys^{351}Ile$ ($C^{3}S$) fusion protein in the presence and absence of RGS16-GST

High affinity GTPase activity of membranes expressing the α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{3}Ser$, $Cys^{351}Ile$ (**$C^{3}S$**) fusion protein was assessed for three conditions: basal (black line), 100μM adrenaline-stimulated (red line) and 1μM RGS16 in the presence of 100μM adrenaline-stimulated (blue line). The rate of GTP hydrolysis, V (pmol/mg/min) was assessed for increasing concentrations of GTP as substrate (a). The data generated were converted to Eadie-Hofstee plots to analyse effects on K_m for GTP hydrolysis and V_{max} (b). Results are from triplicate determinations. Analysis is representative of three similar experiments.

Figure 4.17

a)



b)

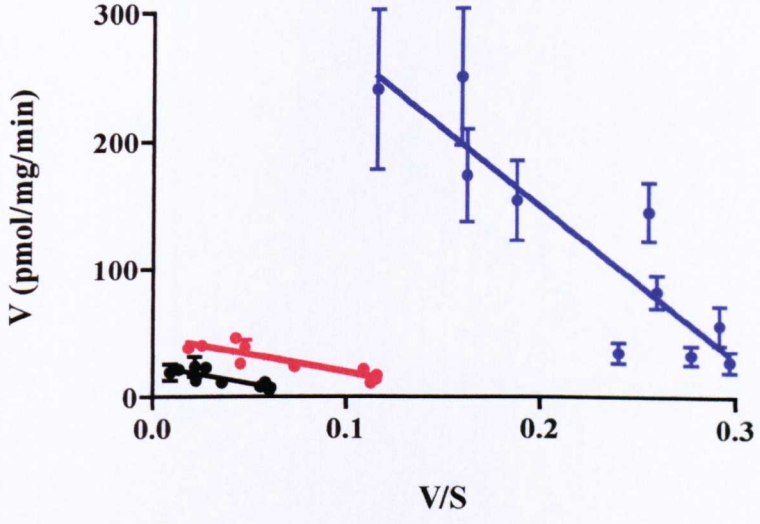


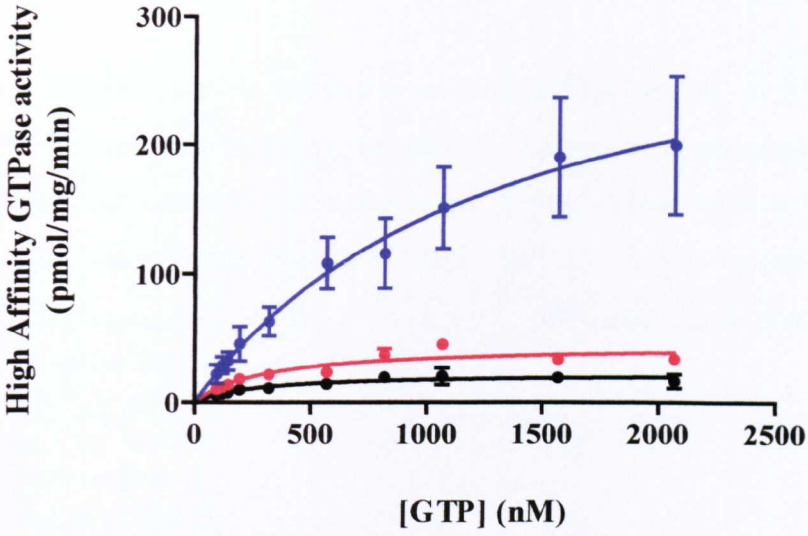
Figure 4.18

Basal and adrenaline-stimulated high affinity GTPase activity of membranes expressing the α_{2A}Cys^{442}Ala-adrenoceptor-$G_{o1}\alpha$Cys^{3}Ser, Cys^{351}Ile (C^{442}A, C^{3}S) fusion protein in the presence and absence of RGS16-GST

High affinity GTPase activity of membranes expressing the α_{2A}Cys^{442}Ala-adrenoceptor-$G_{o1}\alpha$Cys^{3}Ser, Cys^{351}Ile (**C^{442}A, C^{3}S**) fusion protein was assessed for three conditions: basal (black line), 100μM adrenaline-stimulated (red line) and 1μM RGS16 in the presence of 100μM adrenaline-stimulated (blue line). The rate of GTP hydrolysis, V (pmol/mg/min) was assessed for increasing concentrations of GTP as substrate (a). The data generated were converted to Eadie-Hofstee plots to analyse effects on K_m for GTP hydrolysis and V_{max} (b). Results are from triplicate determinations. Analysis is representative of three similar experiments.

Figure 4.18

a)



b)

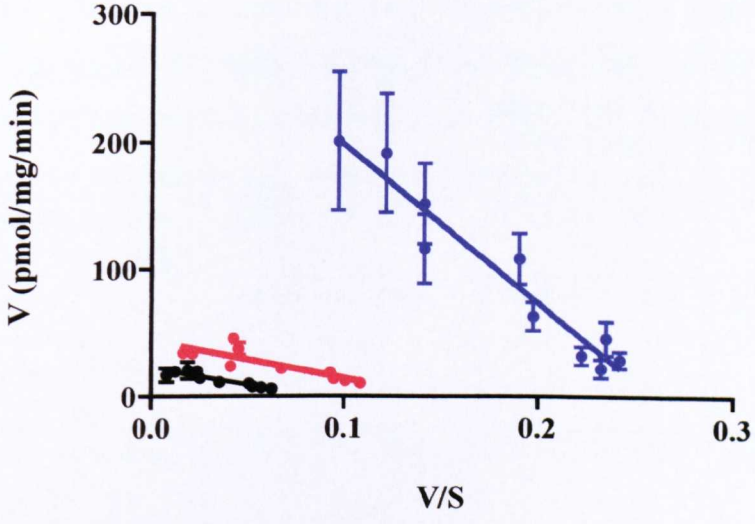


Table 4.9

Comparison of basal and adrenaline-stimulated high affinity GTPase activity V_{max} of membranes expressing the α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ fusion proteins in the presence and absence of RGS16-GST

The results from **Figure 4.15-4.18** were presented in a tabular form for clarity of comparison between the constructs. Results are presented as a mean +/- SEM for basal, adrenaline-stimulated and RGS16 in the presence of adrenaline-stimulated high affinity GTPase activity, V_{max}, of all four palmitoylation-variant α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ fusion proteins.

Table 4.10

Comparison of basal and adrenaline-stimulated K_m for GTP hydrolysis of membranes expressing the α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ fusion proteins in the presence and absence of RGS16-GST

The results from **Figure 4.15-4.18** were presented in a tabular form for clarity of comparison between the constructs. Results are presented as a mean +/- SEM for basal, adrenaline-stimulated and RGS16 in the presence of adrenaline-stimulated K_m for GTP hydrolysis of all four palmitoylation-variant α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ fusion proteins.

Table 4.9

Construct	Basal V_{max} (pmol/mg/min)	Adrenaline-stimulated V_{max} (pmol/mg/min)	RGS16 + Adrenaline-stimulated V_{max} (pmol/mg/min)
α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ (WT)	21.3 +/- 2.5	45.2 +/- 2.2	358.9 +/- 19.7
$\alpha_{2A}Cys^{442}Ala$-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ ($C^{442}A$)	18.8 +/- 1.9	38.8 +/- 4.6	293.7 +/- 25.6
α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{3}Ser$, $Cys^{351}Ile$ ($C^{3}S$)	24.1 +/- 2.6	47.0 +/- 4.1	390.0 +/- 48.7
$\alpha_{2A}Cys^{442}Ala$-adrenoceptor-$G_{o1}\alpha Cys^{3}Ser$, $Cys^{351}Ile$ ($C^{442}A$, $C^{3}S$)	22.2 +/- 1.5	43.6 +/- 4.7	321.8 +/- 21.8

Table 4.10

Construct	Basal K_m GTP (nM)	Adrenaline-stimulated K_m GTP (nM)	RGS16 + Adrenaline-stimulated K_m GTP (nM)
α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ (WT)	252.0 +/- 25.4	275.2 +/- 30.9	1353 +/- 100
$\alpha_{2A}Cys^{442}Ala$-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ ($C^{442}A$)	235.9 +/- 24.4	277.4 +/- 21.9	1217 +/- 146.5
α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{3}Ser$, $Cys^{351}Ile$ ($C^{3}S$)	271.2 +/- 23.4	277.6 +/- 20.9	1202 +/- 109.2
$\alpha_{2A}Cys^{442}Ala$-adrenoceptor-$G_{o1}\alpha Cys^{3}Ser$, $Cys^{351}Ile$ ($C^{442}A$, $C^{3}S$)	261.0 +/- 28.6	297.0 +/- 36.7	1240 +/- 115.2

Table 4.11

Comparison of basal and adrenaline-stimulated turnover numbers for GTP hydrolysis of membranes expressing the α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ fusion proteins in the presence and absence of RGS16-GST

The results from **Figure 4.15-4.18** were adjusted to take into account the construct expression levels. This resulted in the calculation of turnover numbers (mean +/- SEM) for basal, adrenaline-stimulated and RGS16 in the presence of adrenaline-stimulated GTP hydrolysis of all four palmitoylation-variant α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ fusion proteins (presented in a tabular form for clarity of comparison between the constructs).

Table 4.11

Construct	Basal Turnover Number (min^{-1})	Adrenaline-Stimulated Turnover Number (min^{-1})	RGS16 + Adrenaline-stimulated Turnover Number (min^{-1})
α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ (**WT**)	3.2 +/- 0.4	6.8 +/- 0.3	53.6 +/- 2.9
$\alpha_{2A}Cys^{442}Ala$-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ (**$C^{442}A$**)	3.1 +/- 0.3	6.5 +/- 0.8	49.0 +/- 4.3
α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{3}Ser$, $Cys^{351}Ile$ (**$C^{3}S$**)	3.4 +/- 0.4	6.7 +/- 0.6	55.7 +/- 7.0
$\alpha_{2A}Cys^{442}Ala$-adrenoceptor-$G_{o1}\alpha Cys^{3}Ser$, $Cys^{351}Ile$ (**$C^{442}A$, $C^{3}S$**)	3.6 +/- 0.3	7.0 +/- 0.8	51.9 +/- 3.5

Figure 4.19

Biotin labelling of all four palmitoylation-variant α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ fusion proteins

HEK293T cells were transiently transfected to express the four palmitoylation-variant α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ fusion proteins. Cell surface glycoproteins were labelled with biotin and the receptors were immunoprecipitated and visualised as detailed in section 2.7.7. In this figure α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ **(WT)** is in lane 1, α_{2A}-adrenoceptor $Cys^{442}Ala$-$G_{o1}\alpha Cys^{351}Ile$ **($C^{442}A$)** is in lane 2, α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{3}Ser$, $Cys^{351}Ile$ **($C^{3}S$)** is in lane 3 and α_{2A}-adrenoceptor $Cys^{442}Ala$-$G_{o1}\alpha Cys^{3}Ser$, $Cys^{351}Ile$ **($C^{442}A$, $C^{3}S$)** is in lane 4. A representative blot from three individual experiments is shown.

Figure 4.19

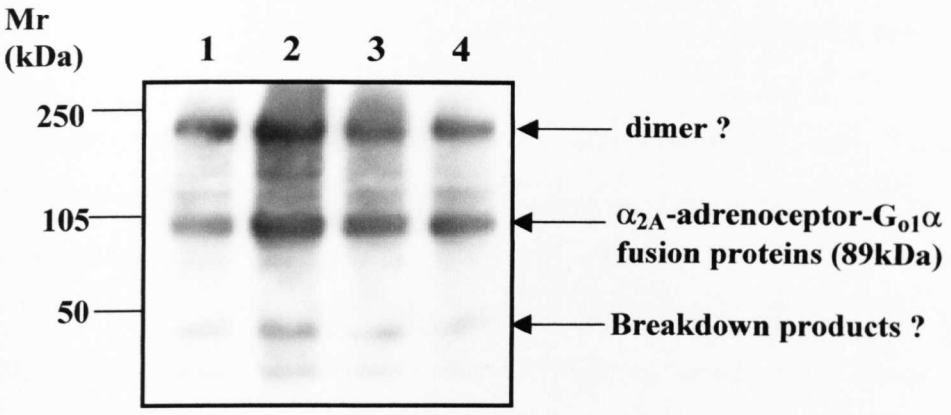

Mr
(kDa)
1
2
3
4
250
105
50
dimer ?
α_{2A}-adrenoceptor-$G_{o1}\alpha$
fusion proteins (89kDa)
Breakdown products ?

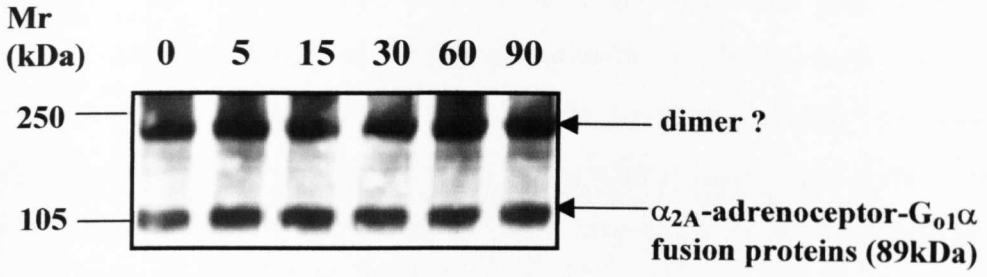
Figure 4.20

Lack of adrenaline-stimulated internalisation of the α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ (WT) and $\alpha_{2A}Cys^{442}Ala$-adrenoceptor-$G_{o1}\alpha Cys^{3}Ser$, $Cys^{351}Ile$ ($C^{442}A$, $C^{3}S$) fusion proteins

HEK293T cells were transiently transfected to express the α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ (**WT, a**) or $\alpha_{2A}Cys^{442}Ala$-adrenoceptor-$G_{o1}\alpha Cys^{3}Ser$, $Cys^{351}Ile$ (**$C^{442}A$, $C^{3}S$, b**) fusion proteins. The cells were either treated with vehicle or 100μM adrenaline for the indicated times (min). Cell surface glycoproteins were labelled with biotin and the receptors were immunoprecipitated and visualised as detailed in section 2.7.5. A representative blot from three individual experiments is shown.

Figure 4.20

a)



b)

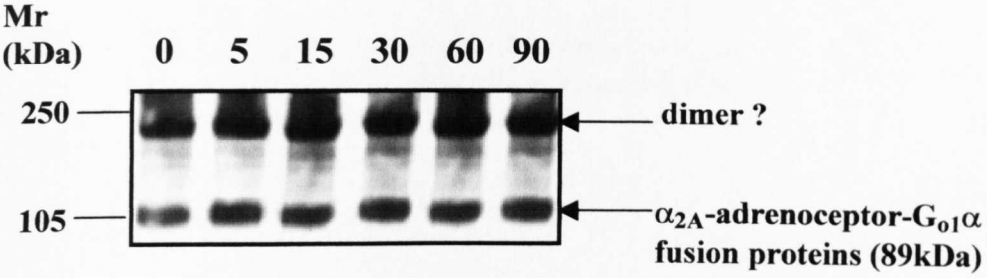


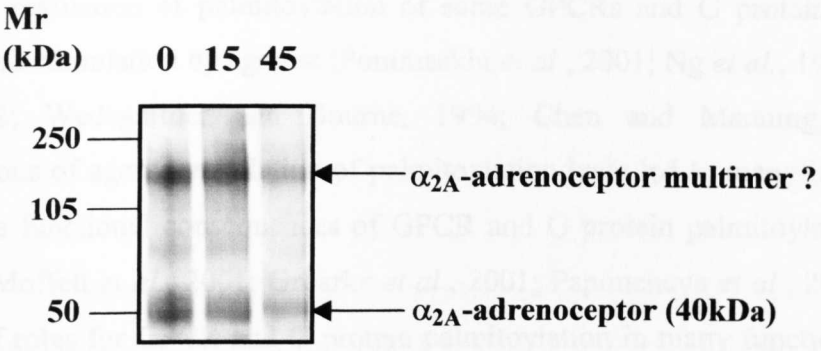
Figure 4.21

Internalisation of adrenaline-stimulated α_{2A}-adrenoceptor alone but not the α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ (WT) fusion protein

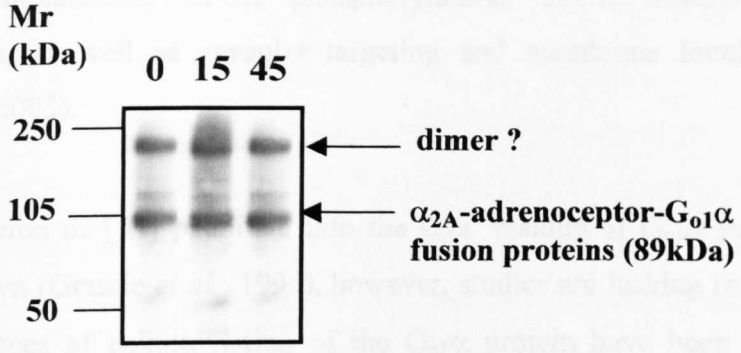
HEK293T cells were transiently transfected to express the α_{2A}-adrenoceptor alone **(a)** or the α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ (**WT, b**) fusion protein. The cells were either treated with vehicle or 100μM adrenaline for the indicated times (min). Cell surface glycoproteins were labelled with biotin and the receptors were immunoprecipitated and visualised as detailed in section 2.7.5. A representative blot from three individual experiments is shown.

Figure 4.21

a)



b)



4.3 Discussion

Dynamic regulation of palmitoylation of some GPCRs and G proteins can occur in response to stimulation by agonist (Ponimaskin *et al.*, 2001; Ng *et al.*, 1994; Mouillac *et al.*, 1992; Wedegartner and Bourne, 1994; Chen and Manning, 2000). Such observations of agonist regulation of palmitoylation have led to many investigations in which the functional consequences of GPCR and G protein palmitoylation have been studied (Moffett *et al.*, 2001; Groarke *et al.*, 2001; Papoucheva *et al.*, 2004). There are reports of roles for GPCR and G protein palmitoylation in many functional properties. Examples of such functional properties are GPCR-G protein interactions, RGS-G protein interactions, GPCR phosphorylation, GPCR desensitisation and down-regulation, as well as caveolar targeting and membrane localisation (Qanbar and Bouvier, 2003).

Incorporation of [^{3}H] palmitate into the Cys^3 residue of $G_{o1}\alpha$ protein has previously been shown (Grassie *et al.*, 1994), however, studies are lacking in which the functional consequences of palmitoylation of the $G_{o1}\alpha$ protein have been studied. In addition, although the porcine α_{2A}-adrenoceptor was one of the first GPCRs demonstrated to be a target for post-translational palmitoylation (Kennedy and Limbird, 1993, 1994), the studies of the functional consequences of palmitoylation for this receptor are limited and have been performed in excess of ten years ago (Kennedy and Limbird, 1993, 1994; Eason and Liggett, 1992; Eason *et al.*, 1994).

The current study made use of fusion proteins in which the N-terminus of the G_{o1} protein α subunit was linked in-frame to the C-terminal tail of the α_{2A}-adrenoceptor. This type of approach is now routinely used in a number of labs (Bertin *et al.*, 1994; Wise and Milligan, 1997; Seifert *et al.*, 1998; Guo *et al.*, 2001) on account of the many advantageous features afforded by fusion proteins (previously discussed in **Chapter 3**). A number of these advantages were also useful for the current study. In addition to the ensured 1:1 stoichiometry of GPCR to G protein and co-localisation of the two proteins our fusion protein approach (in which the fused Gα protein is a pertussis-toxin resistant version, $G_{o1}\alpha Cys^{351}Ile$) allowed us to inactivate the endogenous G_i class of G proteins and hence ensure all functional signal observed reflected only that arising from the

fused Gα protein (Loisel *et al.*, 1999; Stevens *et al.*, 2001; Jones and Reed, 1987; Lochrie and Simon, 1988; Burt *et al.*, 1998). This feature was of particular importance for the current study when GTPγS binding and high affinity GTPase activity were assessed.

All four possible palmitoylation-variant α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ fusion constructs were used herein to assess the importance of α_{2A}-adrenoceptor and $G_{o1}\alpha Cys^{351}Ile$ protein palmitoylation for a selection of functional properties. These were the α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ (**WT**) construct (which has the ability to undergo palmitoylation on two sites, one site in the GPCR and one site in the G protein), the $\alpha_{2A}Cys^{442}Ala$-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ (**$C^{442}A$**) construct (which has the ability to undergo palmitoylation only in the G protein), the α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{3}Ser,Cys^{351}Ile$ (**$C^{3}S$**) construct (which has the ability to undergo palmitoylation only in the GPCR) and the $\alpha_{2A}Cys^{442}Ala$-adrenoceptor-$G_{o1}\alpha Cys^{3}Ser$, $Cys^{351}Ile$ (**$C^{442}A$, $C^{3}S$**) construct (which has no potential palmitoylation sites). In the current study, the role of palmitoylation in fusion protein expression levels, affinity for antagonist and agonist molecules, the ability to bind and also to hydrolyse GTP, as well as the ability of the proteins to internalise were all studied.

The first functional property to be studied was the expression levels of the four palmitoylation-variant fusion proteins. Some previous studies had suggested a possible role for GPCR palmitoylation in determining receptor expression levels. For the LH/hCG receptor (Zhu *et al.*, 1995), the vasopressin V_2 receptor (Schulein *et al.*, 1996) and the CCR_5 receptor (Percherancier *et al.*, 2001) reduced expression levels were reported when receptors were mutated to remove palmitoylation sites. In the current study, fusion protein expression levels were assessed both by Western blot analysis and antagonist binding studies of membranes expressing the fusion proteins. Western blot analysis by use of either ON1 antiserum (against residues 1-16 of $G_{o1}\alpha$) or OC2 antiserum (against residues 345-354 of $G_{o1}\alpha$) revealed similar expression levels for all four palmitoylation-variant α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ fusion proteins. This observation was supported by the results from binding of a near-saturating (~5nM) concentration of [^{3}H]-RS-79948-197, as well as by saturation binding analyses (of 0.05-5nM) of the same radioligand to the fusions. From the antagonist binding studies, the

expression levels of all four palmitoylation-variant fusion proteins were found to be in the region of 6.5 pmol/mg. This observation, although in contrast to the results for the LH/hCG receptor, the vasopressin V_2 receptor and the CCR_5 receptor, is similar to the findings for the TRH receptor (Tanaka *et al.*, 1998). For this receptor, expression levels were unaffected by the ability to be palmitoylated.

The next functional property to be studied for the four palmitoylation-variant fusion proteins was the affinity for antagonist and agonist molecules. A number of reports published to date have assessed the ligand binding capacity for palmitoylation-deficient mutant receptors for comparison with the wild type versions. For the rhodopsin receptor (Karnik *et al.*, 1993), the TRH receptor (Tanaka *et al.*, 1998), the vasopressin V_{1a} receptor (Hawtin *et al.*, 2001), the vasopressin V_2 receptor (Schulein *et al.*, 1996) and the dopamine D_1 receptor (Jin *et al.*, 1997) the ability to become palmitoylated did not affect ligand binding. In agreement with these findings, the current study demonstrated that neither the affinity for the agonist adrenaline (K_i=2nM and 0.25μM: high and low affinity binding) nor the antagonist [^{3}H]-RS-79948-197 (K_d=0.32nM) was different for the four palmitoylation-variant α_{2A}-adrenoceptor-$G_{o1}\alpha$Cys^{351}Ile fusion proteins.

Another functional property studied in the current investigation was the importance of GPCR and G protein palmitylation for the activation of signalling. This was assessed in terms of two properties, the ability to bind GTP (from GTPγS binding studies) and the ability to hydrolyse GTP (from high affinity GTPase assays). For all four fusion proteins, GTPγS binding (using 10fmol per fusion, 2.5 min incubation and using 10^{-4}M adrenaline where appropriate) was stimulated approximately 16-fold over basal levels in the presence of the agonist adrenaline. However, neither the levels of basal nor adrenaline-stimulated GTPγS binding were found to be significantly different when compared between the four palmitoylation-variant fusion proteins. Similarly, high affinity GTPase activity (using 1.5μg of membranes expressing the fusions, 40 min incubation and using 10^{-4}M adrenaline where appropriate) was stimulated approximately 3-fold over basal levels in the presence of the agonist adrenaline. Once again, neither the levels of basal nor adrenaline-stimulated high affinity GTPase activity were found to be significantly different when compared for the four palmitoylation-variant fusion proteins. It is therefore concluded that the ability for the α_{2A}-adrenoceptor

and the $G_{o1}\alpha Cys^{351}Ile$ protein parts of the α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ fusion protein to be palmitoylated has no bearing on the ability of this fusion protein to signal effectively.

Currently in the literature there are many varied reports of the relative importance of GPCR and G protein palmitoylation for signalling. Most studies have concerned GPCR palmitoylation rather than G protein palmitoylation. Nevertheless, there have been a few studies focusing on the functional effects of mutating Gα protein palmitoylation sites. In one such study, mutating the palmitoylation site of $G_s\alpha$ seemed to decrease the efficiency of signalling via this G protein (Wedegaertner *et al.*, 1993). However, later reports suggested that the apparent alterations in efficiency of signalling were due to absence of membrane localisation of the palmitoylation-deficient G protein α-subunit (and subsequent inability to be activated), given that normal signalling efficiency was recovered upon fusion of the Gα subunit to the β_2-adrenoceptor (Ugur *et al.*, 2003). In terms of GPCR palmitoylation, the current observation about the importance of palmitoylation for signalling via the fused α_{2A}-adrenoceptor is consistent with a previous observation for the non-fused α_{2A}-adrenoceptor (Kennedy and Limbird, 1993, 1994). In both cases, mutating potential palmitoylation sites had no effect on coupling to G_i/G_o proteins. Similar observations have also been made for a number of other GPCRs, including the rhodopsin receptor (Karnik *et al.*, 1993), the LH/hCG receptor (Kawate *et al.*, 1997), the vasopressin V_{1a} receptor (Hawtin *et al.*, 2001) and the dopamine D_1 receptor (Jin *et al.*, 1997). Interestingly, there have also been a number of contrasting results, where the ability of the GPCR to be palmitoylated was reported to affect the efficiency of signalling. For the β_2-adrenoceptor (O'Dowd *et al.*, 1989; Moffett *et al.*, 1996, 2001) and the m_2 muscarinic receptor (Hayashi and Haga, 1997), mutating palmitoylation sites led to a reduced ability to signal.

There have also been some reports in the literature that palmitoylation of Gα proteins may affect the ability of RGS proteins to accelerate the GTPase activity of activated Gα proteins (Ross and Wilkie, 2000). In light of this, we decided to use our α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ fusion proteins to assess the importance of both GPCR and G protein palmitoylation for this functional property. This was done by analysis of the

basal and agonist-stimulated high affinity GTPase activities in the presence of GST purified RGS16 protein.

In an initial experiment, levels of adrenaline-stimulated high affinity GTPase activity were stimulated approximately 3-fold compared with basal levels whereas the levels of adrenaline-stimulated high affinity GTPase activity in the presence of RGS16 were stimulated approximately 11-fold compared with basal levels. In addition, a very small (approximately ~1.3-fold) stimulation of basal GTPase activity in the presence of RGS16 was also observed. For all conditions tested similar levels were obtained for the four α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ fusion constructs.

However, in order to accurately compare the results for all four palmitoylation-variant constructs and determine whether palmitoylation on either the GPCR or G protein parts of the fusion affected the ability of RGS16 to accelerate the GTPase activity, an alternative approach was required. The high affinity GTPase activity for each fusion protein was re-determined in the presence of various concentrations of GTP (substrate). This allowed conversion of the generated data to Eadie-Hofstee plots for easier visualisation of GTPase activity (V_{max}) and the K_m for GTP hydrolysis.

This time, levels of adrenaline-stimulated high affinity GTPase activity (~44 pmol/mg/min) were approximately 2-fold greater than basal levels (~22 pmol/mg/min) whereas the levels of adrenaline-stimulated high affinity GTPase activity in the presence of RGS16 (~340pmol/mg/min) were approximately 15-fold higher than basal levels. Such values for GTPase activity were similar to those previously reported in a study with RGS16 and the α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ (**WT**) fusion protein (Hoffman *et al.*, 2001) and once again for all conditions tested, similar levels were obtained for the four α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ fusion constructs.

Before the effect of GPCR and G protein palmitoylation on RGS-accelarated GTPase activity could be determined it was necessary to take into account the slight differences in expression levels for each construct. This was done by calculation of the turnover number for GTP (min^{-1}). Calculation of essentially equal turnover numbers for basal (~3.6 min^{-1}), adrenaline-stimulated (~6.7 min^{-1}) and adrenaline plus RGS16-stimulated

(~60 min^{-1}) GTPase activity for all four palmitoylation-variant fusions, determined that the ability of the RGS16 protein to accelerate the GTPase activity of α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ fusion proteins was not affected by the ability of either the GPCR or the G protein parts of the fusion to be palmitoylated.

The final functional property tested in the current study was the ability of the palmitoylation-variant α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ fusion proteins to internalise in response to adrenaline. This property was tested for comparision with the results of internalisation studies for other GPCRs (Schulein *et al.*, 1996; Kawate *et al.*, 1997; Gao *et al.*, 1999; Groarke *et al.*, 2001; Charest and Bouvier, 2003) and another GPCR-G protein fusion (Loisel *et al.*, 1999). The ability of a number of GPCRs to be palmitoylated has been shown to affect their internalisation properties. Mutating the palmitoylation sites of the vasopressin V_2 receptor (Schulein *et al.*, 1996; Charest and Bouvier, 2003), the CCR_5 receptor (Kraft *et al.*, 2001) and the TRH receptor (Groarke *et al.*, 2001) were all shown to result in decreased internalisation of these receptors in response to agonist. In contrast, mutating the palmitoylation site of the LH/hCG receptor (Kawate *et al.*, 1997) resulted in increased levels of agonist-promoted internalisation. There are also some GPCRs, such as the A_1 adenosine receptor (Gao *et al.*, 1999), for which there is no difference in internalisation properties upon mutation of palmitoylation sites. In the current study, it was uncertain whether any internalisation would be observed for the α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ fusion proteins, since the fusion of an entire $G_{o1}\alpha Cys^{351}Ile$-subunit could, very likely, disrupt the normal internalisation properties of the α_{2A}-adrenoceptor (Olli-Lahdesmaki *et al.*, 2003). Such disruption had previously been suggested from the internalisation studies of a β_2-adrenoceptor-$G_s\alpha$ fusion protein compared with the β_2-adrenoceptor alone (Loisel et al., 1999). In this study it was found that unlike the receptor alone, the fusion protein could not internalise, a restriction found to coincide with alterations in the patterns of incorporation of [^{3}H] palmitate into the two constituent parts of the fusion protein. The results from the current study using α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ fusion proteins were therefore expected to be useful in terms of internalisation properties and for further insight into the regulation of palmitoylation results.

After an initial test to check that all α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ fusion proteins could be biotin labelled and successfully detected the α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ (**WT**) and $\alpha_{2A}Cys^{442}Ala$-adrenoceptor-$G_{o1}\alpha Cys^{3}Ser$, $Cys^{351}Ile$ (**$C^{442}A$, $C^{3}S$**) fusion proteins were assessed in terms of internalisation. Akin to the results with the β_2-adrenoceptor-$G_s\alpha$ fusion protein, neither palmitoylation variant α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ fusion protein was able to internalise over the 90 min incubation period. In contrast, the α_{2A}-adrenoceptor alone was found capable of internalisation (just as for the β_2-adrenoceptor alone). Taken together these results demonstrate that the adrenaline-stimulated internalisation properties of the α_{2A}-adrenoceptor appear to be lost upon constraint of the receptor in the α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ fusion protein. One possible explanation for this observation may be that upon fusion of the G-protein to the receptor, the binding of important molecules for internalisation, such as β-arrestins, may be abolished. Consequently, if the effect of α_{2A}-adrenoceptor palmitoylation on internalisation properties is to be assessed in future studies, this will have to be done with the non-fused receptor.

These observations led us to consider a few points concerning the advantages and disadvantages of GPCR-G protein fusion proteins in the current study. In terms of advantages, aside from the general benefits already discussed, fusions had previously been used successfully to study not only the regulation of palmitoylation (Stevens *et al.*, 2001) but also to study many of the functional properties which were tested in the current investigation. A number of groups had previously shown that the agonist and antagonist binding properties, the intrinsic GTPase activity (Wise and Milligan, 1997; Hoffman *et al.*, 2001) and the ability of RGS proteins to act as GTPase activating proteins (Cavalli *et al.*, 2000) were all as expected for the fusion proteins. Therefore a fusion-based approach to the current study was deemed appropriate. However, there are a few possible limitations for the use of GPCR-G protein fusion proteins, one of which was directly highlighted in the current study. The study of receptor internalisation properties was not possible within the fusion protein context, since none of the fusion proteins were able to internalise unlike the receptor alone. Another possible limitation of fusions must be considered. We have observed from the current results that although Gα subunit palmitoylation is regulated by agonist (**Chapter 3**) this palmitoylation does not seem to affect any of the functional properties tested here in **Chapter 4**. From the

literature, palmitoylation was suggested to play a role either in the regulation of the ability of RGS proteins to accelerate the GTPase reaction or in determining the membrane localisation/targeting of the Gα subunits. Our results show that for the $G_{o1}\alpha Cys^{351}Ile$ portion of the α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ fusion proteins, palmitoylation has no effect on the ability of RGS16 to accelerate the GTPase reaction. This leaves the possibility that palmitoylation may be important for the membrane localisation of the $G_{o1}\alpha Cys^{351}Ile$ protein. It had previously been observed for the $G_s\alpha$ protein that lack/loss of palmitoylation resulted in a translocation of the α-subunit from the membrane to the cytosol (Wedegaertner *et al.*, 1993). Such an observation made for $G_s\alpha$ may have arisen because palmitoylation is the only lipid modification present on this Gα protein and may not occur for G proteins such as $G_{o1}\alpha Cys^{351}Ile$ which also have a 14 carbon myristate group attached. However, if the role of dynamic palmitoylation of the $G_{o1}\alpha Cys^{351}Ile$ protein is in determining the degree of physical separateness of the GPCR and G protein, then the fusion proteins where the two proteins are constrained together may cause altered signalling. This point must be addressed in future work by use of non-fused GPCRs and G proteins. It is important however, to note that such non-fusion studies may also entail problems/limitations of their own. In conclusion, there are advantages and disadvantages associated with any experimental system. The results of this investigation have shown that GPCR-G protein fusion proteins can be successfully used to study a number of aspects of GPCR and G protein signalling. Simultaneously they have highlighted that other functional properties are better suited to non-fusion studies.

To summarise, the current study aimed to assess the importance of GPCR and G protein palmitoylation for a variety of functional properties. Previous studies had suggested roles for Gα protein palmitolyation in the efficiency of signalling and in regulating the ability of RGS proteins to accelerate the GTPase reaction. Previous studies had also suggested roles for GPCR palmitoylation in regulating receptor expression levels, the affinity for agonist and antagonist molecules, and the efficiency of signalling. Herein, the effect of the α_{2A}-adrenoceptor and the $G_{o1}\alpha Cys^{351}Ile$ portions of the α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ fusion protein were assessed in terms of all these functional properties. It has been observed that, at least in the fusion protein context, neither the palmitoylation of the α_{2A}-adrenoceptor nor of the $G_{o1}\alpha Cys^{351}Ile$ portions of the α_{2A}-

adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ fusion protein seem to have any effect on protein expression levels, the affinity for agonist and antagonist molecules, the efficiency of signalling, or the efficiency of RGS proteins to accelerate the GTPase reaction. Given the lack of previous information regarding the functional importance of palmitoylation for the α_{2A}-adrenoceptor and the $G_{o1}\alpha Cys^{351}Ile$ protein, the current results were compared to information gathered from studies on other GPCRs and G proteins. However, such was the variety of information for other GPCRs and G proteins, that the main conclusion which can be drawn from the current study is that in general, the roles of GPCR and G protein palmitoylation appear to be different dependent on the GPCR and G protein in question. Perhaps this is not altogether surprising given the variety of signalling effects observed with different GPCR and G protein pairs. Altogether these results highlight the need for further investigation into a variety of other functional consequences before a clearer picture of the role(s) of palmityolation can be obtained.

Chapter 5

Regulation of palmitoylation in

5-HT_{1A}-receptor-$G_{o1}\alpha Cys^{351}Ile$ fusion proteins

5.1 Introduction

The 5-HT_{1A}-receptor used in this study is responsible for signal transduction across the plasma membrane via interaction with G_i/G_o class of heterotrimeric G proteins, leading to inhibition of adenylyl cyclase and L-type Ca^{2+} channels and activation of K^+ channels. The natural ligand for the 5-HT_{1A}-receptor is serotonin: an important neurotransmitter and local hormone in the CNS and intestine. Clinically, 5-HT_{1A} receptor ligands represent potential anxiolytic (Tunnicliff, 1991) and anti-hypertensive agents (Lundberg, 1996).

This study concerned the attachment of the 16-carbon fatty acid moiety, palmitate, to both the 5-HT_{1A}-receptor and the G_{o1} G protein. As previously described in **Chapter 3**, a wide variety of cellular proteins including GPCRs and G proteins undergo palmitoylation via a labile thioester linkage to cysteine residues (Mumby, 1997). Given that the lability of the thioester bond allows the attachment of palmitate to be both dynamic and regulated (Mumby, 1997; Qanbar and Bouvier, 2003), the study of the regulation of palmitoylation is expected to give some indication of the functional significance of the role of palmitoylation in the protein.

Both the 5-HT_{1A}-receptor (Papoucheva *et al.*, 2004) and the $G_{o1}\alpha$ (Grassie *et al.*, 1994) protein used in this study have previously been shown to undergo post-translational palmitoylation. In the work of Papoucheva *et al.* (2004) palmitate attached to the 5-HT_{1A}-receptor was not regulated by the agonist serotonin. In addition, these authors reported that cycloheximide treatment of cells expressing the 5-HT_{1A}-receptor led to abolished incorporation of [^{3}H] palmitate into the receptor, indicating no significant turnover of receptor-bound palmitate. This result was supported by their observation from pulse-chase experiments that the lifetime of [^{3}H] palmitate attached to the 5-HT_{1A}-receptor corresponded with the lifetime of the receptor itself (Papoucheva *et al.*, 2004). Such observations for 5-HT_{1A}-receptor palmitoylation appear similar to those previously reported for the α_{2A}-adrenoceptor, whereby palmitate was relatively stably attached to this GPCR and (in the case of the α_{2A}-adrenoceptor) agonist did not have a

very dramatic effect on the regulation of this modification (Kennedy and Limbird, 1994). However they are in distinct contrast to the observations for many other protein targets for thio-acylation where rapid cycles of palmitoylation and depalmitoylation are thought to occur (Qanbar and Bouvier, 2003).

In the current study, regulation of 5-HT_{1A} and $G_{o1}\alpha$ G protein palmitoylation were assessed by use of palmitoylation-variant 5-HT_{1A}-receptor-$G_{o1}\alpha Cys^{351}$Ile fusion proteins. As in the study of regulation of α_{2A}-adrenoceptor and $G_{o1}\alpha$ G protein palmitoylation by use of α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}$Ile fusion proteins (**Chapter 3**), there were clear reasons for employing a fusion-protein approach in this work. To recapitulate, a fusion-based approach provided the advantages of defined stoichiometry of expression of the two substituent entities as 1:1 and also ensured co-localisation following expression. In terms of palmitoylation studies these features ensured every copy of the $G_{o1}\alpha Cys^{351}$Ile protein had the potential to be activated upon agonist stimulation of the 5-HT_{1A} receptor.

Previous studies had identified the Cys^{417} and Cys^{420} residues of the 5-HT_{1A}-receptor (Papoucheva *et al.*, 2004) and the Cys^{3} residue of the $G_{o1}\alpha$ protein (Grassie *et al.*, 1994) to be the sites for incorporation of palmitate. Therefore in this study, the eight possible palmitoylation–variant 5-HT_{1A}-receptor-$G_{o1}\alpha Cys^{351}$Ile fusion proteins (5-HT_{1A}-receptor-$G_{o1}\alpha Cys^{351}$Ile (**WT**), 5-$HT_{1A}Cys^{417}$Ser-receptor-$G_{o1}\alpha Cys^{351}$Ile (**$C^{417}S$**), 5-$HT_{1A}Cys^{417}$Ser-receptor-$G_{o1}\alpha Cys^{3}$Ser, Cys^{351}Ile (**$C^{417}S$, $C^{3}S$**), 5-$HT_{1A}Cys^{420}$Ser-receptor-$G_{o1}\alpha Cys^{351}$Ile (**$C^{420}S$**), 5-$HT_{1A}Cys^{420}$Ser-receptor-$G_{o1}\alpha Cys^{3}$Ser, Cys^{351}Ile (**$C^{420}S$, $C^{3}S$**), 5-$HT_{1A}Cys^{417}$Ser, Cys^{420}Ser-receptor-$G_{o1}\alpha Cys^{351}$Ile (**$C^{417}S$, $C^{420}S$**), 5-HT_{1A}-receptor-$G_{o1}\alpha Cys^{3}$Ser, Cys^{351}Ile (**$C^{3}S$**), and 5-$HT_{1A}Cys^{417}$Ser, Cys^{420}Ser-receptor-$G_{o1}\alpha Cys^{3}$Ser, Cys^{351}Ile (**$C^{417}S$, $C^{420}S$, $C^{3}S$**) were created and subjected to a series of palmitoylation assays. The specific objective for the work contained in this chapter was to investigate the regulation of palmitate attached to the GPCR and G protein parts of these fusions and to ascertain whether regulation of acylation was co-ordinated in these two proteins.

It is necessary at this stage to provide the reader with a cautionary note that interpreting the regulation of palmitoylation data from the 5-HT_{1A}-receptor-$G_{o1}\alpha Cys^{351}Ile$ fusions will not be as easy as for the α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ fusion proteins. This is largely due to the presence of three potential palmitoylation sites on these fusions (in contrast to only two in the α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ constructs), meaning eight palmitoylation-variant fusions are possible. With so many constructs it is therefore diffult to keep in mind exactly which sites are being studied when each mutation is studied. In relation to this, it may be of use for the reader to refer to **Table 5.1** when interpreting each result. This table gives the abbreviated name for each construct (corresponding to the mutated residue) and the resultant potential palmitoylation sites which are left unaltered.

5.2 Results

Construction and expression of 5-HT_{1A}-receptor-$G_{o1}\alpha Cys^{351}Ile$ fusion proteins

PCR was used to mutate a previously existing 5-HT_{1A}-receptor-$G_{o1}\alpha Cys^{351}Ile$ fusion protein, available in-house, in order to remove the predicted palmitoylation sites from the GPCR (Papoucheva *et al.*, 2004) or the G protein (Grassie *et al.*, 1994) or both parts of the fusion protein. These cDNA constructs were transiently transfected into HEK293T cells and a number of palmitoylation assays were performed to investigate the regulation of GPCR and G protein acylation in the fusion proteins. In order to assure equal construct expression level in all 6cm dishes used within one palmitoylation assay, it was necessary to transfect one initial plate of HEK 293T cells with each desired fusion construct DNA, then to split these transfected cells into multiple duplicate 6cm dishes to be used in the palmitoylation assay. In **Figure 5.1** it is shown that such an approach led to equal expression level of the wild type **(WT)** 5-HT_{1A}-receptor-$G_{o1}\alpha Cys^{351}Ile$ construct. In this and in subsequent experiments, the expression levels of 5-HT_{1A}-receptor-$G_{o1}\alpha Cys^{351}Ile$ constructs were determined by performing receptor binding studies using the tritiated antagonist [^{3}H]-WAY100635 (Fletcher *et al.*, 1996). In these receptor binding studies non-radiolabelled WAY100635 was used to allow calculation of non-specific binding (Langer and Hicks, 1984).

Confirmation of the Cys^{417} site on the GPCR and the Cys^3 site on the G protein as sites for incorporation of [^{3}H] palmitic acid in the 5-HT_{1A}-receptor-$G_{o1}\alpha Cys^{351}Ile$ fusion proteins

The first objective for the current study was to confirm whether the Cys^{417} and Cys^{420} sites on the GPCR and the Cys^3 site on the G protein were the sites for incorporation of palmitate. In order to do this a one time-point, pulse-labelling palmitoylation assay was performed for all eight palmitoylation-variant fusion proteins plus pcDNA3 control. The constructs were expressed transiently in HEK293T cells. [9,10(n)-^{3}H] palmitic acid was added to the cells in the presence or absence of 100μM 8-OH-DPAT for 30 min. Following labelling of cells, immunoprecipitation using an antiserum (ON1) that

identifies the N-terminal region of $G_{o1}\alpha$ (Mullaney and Milligan, 1990), SDS-PAGE and autoradiography, the incorporation of [^{3}H] palmitate into a band with apparent molecular mass of some 85 kDa (corresponding to 5-HT_{1A}-receptor-$G_{o1}\alpha Cys^{351}$Ile fusions) was assessed. The **WT**, the **$C^{417}S$**, the **$C^{420}S$**, the **$C^{420}S$, C^3S**, the **$C^{417}S$, $C^{420}S$** and the **C^3S** fusions but not the pcDNA3 control, the **$C^{417}S$, $C^{420}S$, C^3S** fusion or, surprisingly, the **$C^{417}S$, C^3S** forms of the fusion protein incorporated [^{3}H] palmitate (**Figure 5.2 a**, upper panels). The lack of incorporation of [^{3}H] palmitate into the **$C^{417}S$, $C^{420}S$, C^3S** and the **$C^{417}S$, C^3S** forms of the fusion protein confirmed that the Cys^{417} site on the GPCR and the Cys^3 site on the G protein are the only two sites for dynamic incorporation of [^{3}H] palmitic acid into the 5-HT_{1A}-receptor-$G_{o1}\alpha Cys^{351}$Ile fusion proteins.

It was very interesting to note that in the absence of agonist and with the same period of exposure to [^{3}H] palmitate, the extent of incorporation of radioactivity into the fusions did not appear to be equal (**Figure 5.2 b, Table 5.1**). In addition, the presence of 8-OH-DPAT had a variety of effects on the incorporation of [^{3}H] palmitate into the different 5-HT_{1A}-receptor-$G_{o1}\alpha Cys^{351}$Ile fusions (**Figure 5.2, Table 5.1**). The observed effects were not attributable to unequal amounts of the fusion protein in each sample since, in parallel with these studies, samples of the cell lysates were resolved directly by SDS-PAGE and immunoblotted with antiserum ON1 (**Figure 5.2 a**, lower panels).

When the data from **Figure 5.2** was analysed, three seemingly distinct patterns of palmitoylation were observed. Firstly, statistically similar patterns (for both unstimulated and stimulated, $p>0.05$) were observed for the **WT** and the **$C^{420}S$** fusions, wherein 8-OH-DPAT-stimulation led to approximately 20% higher levels of incorporation of [^{3}H] palmitate than for the basal levels ($p<0.05$).

Secondly, a contrasting pattern was observed for both the **$C^{417}S$** and the **$C^{417}S$, $C^{420}S$** fusions. Once again the two fusions were assessed to be statistically similar (for both unstimulated and stimulated, $p>0.05$). For these two fusions, 8-OH-DPAT led to approximately half the amount of [^{3}H] palmitate incorporation as for the basal levels ($p<0.05$).

The third pattern was observed for the **$C^{3}S$** and the **$C^{420}S$, $C^{3}S$** fusions. These two fusions were also assessed to be statistically similar (for both unstimulated and stimulated, $p>0.05$) and 8-OH-DPAT stimulation of these fusions led to approximately 50% higher levels of incorporation of [^{3}H] palmitate than for basal levels ($p<0.05$).

The first and third patterns however, were assessed not to be statistically significant. As a result, only two real (and opposing) patterns of palmitoylation could be concluded. Firstly, the pattern observed for both the **$C^{417}S$** and the **$C^{417}S$, $C^{420}S$** fusions, where agonist led to a decrease in palmitate incorporation and secondly, the pattern observed for the other four fusions, where agonist led to increased levels of incorporated palmitate.

Given the observation that palmitate did not become dynamically incorporated into the C^{420} residue of our 5-HT_{1A}-receptor-$G_{o1}\alpha Cys^{351}$Ile fusion proteins (as assessed by use of the **$C^{417}S$, $C^{3}S$** fusion protein) it is perhaps not surprising that pairs of fusions have been observed to display similar palmitoylation characteristics. If the palmitate contribution of the **C^{420}** site is zero then the study of such "pairs" effectively should involve analysis of the same site(s).

To explore the different patterns of palmitoylation further, time course palmitoylations were performed for of the incorporation of [^{3}H] palmitate into some of the constructs. The **WT** fusion (to explore all sites together), the **$C^{417}S$, $C^{420}S$** fusion (to explore the G protein site only), the **$C^{3}S$** fusion (to explore both receptor sites) and the **$C^{420}S$, $C^{3}S$** fusion (to explore only the **C^{417}** site of the receptor) were all selected for these studies.

Analysis of the time courses of incorporation of [^{3}H] palmitic acid into the 5-HT_{1A}-receptor-$G_{o1}\alpha Cys^{351}$Ile fusion proteins

The 5-HT_{1A}-receptor-$G_{o1}\alpha Cys^{351}$Ile (**WT**) fusion protein was expressed transiently in HEK293T cells. [^{3}H] palmitic acid was added to the cells in the presence or absence of the 5-HT_{1A}-receptor agonist 8-OH-DPAT (100μM) for times varying between 5-120 min. After cell lysate generation, immunoprecipitation, SDS-PAGE and autoradiography, in the absence of 8-OH-DPAT, radioactivity was incorporated into bands with apparent molecular mass of approximately 85 kDa (**Figure 5.3 a**, upper panels). This occurred in a time-dependent manner with maximal incorporation being achieved between 60-120 min. In the presence of 8-OH-DPAT, incorporation of [^{3}H] palmitate into the fusion protein was substantially increased ($p<0.05$) over this time scale (**Figure 5.3 a**, upper panels, **Figure 5.3 b**) and once again, maximal incorporation was achieved in a similar timescale, between 60-120 min ($p>0.05$). The effects observed were not attributable to unequal amounts of the fusion protein in each sample since, in parallel with these studies, samples of the cell lysates were resolved directly by SDS-PAGE and immunoblotted with antiserum ON1 (**Figure 5.3 a**, lower panels).

The time course of incorporation of [^{3}H] palmitate into the $G_{o1}\alpha Cys^{351}$Ile protein was then assessed by use of the 5-$HT_{1A}Cys^{417}$Ser, Cys^{420}Ser-receptor-$G_{o1}\alpha Cys^{351}$Ile (**$C^{417}S$, $C^{420}S$**) fusion protein. After labelling, cell lysate generation, immunoprecipitation, SDS-PAGE and autoradiography, radioactivity was again incorporated into bands with apparent molecular mass of approximately 85 kDa (**Figure 5.4 a**, upper panels). In the absence of 8-OH-DPAT, this occurred in a time-dependent manner with maximal incorporation being achieved between 30-60 min. In the presence of 8-OH-DPAT, incorporation of [^{3}H] palmitate into the fusion protein was substantially reduced ($p<0.05$) over this time scale (**Figure 5.4 a**, upper panels, **Figure 5.4 b**). In addition, maximal incorporation of [^{3}H] palmitate in the presence of the agonist occurred at a similar rate ($p>0.05$). Once again the amounts of the fusion protein in each sample were confirmed in Western blots performed in parallel with these studies (**Figure 5.4 a**, lower panels).

Upon analysis of the time course of incorporation of [^{3}H] palmitate into the two potential 5-HT$_{1A}$ receptor palmitoylation sites (by use of the 5-HT$_{1A}$-receptor-G$_{o1}\alpha$Cys^{3}Ser, Cys^{351}Ile (**C^{3}S**) construct), a similar pattern of [^{3}H] palmitate incorporation was observed as for the 5-HT$_{1A}$-receptor-G$_{o1}\alpha$Cys^{351}Ile (**WT**) fusion. Once more radioactivity was incorporated into bands with apparent molecular mass of approximately 85 kDa (**Figure 5.5 a**, upper panels). In the absence of 8-OH-DPAT, incorporation occurred in a time-dependent manner with maximal incorporation not being reached within the 120 min time-course. Similarly, in the presence of 8-OH-DPAT, maximal incorporation was again not achieved within the 120 min time-course and the rate of labelling for the two conditions was not different ($p>0.05$). However the overall level of incorporation of [^{3}H] palmitate into the fusion protein was substantially increased ($p<0.05$) (**Figure 5.5 a**, upper panels, **Figure 5.5 b**). Immunoblots carried out in parallel once again ensured equal amounts of the fusion protein were present in each sample (**Figure 5.5 a**, lower panels).

When the time course of incorporation of [^{3}H] palmitate into only the **Cys^{417}** residue of the 5-HT$_{1A}$ receptor (by use of the 5-HT$_{1A}$Cys^{420}Ser-receptor-G$_{o1}\alpha$Cys^{3}Ser, Cys^{351}Ile (**C^{420}S, C^{3}S**) construct) is analysed, a pattern of [^{3}H] palmitate incorporation was observed which was not significantly different ($p>0.05$) from that observed for the 5-HT$_{1A}$-receptor-G$_{o1}\alpha$Cys^{3}Ser, Cys^{351}Ile (**C^{3}S**) fusion protein. Once again in the absence of 8-OH-DPAT, [^{3}H] palmitate incorporation into bands with apparent molecular mass of approximately 85 kDa (**Figure 5.6 a**, upper panels) occurred in a time-dependent manner with maximal incorporation not being reached within the 120 min time-course. In the presence of 8-OH-DPAT, maximal incorporation was again not achieved within the 120 min time-course and the rate of labelling for the two conditions was not different ($p>0.05$). However, the overall level of incorporation of [^{3}H] palmitate into the fusion protein was substantially increased ($p<0.05$) (**Figure 5.6 a**, upper panels, **Figure 5.6 b**). Immunoblots carried out in parallel ensured equal amounts of the fusion protein were present in each sample (**Figure 5.6 a**, lower panels).

It is useful, for clarity, to take all these results together for interpretation (**Table 5.2**). In order to compare the results obtained with different constructs, the incorporation levels of [^{3}H] palmitate into each construct were expressed as a percentage of the value

obtained for that construct's 60 min unstimulated incubation. For the $G_{o1}\alpha Cys^{351}Ile$ protein (as assessed by use of the $C^{417}S$, $C^{420}S$ fusion), stimulation by 8-OH-DPAT was found to lead to reduced levels of incorporated palmitate into this fusion. In contrast, an alternative pattern was observed for the incorporation of palmitate into the receptor part of the fusions (as assessed by use of $C^{3}S$; representing incorporation into both receptor sites together, or by use of $C^{420}S$, $C^{3}S$; representing incorporation into the receptor C^{417} site alone). For these two constructs, stimulation by 8-OH-DPAT led to increased levels of incorporated palmitate into the fusions. These results were in support of the findings from **Figure 5.2**, suggesting different regulation of palmitate attached to the GPCR and the G protein parts of our fusions. Interestingly the observed levels of incorporation of palmitate into all three potential palmitoylation sites (as assessed by use of the **WT** fusion) was found to exhibit a similar pattern to those of the $C^{3}S$ and $C^{420}S$, $C^{3}S$ fusions (once again in agreement with the results from **Figure 5.2**). This result was somewhat unexpected given the two opposing pattern of palmitoylation. Instead, one might have expected similar levels of incorporation of palmitate in the presence and absence of agonist with this **WT** fusion. However, an influence of the two opposing patterns can be observed upon analysis of the kinetic data generated by these results.

Non-linear regression analysis allowed the calculation of $t_{1/2}$ (min) for incorporation of palmitate, as well as the maximal incorporation level, B_{max} (%) for each construct. Upon analysis of B_{max} in the absence of 8-OH-DPAT, we can see that the levels of incorporation into the **WT**, the $C^{3}S$ and the $C^{420}S$, $C^{3}S$ fusions were not significantly different ($p>0.05$). However, the B_{max} for all three were significantly different from that of the $C^{417}S$, $C^{420}S$ fusion ($p<0.05$). When the B_{max} in the presence of 8-OH-DPAT was compared for all four constructs, only the values for the $C^{3}S$ and the $C^{420}S$, $C^{3}S$ fusions were not significantly different ($p>0.05$). Once again the B_{max} for these two fusions were significantly different from that of the $C^{417}S$, $C^{420}S$ construct ($p<0.05$). In the presence of agonist the B_{max} for **WT** was found to be significantly lower than for the $C^{3}S$ and the $C^{420}S$, $C^{3}S$ fusions as well as significantly higher than for the $C^{417}S$, $C^{420}S$ fusion ($p<0.05$ in both cases).

When the $t_{1/2}$ for incorporation of palmitate was compared for all four fusions, the results mirrored those obtained for the B_{max} levels. In the absence of 8-OH-DPAT the

$t_{1/2}$ for incorporation into the **WT**, the **C^3S** and the **$C^{420}S$, C^3S** fusions were not significantly different ($p>0.05$). In addition the $t_{1/2}$ values for all three were significantly higher than the $t_{1/2}$ for incorporation for the **$C^{417}S$, $C^{420}S$** fusion ($p<0.05$). Similarly, upon comparison of the 8-OH-DPAT-stimulated $t_{1/2}$ for incorporation values only the values for the **C^3S** and the **$C^{420}S$, C^3S** fusions were not significantly different ($p>0.05$). Once again the $t_{1/2}$ values for these two fusions were significantly different from that of the **$C^{417}S$, $C^{420}S$** construct ($p<0.05$). In the presence of agonist the $t_{1/2}$ for incorporation for **WT** was found to be significantly lower than for the **C^3S** and the **$C^{420}S$, C^3S** fusions as well as significantly higher than for the **$C^{417}S$, $C^{420}S$** fusion ($p<0.05$ in both cases).

In summary, these studies indicated clear differences in the levels and kinetics of palmitoylation (and the effects of 8-OH-DPAT on these properties) in the GPCR and G protein elements of the fusion proteins. 8-OH-DPAT resulted in increased levels of palmitate incorporation into the receptor portion of our fusion proteins, whilst it led to decreased levels of incorporation into the G protein. It was also noted that incorporation of palmitate into the G protein was significantly faster than the rate of incorporation into the GPCR. In light of the fact that pulse-labelling results for the effects of 8-OH-DPAT could potentially represent altered rates of palmitoylation or de-palmitoylation of the G protein, the dynamics of de-palmitoylation of GPCR-G protein fusions were subsequently studied in pulse-chase format experiments.

Analysis of the depalmitoylation rates of the 5-HT_{1A}-receptor-$G_{o1}\alpha Cys^{351}Ile$ fusion proteins

Following transfection of HEK293T cells with the 5-HT_{1A}-receptor-$G_{o1}\alpha Cys^{351}Ile$ (**WT**) fusion construct and labelling of the cells with [^{3}H] palmitate for 30 min, the radiolabel was removed and replaced with non-radioactive palmitate. Samples were taken for analysis at times up to 180 min and parallel immunoblots of cell lysates confirmed equal loading of the gel lanes. [^{3}H] palmitate was found to be removed from the immunoprecipitated (85kDa) fusion protein with t½ = 45.5 +/- 15.6 min (mean +/- SEM, n = 3) (**Figure 5.7 a** and **b**). When the chase was conducted in the presence of 8-OH-DPAT removal of [^{3}H] palmitate from the fusion appeared to occur at a similar rate, with t½ = 48.4 +/- 23.5 min (mean +/- SEM, n = 3, $p>0.05$).

To assess the contribution of the G protein to these effects, HEK293T cells were transfected to express the 5-$HT_{1A}Cys^{417}Ser$, $Cys^{420}Ser$-receptor-$G_{o1}\alpha Cys^{351}Ile$ ($\mathbf{C^{417}S}$, $\mathbf{C^{420}S}$) fusion protein, in which only the G protein element is a target for thio-acylation. Using the same protocol, the rate of disappearance of [^{3}H] palmitate from immunoprecipitated samples was rapid ($t½$ = 15.3 +/- 6.5 min, mean +/- SEM, n = 3) and appeared to be accelerated somewhat by the presence of agonist ($t½$ = 6.3 +/- 2.4 min, mean +/- SEM, n = 3) (**Figure 5.8 a** and **b**). However, in both experiments the errors were relatively large, meaning that the effect of agonist in each case was not assessed to be statistically significant ($p>0.05$). In contrast, the data was of sufficient quality to assess that both the unstimulated ($p<0.05$) and the 8-OH-DPAT-stimulated ($p<0.05$) rates of $G_{o1}\alpha Cys^{351}Ile$ depalmitoylation (as assessed by the use of $\mathbf{C^{417}S}$, $\mathbf{C^{420}S}$) were significantly faster than the rates observed for the **WT** fusion (reflecting all three potential palmitoylation sites).

To assess the contribution of the 5-HT_{1A} receptor to these effects, HEK293T cells were transfected to express the 5-HT_{1A}-receptor-$G_{o1}\alpha Cys^{3}Ser$,$Cys^{351}Ile$ ($\mathbf{C^{3}S}$) fusion protein. These results would provide a reflection of the combined depalmitoylation effects of the two receptor residues, $\mathbf{C^{417}}$ and $\mathbf{C^{420}}$. In such experiments (**Figure 5.9 a** and **b**) following labelling then chase, it was found that [^{3}H] palmitate, whilst effectively incorporated, was not removed at all from the immunoprecipitated fusion protein during a three-hour chase period. The same observation was made whether the chase was conducted in the presence or absence of 8-OH-DPAT. These results therefore indicate that palmitate, once incorporated into this fusion, remains stably attached.

One further depalmitoylation experiment was then performed with the 5-$HT_{1A}Cys^{420}Ser$-receptor-$G_{o1}\alpha Cys^{3}Ser$, $Cys^{351}Ile$ ($\mathbf{C^{420}S}$, $\mathbf{C^{3}S}$) fusion protein in which only the $\mathbf{C^{417}}$ residue could be a potential target for palmitoylation. In this experiment (**Figure 5.10 a** and **b**), the results were essentially identical to those from the previous study with the $\mathbf{C^{3}S}$ fusion and once again no depalmitoylation was observed.

The depalmitoylation data for all four experiments have been presented together in **Table 5.3** for clarity of comparison between constructs. In summary, we can conclude

that only the $G_{o1}\alpha Cys^{351}Ile$ protein part of our 5-HT_{1A}-receptor-$G_{o1}\alpha Cys^{351}Ile$ fusion proteins can undergo dynamic depalmitoylation and that this process may (currently unclear from the present results) be stimulated by the presence of the agonist, 8-OH-DPAT. The current investigation also suggests that palmitate, once incorporated into the 5-HT_{1A}-receptor portion of our 5-HT_{1A}-receptor-$G_{o1}\alpha Cys^{351}Ile$ fusion proteins, is stably attached, regardless of the presence or absence of agonist.

Analysis of concentration-dependent effects of 8-OH-DPAT on palmitoylation of 5-HT_{1A}-receptor-$G_{o1}\alpha Cys^{351}Ile$ fusion proteins

The next set of palmitoylation experiments aimed to assess whether agonist-stimulated regulation of palmitoylation was concentration-dependent.

When labelling of the 5-HT_{1A}-receptor-$G_{o1}\alpha Cys^{351}Ile$ **(WT)** fusion protein with [^{3}H] palmitate was allowed to proceed for 30 min in the presence of varying concentrations of 8-OH-DPAT, it was found that a half-maximal increase in incorporation of [^{3}H] palmitate into the immunoprecipitated 85kDa fusion protein was observed with 63 +/- 48 nM 8-OH-DPAT (mean +/- SEM, n = 3) (**Figure 5.11 a** and **b, Table 5.4**).

In contrast, when labelling of the 5-$HT_{1A}Cys^{417}Ser$, $Cys^{420}Ser$-receptor-$G_{o1}\alpha Cys^{351}Ile$ **($C^{417}S$, $C^{420}S$)** fusion protein was carried out using the same protocol, the agonist 8-OH-DPAT caused a half-maximal reduction in levels of [^{3}H] palmitate incorporated into the immunoprecipitated fusion protein with 14 +/- 8 nM 8-OH-DPAT (mean +/- SEM, n = 3) (**Figure 5.12 a** and **b, Table 5.4**).

When labelling of the 5-$HT_{1A}Cys^{420}Ser$-receptor-$G_{o1}\alpha Cys^{3}Ser$, $Cys^{351}Ile$ **($C^{420}S$, $C^{3}S$)** fusion protein fusion protein was carried out using the same protocol, the agonist 8-OH-DPAT caused a half-maximal increase in levels of [^{3}H] palmitate incorporated into the immunoprecipitated fusion protein with 12 +/- 7 nM 8-OH-DPAT (mean +/- SEM, n = 3) (**Figure 5.13 a** and **b, Table 5.4**).

We can therefore conclude that 8-OH-DPAT was capable of producing concentration-dependent regulation of palmitoylation of both the GPCR and G protein parts of the 5-HT_{1A}-receptor-$G_{o1}\alpha Cys^{351}Ile$ fusion proteins with a statistically similar ($p>0.05$) EC_{50} of ~10-60nM.

Analysis of the incorporation of [^{3}H] palmitic acid into endogenously expressed $G_{o1}\alpha$ proteins

In order to assess how agonist-regulated G protein palmitoylation results obtained using the 5-$HT_{1A}Cys^{417}Ser$, $Cys^{420}Ser$-receptor-$G_{o1}\alpha Cys^{351}Ile$ (**$C^{417}S$, $C^{420}S$**) fusion protein compared with those for the non-fused G protein, we examined the effects of 8-OH-DPAT on the palmitoylation status of the small amount of $G_{o1}\alpha$ that is expressed endogenously in HEK293 cells. It has previously been noted that when expressed at high levels the receptor element of GPCR-G protein fusions can activate endogenous G proteins as well as the G protein fused to it (Burt *et al.*, 1998). HEK293T cells were transfected with the 5-HT_{1A}-receptor-$G_{o1}\alpha Cys^{351}Ile$ (**WT**) fusion protein and incubated with [^{3}H] palmitate in the absence or presence of 8-OH-DPAT. After cell lysate production, immunoprecipitation with antiserum ON1, SDS-PAGE and autoradiography, the incorporation of [^{3}H] palmitate into a band with apparent molecular mass of some 40 kDa (corresponding to endogenous $G_{o1}\alpha$) was assessed (**Figure 5.14**, upper panel). Expression levels of endogenous $G_{o1}\alpha$ were also assessed by parallel immunoblots with antiserum ON1 (**Figure 5.14**, lower panel). The endogenous $G_{o1}\alpha$ incorporated [^{3}H] palmitate in a time-dependent manner but in contrast to the fused $G_{o1}\alpha$, the presence of 100μM 8-OH-DPAT enhanced labelling (**Figure 5.14**, upper panel), suggesting some inherent difference for the receptor-linked G protein.

Analysis of the ability of the 5-$HT_{1A}$$Cys^{417}$Ser, Cys^{420}Ser-receptor-$G_{o1}\alpha$$Cys^{351}$Ile ($C^{417}S$, $C^{420}S$) fusion protein to be repalmitoylated

When the agonist-promoted regulation of palmitoylation is compared for GPCR-G protein fusion proteins and their non-fused components some differences have been observed (current study and Loisel *et al.*, 1999). As explained previously, in the work of Loisel *et al.* (1999), the differences in agonist-promoted palmitate regulation observed for the β_2-adrenoceptor-$G_s\alpha$ fusion protein as opposed to the non-fused GPCR and G protein were thought to be as a result of the inability of the fusion protein to be repalmitoylated. For comparison with the findings of Loisel *et al.* (1999), the ability of the 5-$HT_{1A}$$Cys^{417}$Ser, Cys^{420}Ser-receptor-$G_{o1}\alpha$$Cys^{351}$Ile (**$C^{417}S$, $C^{420}S$**) fusion protein to be repalmitoylated was assessed. HEK293T cells were transfected with the 5-$HT_{1A}$$Cys^{417}$Ser, Cys^{420}Ser-receptor-$G_{o1}\alpha$$Cys^{351}$Ile (**$C^{417}S$, $C^{420}S$**) fusion protein and incubated for 30 min with [^{3}H] palmitate in the absence of agonist (to allow approximately steady-state levels of [^{3}H] palmitate incorporation to be reached). Subsequent to this, cells were incubated with [^{3}H] palmitate in the absence or presence of 100μM 8-OH-DPAT. After cell lysate production, immunoprecipitation with antiserum ON1, SDS-PAGE and autoradiography, the incorporation of [^{3}H] palmitate into a band of some 85kDa was monitored (**Figure 5.15 a** and **b**). In the absence of agonist the levels of [^{3}H] palmitate incorporated into the 5-$HT_{1A}$$Cys^{417}$Ser,$Cys^{420}$Ser-receptor-$G_{o1}\alpha$$Cys^{351}$Ile (**$C^{417}S$, $C^{420}S$**) fusion protein did not decrease with increasing time (**Figure 5.15 a** lanes 1, 2 and 4). In contrast, in the presence of 100μM 8-OH-DPAT (**Figure 5.15 a** lanes 3 and 5) the levels of [^{3}H] palmitate incorporated into the 5-$HT_{1A}$$Cys^{417}$Ser, Cys^{420}Ser-receptor-$G_{o1}\alpha$$Cys^{351}$Ile (**$C^{417}S$, $C^{420}S$**) fusion protein were markedly reduced ($p<0.05$). These results are interpreted as follows. In the presence of 8-OH-DPAT, depalmitoylation of the 5-$HT_{1A}$$Cys^{417}$Ser, Cys^{420}Ser-receptor-$G_{o1}\alpha$$Cys^{351}$Ile (**$C^{417}S$, $C^{420}S$**) fusion occurs (as previously observed from our depalmitoylation studies) and subsequent repalmitoylation of this fusion is inhibited (as reflected by the decrease in overall [^{3}H] palmitate incorporated into this protein).

Figure 5.1

Analysis of expression levels of a 5-HT_{1A}-receptor-$G_{o1}\alpha Cys^{351}$Ile fusion protein after sub-culture into multiple duplicate culture plates (determined from binding of a near-saturating concentration (~5nM) of [^{3}H]-WAY100635 to membranes expressing the 5-HT_{1A}-receptor-$G_{o1}\alpha Cys^{351}$Ile fusion protein)

HEK293T cells were transfected to express 5-HT_{1A}-receptor-$G_{o1}\alpha Cys^{351}$Ile fusion protein. Membranes were prepared and binding of a near-saturating concentration (~5nM) of [^{3}H]-WAY100635 to membranes expressing the 5-HT_{1A}-receptor-$G_{o1}\alpha Cys^{351}$Ile fusion protein was assessed for multiple duplicate culture plates (labelled 1-6). Results are from triplicate determinations. Analysis is representative of three similar experiments.

Figure 5.1

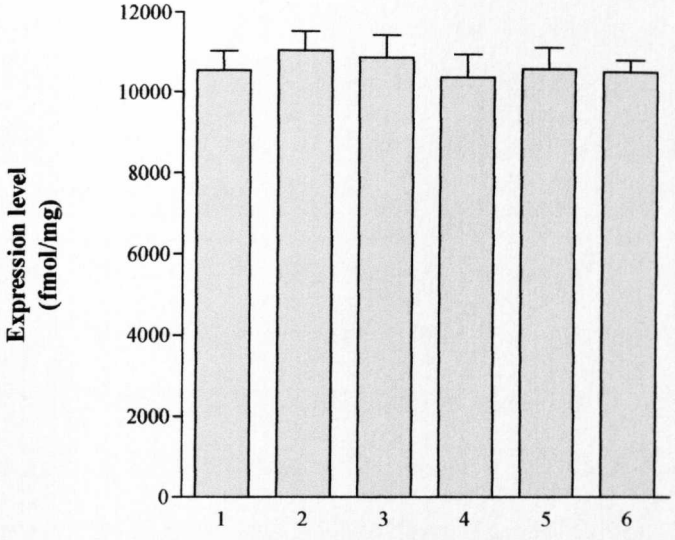

Expression level
(fmol/mg)
12000
10000
8000
6000
4000
2000
0
1
2
3
4
5
6

Figure 5.2

Incorporation of [^{3}H] palmitate into the palmitoylation-variant 5-HT$_{1A}$-receptor-G$_{o1}\alpha$Cys^{351}Ile fusion proteins in the presence and absence of 8-OH-DPAT.

HEK293T cells were transfected with empty vector (**pcDNA3, lanes 1 and 10**) or to express 5-HT$_{1A}$-receptor-G$_{o1}\alpha$Cys^{351}Ile (**WT,** lanes **2** and **11**), 5-HT$_{1A}$Cys^{417}Ser-receptor-G$_{o1}\alpha$Cys^{351}Ile (**C^{417}S,** lanes **3** and **12**), 5-HT$_{1A}$Cys^{417}Ser-receptor-G$_{o1}\alpha$Cys^{3}Ser,Cys^{351}Ile (**C^{417}S, C^{3}S,** lanes **4** and **13**), 5-HT$_{1A}$Cys^{420}Ser-receptor-G$_{o1}\alpha$Cys^{351}Ile (**C^{420}S,** lanes **5** and **14**), 5-HT$_{1A}$Cys^{420}Ser-receptor-G$_{o1}\alpha$Cys^{3}Ser,Cys^{351}Ile (**C^{420}S, C^{3}S,** lanes **6** and **15**), 5-HT$_{1A}$Cys^{417}Ser, Cys^{420}Ser-receptor-G$_{o1}\alpha$Cys^{351}Ile (**C^{417}S, C^{420}S,** lanes **7** and **16**), 5-HT$_{1A}$-receptor-G$_{o1}\alpha$Cys^{3}Ser,Cys^{351}Ile (**C^{3}S,** lanes **8** and **17**), and 5-HT$_{1A}$Cys^{417}Ser, Cys^{420}Ser-receptor-G$_{o1}\alpha$Cys^{3}Ser,Cys^{351}Ile (**C^{417}S, C^{420}S, C^{3}S,** lanes **9** and **18**) fusion proteins. Cells were incubated with [^{3}H] palmitate for 30 min in the absence (lanes **1-9**) or presence (lanes **10-18**) of 100μM 8-OH-DPAT. Samples were harvested and cell lysates produced. These were either immunoprecipitated with antiserum ON1 prior to SDS-PAGE and autoradiography for 1 month (**a**, upper panels) or resolved directly by SDS-PAGE and immunoblotted with antiserum ON1 (**a**, lower panels). **Figure 5.2 (a)** shows one representative palmitoylation experiment with corresponding western blot analysis. Similar results were obtained for three separate experiments.

Autoradiographs as in the upper panels of **a** were scanned and signals quantitated (**b**) in the area of the film shown. Bars 1-9 = absence, bars 10-18= presence of 8-OH-DPAT. **WT** = turquoise bars, **C^{417}S** = green bars, **C^{417}S, C^{3}S** = purple bars, **C^{420}S** = brown bars, **C^{420}S, C^{3}S** = red bars, **C^{417}S, C^{420}S** = yellow bars, **C^{3}S** = blue bars and **C^{417}S, C^{420}S, C^{3}S** = pink bars. Results for three separate experiments were quantified and data is shown as mean +/- S.E.M., n= 3. In order to compare levels of incorporation from separate experiments it was necessary to express the levels of incorporation for each sample as a percentage of the incorporation observed for the unstimulated **WT** construct.

Figure 5.2


a)
1 2 3 4 5 6 7 8 9
10 11 12 13 14 15 16 17 18
M_r (x10^{-3})
105
75
105
75

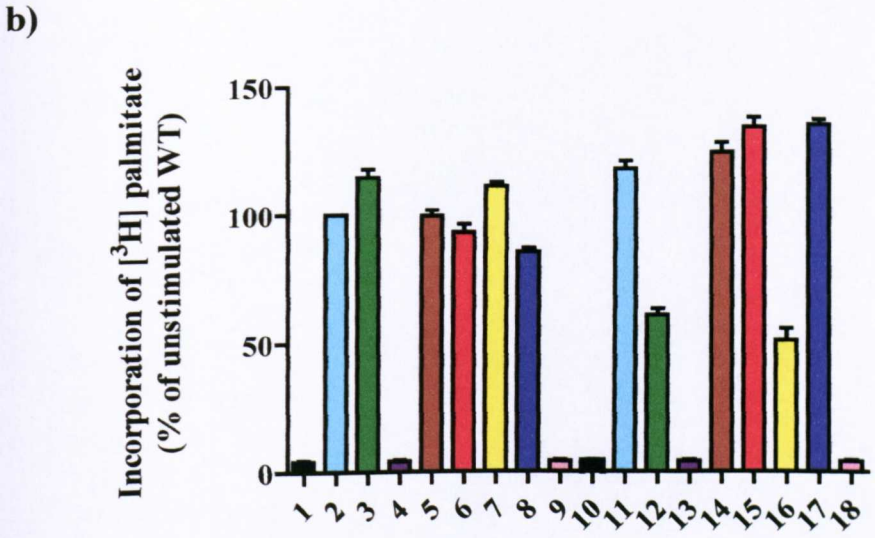

b)
Incorporation of [^{3}H] palmitate
(% of unstimulated WT)
150
100
50
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

Table 5.1

Comparison of the incorporation of [^{3}H] palmitate into all eight palmitoylation-variant fusion proteins in the presence and absence of 8-OH-DPAT

The results from **Figures 5.2** are presented in a tabular form for clarity of comparison between the constructs. Incorporation level into each 5-HT_{1A}-receptor-$G_{o1}\alpha Cys^{351}Ile$ fusion protein in the presence and absence of 8-OH-DPAT is given as a percentage (mean +/- SEM, n=3) of the incorporation observed for the unstimulated **WT** construct.

Table 5.1

Construct	Potential Site of [^{3}H] Palmitate Incorporation	Incorporation of [^{3}H] Palmitate in Absence of 8-OH-DPAT (%)	Incorporation of [^{3}H] Palmitate in Presence of 8-OH-DPAT (%)
5-HT_{1A}-receptor-$G_{o1}\alpha Cys^{351}Ile$ (**WT**)	GPCR $Cys^{417}$and Cys^{420} residues and G protein Cys^3 residue	100	118 +/- 5.3
5-$HT_{1A}Cys^{417}Ser$-receptor-$G_{o1}\alpha$ $Cys^{351}Ile$ (**$C^{417}S$**)	GPCR Cys^{420} residue and G protein Cys^3 residue	114.5 +/- 5.9	61.0 +/- 4.1
5-$HT_{1A}Cys^{417}Ser$-receptor-$G_{o1}\alpha Cys^3Ser$, $Cys^{351}Ile$ (**$C^{417}S$, C^3S**)	GPCR Cys^{420} residue	None	None
5-$HT_{1A}Cys^{420}Ser$-receptor-$G_{o1}\alpha$ $Cys^{351}Ile$ (**$C^{420}S$**)	GPCR Cys^{417} residue and G protein Cys^3 residue	99.6 +/- 3.8	124 +/- 6.7
5-$HT_{1A}Cys^{420}Ser$-receptor-$G_{o1}\alpha Cys^3Ser$, $Cys^{351}Ile$ (**$C^{420}S$, C^3S**)	GPCR Cys^{417} residue	93.2 +/- 5.4	134.0 +/- 6.6
5-$HT_{1A}Cys^{417}Ser$, $Cys^{420}Ser$-receptor-$G_{o1}\alpha Cys^{351}Ile$ (**$C^{417}S$, $C^{420}S$**)	G protein Cys^3 residue	111.2 +/- 2.6	50.9 +/- 8.2
5-HT_{1A}-receptor - $G_{o1}\alpha Cys^3Ser$, $Cys^{351}Ile$ (**Cys^3Ser**)	GPCR $Cys^{417}$and Cys^{420} residues	85.8 +/- 2.6	135.1 +/- 3.5
5-$HT_{1A}Cys^{417}Ser$, $Cys^{420}Ser$-receptor-$G_{o1}\alpha$ Cys^3Ser, $Cys^{351}Ile$ (**$C^{417}S$ $C^{420}S$, C^3S**)	None	None	None

Figure 5.3

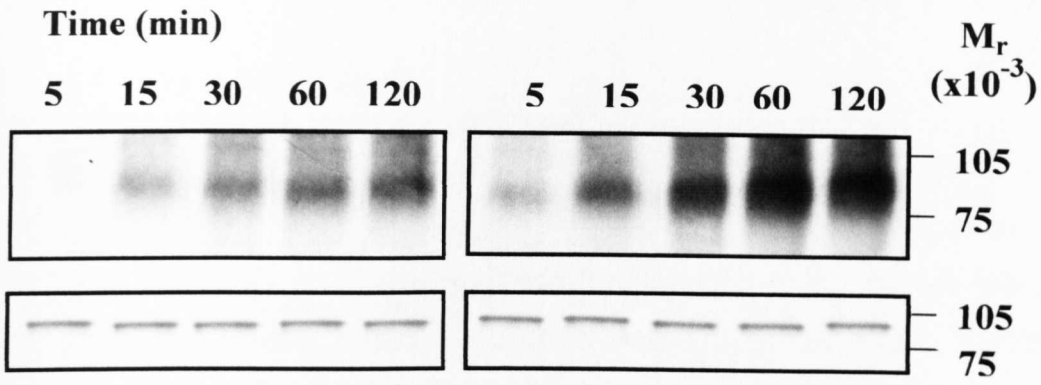
Incorporation of [^{3}H] palmitate into the 5-HT$_{1A}$-receptor-G$_{o1}\alpha$Cys^{351}Ile (WT) fusion protein in the presence and absence of 8-OH-DPAT.

A 5-HT$_{1A}$-receptor-G$_{o1}\alpha$Cys^{351}Ile (**WT**) fusion protein was expressed in HEK293T cells. Cells were incubated with [^{3}H] palmitate for the indicated times in the absence (left panels) or presence (right panels) of 100μM 8-OH-DPAT. Samples were harvested and cell lysates produced. These were either immunoprecipitated with antiserum ON1 prior to SDS-PAGE and autoradiography for 1 month (**a**, upper panels) or resolved directly by SDS-PAGE and immunoblotted with antiserum ON1 (**a**, lower panels). **Figure 5.3** (**a**) shows one representative palmitoylation experiment with corresponding western blot analysis. Similar results were obtained for three separate experiments.

Autoradiographs as in the upper panels of **a** were scanned and signals quantitated (**b**) in the area of the film shown. Open circles = absence, filled circles = presence of 8-OH-DPAT. Results for three separate experiments were quantified and data is shown as mean +/- S.E.M., n= 3. In order to compare levels of incorporation from separate experiments it was necessary to express the levels of incorporation for each sample as a percentage of the, near maximal, level of incorporation for one specific timepoint (as in **Chapter 3** pulse-labelling studies, this was consistently taken as the 60 min time-point of the unstimulated construct).

Figure 5.3

a)


Time (min)
5
15
30
60
120
5
15
30
60
120
M_r ($x10^{-3}$)
105
75
105
75

b)

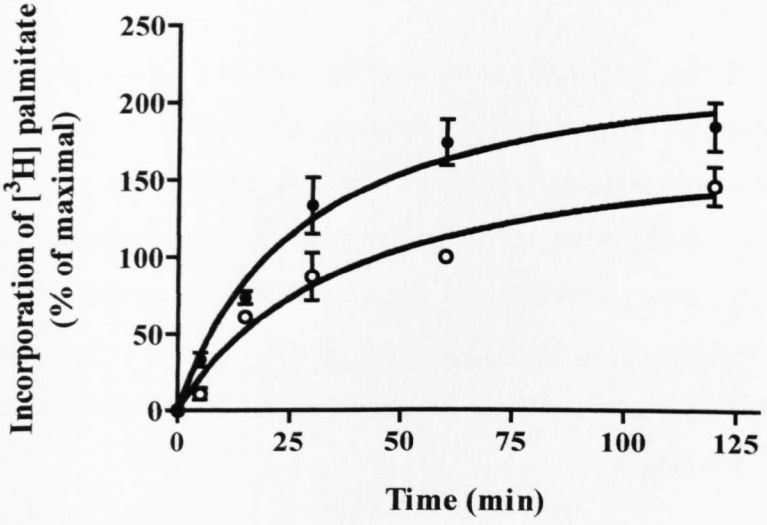

Incorporation of [3H] palmitate (% of maximal)
250
200
150
100
50
0
0
25
50
75
100
125
Time (min)

Figure 5.4

Incorporation of [^{3}H] palmitate into the 5-HT$_{1A}$Cys^{417}Ser, Cys^{420}Ser-receptor-G$_{o1}\alpha$Cys^{351}Ile (C^{417}S, C^{420}S) fusion protein in the presence and absence of 8-OH-DPAT.

A 5-HT$_{1A}$Cys^{417}Ser, Cys^{420}Ser-receptor-G$_{o1}\alpha$Cys^{351}Ile (**C^{417}S, C^{420}S**) fusion protein was expressed in HEK293T cells. Cells were incubated with [^{3}H] palmitate for the indicated times in the absence (left panels) or presence (right panels) of 100μM 8-OH-DPAT. Samples were harvested and cell lysates produced. These were either immunoprecipitated with antiserum ON1 prior to SDS-PAGE and autoradiography for 1 month (**a**, upper panels) or resolved directly by SDS-PAGE and immunoblotted with antiserum ON1 (**a**, lower panels). **Figure 5.4 (a)** shows one representative palmitoylation experiment with corresponding western blot analysis. Similar results were obtained for three separate experiments.

Autoradiographs as in the upper panels of **a** were scanned and signals quantitated **(b)** in the area of the film shown. Open circles = absence, filled circles = presence of 8-OH-DPAT. Results for three separate experiments were quantified and data is shown as mean +/- S.E.M., n= 3. In order to compare levels of incorporation from separate experiments it was necessary to express the levels of incorporation for each sample as a percentage of the, near maximal, level of incorporation for one specific timepoint (as in **Chapter 3** pulse-labelling studies, this was consistently taken as the 60 min time-point of the unstimulated construct).

Figure 5.4

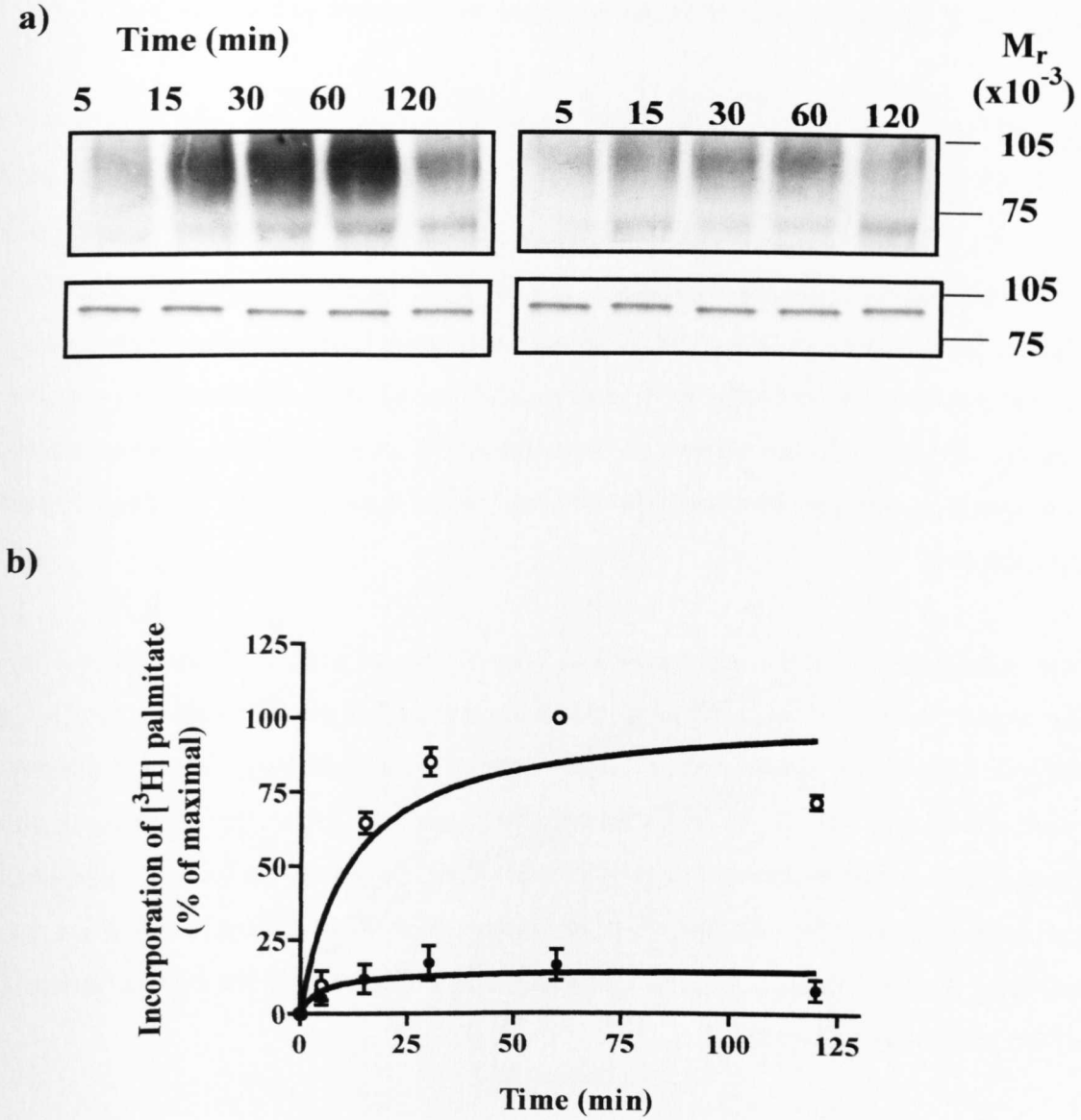

a)
Time (min)
5 15 30 60 120
5 15 30 60 120
M_r ($x10^{-3}$)
105
75
105
75
b)
Incorporation of [^{3}H] palmitate (% of maximal)
125
100
75
50
25
0
0 25 50 75 100 125
Time (min)

Figure 5.5

Incorporation of [^{3}H] palmitate into the 5-HT$_{1A}$-receptor-G$_{o1}\alpha$Cys^{3}Ser, Cys^{351}Ile (C^{3}S) fusion protein in the presence and absence of 8-OH-DPAT.

A 5-HT$_{1A}$-receptor-G$_{o1}\alpha$Cys^{3}Ser,Cys^{351}Ile (**C^{3}S**) fusion protein was expressed in HEK293T cells. Cells were incubated with [^{3}H] palmitate for the indicated times in the absence (left panels) or presence (right panels) of 100μM 8-OH-DPAT. Samples were harvested and cell lysates produced. These were either immunoprecipitated with antiserum ON1 prior to SDS-PAGE and autoradiography for 1 month (**a**, upper panels) or resolved directly by SDS-PAGE and immunoblotted with antiserum ON1 (**a**, lower panels). **Figure 5.5** (**a**) shows one representative palmitoylation experiment with corresponding western blot analysis. Similar results were obtained for three separate experiments.

Autoradiographs as in the upper panels of **a** were scanned and signals quantitated (**b**) in the area of the film shown. Open circles = absence, filled circles = presence of 8-OH-DPAT. Results for three separate experiments were quantified and data is shown as mean +/- S.E.M., n= 3. In order to compare levels of incorporation from separate experiments it was necessary to express the levels of incorporation for each sample as a percentage of the, near maximal, level of incorporation for one specific timepoint (as in **Chapter 3** pulse-labelling studies, this was consistently taken as the 60 min time-point of the unstimulated construct).

Figure 5.5

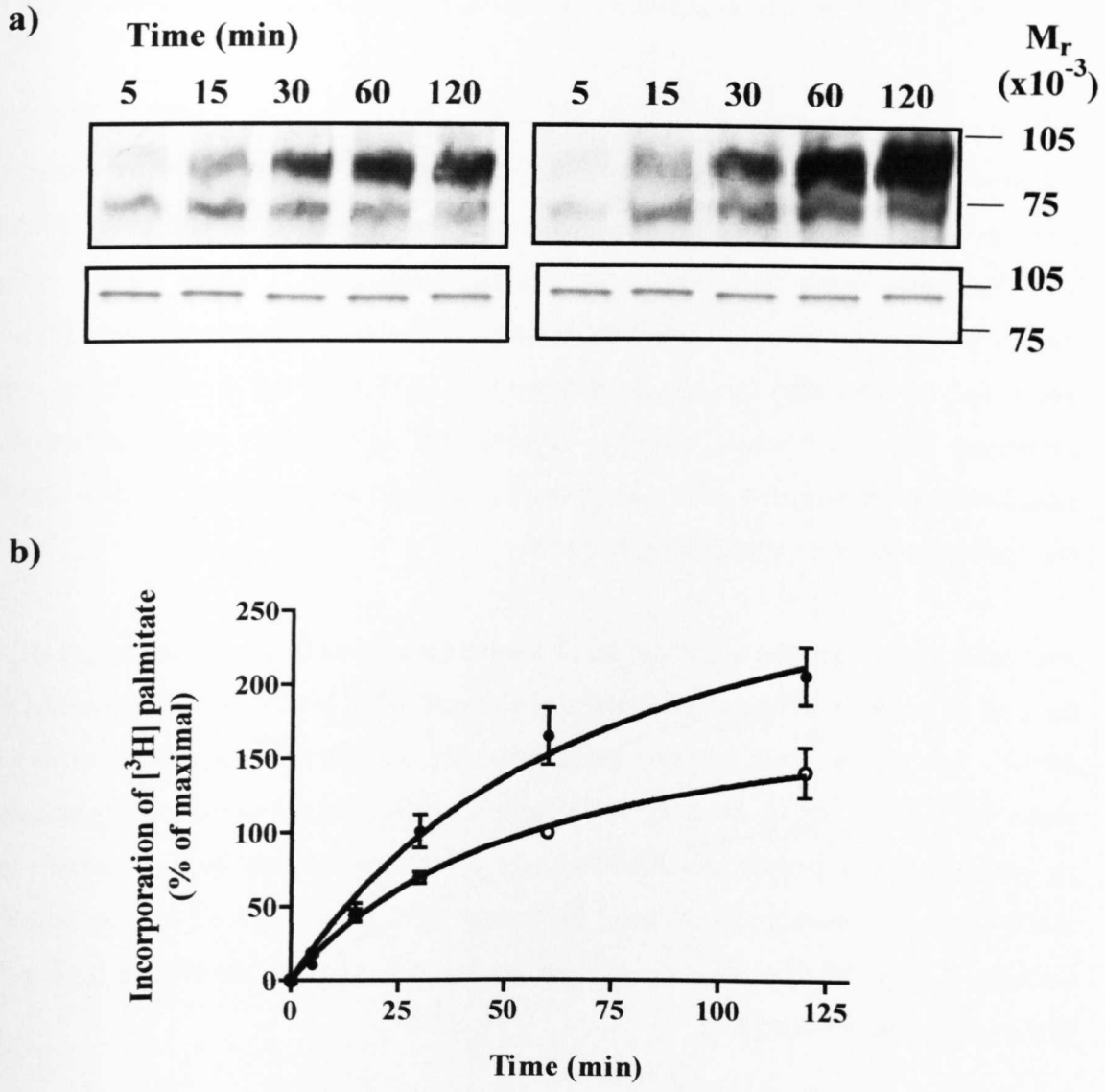

a)
Time (min)
5
15
30
60
120
5
15
30
60
120
M_r ($x10^{-3}$)
105
75
105
75
b)
Incorporation of [^{3}H] palmitate (% of maximal)
250
200
150
100
50
0
0
25
50
75
100
125
Time (min)

Figure 5.6

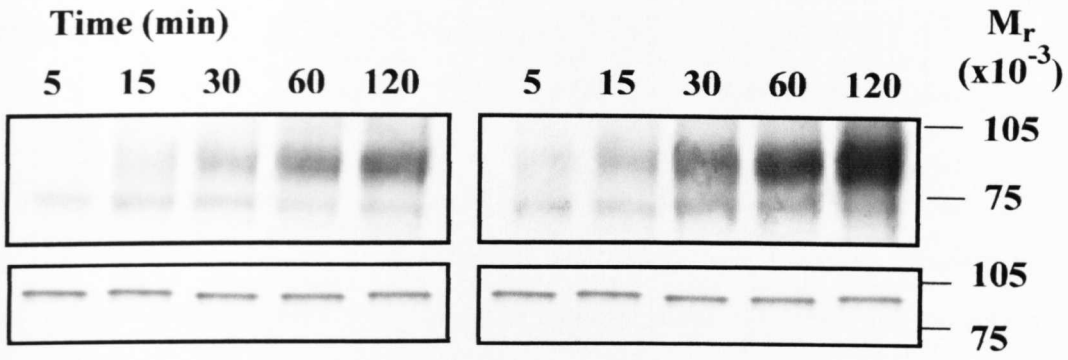
Incorporation of [^{3}H] palmitate into the 5-HT$_{1A}$Cys^{420}Ser-receptor-G$_{o1}\alpha$Cys^{3}Ser, Cys^{351}Ile (C^{420}S, C^{3}S) fusion protein in the presence and absence of 8-OH-DPAT.

A 5-HT$_{1A}$Cys^{420}Ser-receptor-G$_{o1}\alpha$Cys^{3}Ser, Cys^{351}Ile (**C^{420}S, C^{3}S**) fusion protein was expressed in HEK293T cells. Cells were incubated with [^{3}H] palmitate for the indicated times in the absence (left panels) or presence (right panels) of 100μM 8-OH-DPAT. Samples were harvested and cell lysates produced. These were either immunoprecipitated with antiserum ON1 prior to SDS-PAGE and autoradiography for 1 month (**a**, upper panels) or resolved directly by SDS-PAGE and immunoblotted with antiserum ON1 (**a**, lower panels). **Figure 5.6** (**a**) shows one representative palmitoylation experiment with corresponding western blot analysis. Similar results were obtained for three separate experiments.

Autoradiographs as in the upper panels of **a** were scanned and signals quantitated (**b**) in the area of the film shown. Open circles = absence, filled circles = presence of 8-OH-DPAT. Results for three separate experiments were quantified and data is shown as mean +/- S.E.M., n= 3. In order to compare levels of incorporation from separate experiments it was necessary to express the levels of incorporation for each sample as a percentage of the, near maximal, level of incorporation for one specific timepoint (as in **Chapter 3** pulse-labelling studies, this was consistently taken as the 60 min time-point of the unstimulated construct).

Figure 5.6

a)


Time (min)
5
15
30
60
120
5
15
30
60
120
M_r ($x10^{-3}$)
105
75
105
75

b)

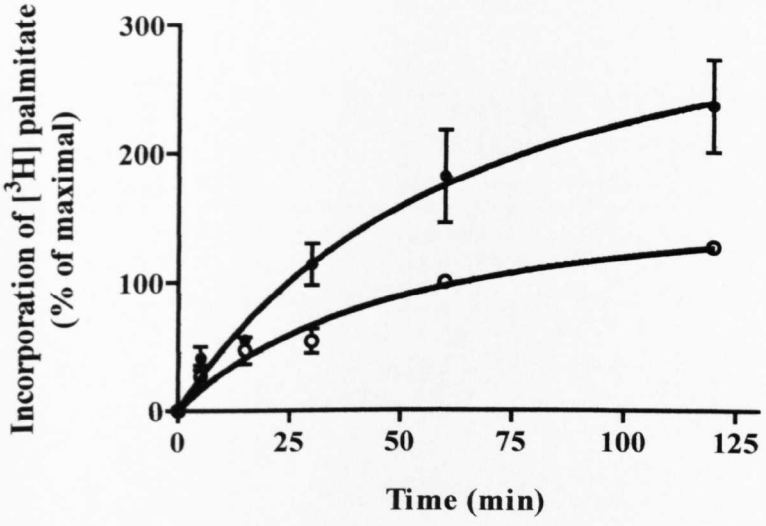

Incorporation of [^{3}H] palmitate (% of maximal)
300
200
100
0
0
25
50
75
100
125
Time (min)

Table 5.2

Comparison of the incorporation of [^{3}H] palmitate results from Figure 5.3-5.6 for the four selected palmitoylation-variant fusion proteins (Figure 5.3: WT, Figure 5.4: $C^{417}S$, $C^{420}S$, Figure 5.5: C^3S, and Figure 5.6: $C^{420}S$, C^3S) in the presence and absence of 8-OH-DPAT

The results from **Figures 5.3-5.6** were presented in a tabular form for clarity of comparison between the constructs. Non-linear regression analysis was used to determine the maximal incorporation level of [^{3}H] palmitate and the $t_{1/2}$ (min) for incorporation into each 5-HT_{1A}-receptor-$G_{o1}\alpha Cys^{351}$Ile fusion protein in the presence and absence of 8-OH-DPAT.

Table 5.2

Construct	Potential Site of [^{3}H] Palmitate Incorp.	Max Palmitate Incorp. - 8-OH-DPAT (%)	$t_{1/2}$ Palmitate Incorp. - 8-OH-DPAT (min)	Max Palmitate Incorp. + 8-OH-DPAT (%)	$t_{1/2}$ Palmitate Incorp. + 8-OH-DPAT (min)
WT	GPCR $Cys^{417}$and Cys^{420} residues and G protein Cys^3 residue	184.2 +/- 20.6	37.8 +/- 10.5	236.7 +/- 20.6	26.9 +/- 6.5
$C^{417}S$, $C^{420}S$	G protein Cys^3 residue	100.8 +/- 10.6	11.3 +/- 4.6	14.6 +/- 3.6	4.4 +/- 5.6
C^3S	GPCR $Cys^{417}$and Cys^{420} residues	206.4 +/- 23.7	59.2 +/- 14.4	345.5 +/- 54.4	75.8 +/- 23.2
$C^{420}S$, C^3S	GPCR Cys^{417} residue	179.5 +/- 22.5	50.9 +/- 14.1	374.1 +/- 79.9	67.8 +/- 29.2

Figure 5.7

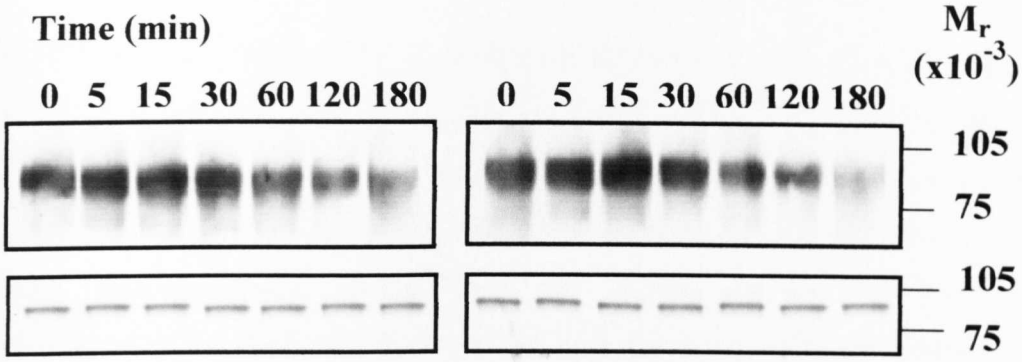
Basal and 8-OH-DPAT-stimulated de-palmitoylation of a 5-HT_{1A}-receptor-$G_{o1}\alpha Cys^{351}Ile$ (WT) fusion protein

HEK293T cells were transfected to express a 5-HT_{1A}-receptor-$G_{o1}\alpha Cys^{351}Ile$ **(WT)** fusion protein. Cells were incubated with [^{3}H] palmitate for 30 min, washed and then excess non-radioactive palmitate was added in the absence (**a**, left panels) or presence (**a**, right panels) of 8-OH-DPAT (100μM). Samples were harvested at varying times and cell lysates produced that were either immunoprecipitated with antiserum ON1 prior to SDS-PAGE and autoradiography for 1 month (**a**, upper panels) or resolved directly by SDS-PAGE and immunoblotted with antiserum ON1 (**a**, lower panels). **Figure 5.7** (**a**) shows one representative depalmitoylation experiment with corresponding western blot analysis. Similar results were obtained for three separate experiments.

Autoradiographs as in the upper panels of **a** were scanned and signals quantitated **(b)** in the area of the film shown. Open circles = absence, filled circles = presence of 8-OH-DPAT. Results for three separate experiments were quantified and data is shown as mean +/- S.E.M., n= 3. In order to compare remaining levels of [^{3}H] palmitate from separate experiments it was necessary to express the remaining levels of [^{3}H] palmitate for each sample as a percentage of the maximal level observed (0 min chase time).

Figure 5.7

a)


Time (min)
0 5 15 30 60 120 180
0 5 15 30 60 120 180
M_r ($x10^{-3}$)
105
75
105
75

b)

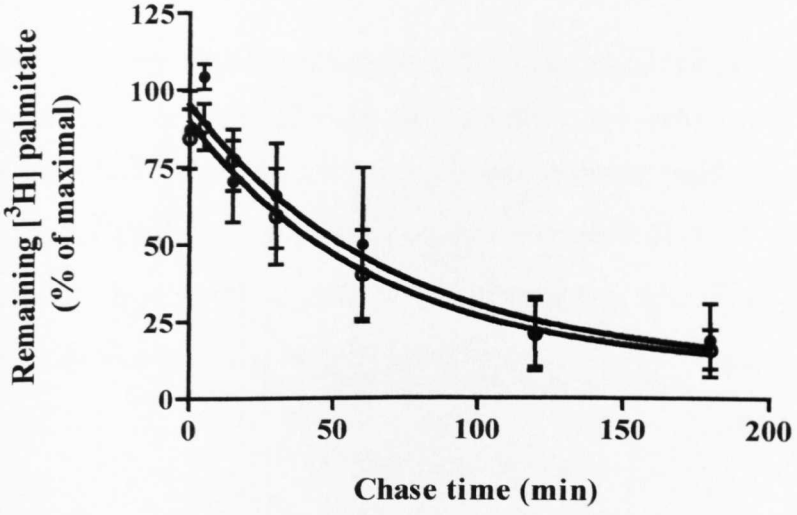

Remaining [^{3}H] palmitate (% of maximal)
125
100
75
50
25
0
0
50
100
150
200
Chase time (min)

Figure 5.8

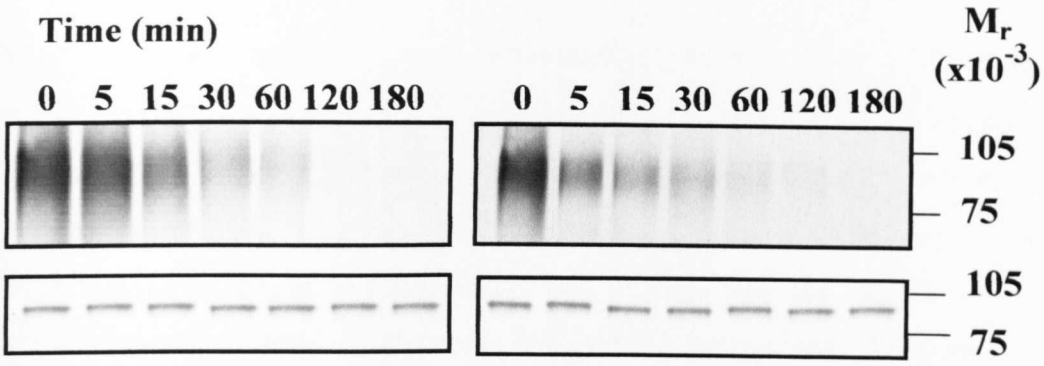
Basal and 8-OH-DPAT-stimulated de-palmitoylation of a 5-$HT_{1A}$$Cys^{417}$Ser, Cys^{420}Ser-receptor-$G_{o1}\alpha Cys^{351}$Ile ($C^{417}S$, $C^{420}S$) fusion protein

HEK293T cells were transfected to express a 5-$HT_{1A}$$Cys^{417}$Ser, Cys^{420}Ser-receptor-$G_{o1}\alpha Cys^{351}$Ile (**$C^{417}S$, $C^{420}S$**) fusion protein. Cells were incubated with [^{3}H] palmitate for 30 min, washed and then excess non-radioactive palmitate was added in the absence (**a**, left panels) or presence (**a**, right panels) of 8-OH-DPAT (100μM). Samples were harvested at varying times and cell lysates produced that were either immunoprecipitated with antiserum ON1 prior to SDS-PAGE and autoradiography for 1 month (**a**, upper panels) or resolved directly by SDS-PAGE and immunoblotted with antiserum ON1 (**a**, lower panels). **Figure 5.8** (**a**) shows one representative depalmitoylation experiment with corresponding western blot analysis. Similar results were obtained for three separate experiments.

Autoradiographs as in the upper panels of **a** were scanned and signals quantitated (**b**) in the area of the film shown. Open circles = absence, filled circles = presence of 8-OH-DPAT. Results for three separate experiments were quantified and data is shown as mean +/- S.E.M., n= 3. In order to compare remaining levels of [^{3}H] palmitate from separate experiments it was necessary to express the remaining levels of [^{3}H] palmitate for each sample as a percentage of the maximal level observed (0 min chase time).

Figure 5.8

a)


Time (min)
0 5 15 30 60 120 180
0 5 15 30 60 120 180
M_r ($x10^{-3}$)
105
75
105
75

b)

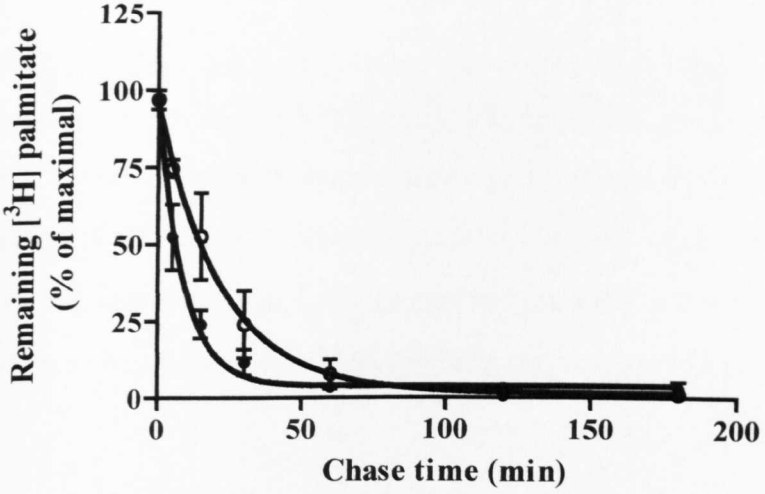

Remaining [^{3}H] palmitate (% of maximal)
125
100
75
50
25
0
0
50
100
150
200
Chase time (min)

Figure 5.9

Lack of basal and 8-OH-DPAT-stimulated de-palmitoylation of a 5-HT_{1A}-receptor-$G_{o1}\alpha Cys^{3}Ser,Cys^{351}Ile$ ($C^{3}S$) fusion protein

HEK293T cells were transfected to express a 5-HT_{1A}-receptor-$G_{o1}\alpha Cys^{3}Ser,Cys^{351}Ile$ (**$C^{3}S$**) fusion protein. Cells were incubated with [^{3}H] palmitate for 30 min, washed and then excess non-radioactive palmitate was added in the absence (**a**, left panels) or presence (**a**, right panels) of 8-OH-DPAT (100μM). Samples were harvested at varying times and cell lysates produced that were either immunoprecipitated with antiserum ON1 prior to SDS-PAGE and autoradiography for 1 month (**a**, upper panels) or resolved directly by SDS-PAGE and immunoblotted with antiserum ON1 (**a**, lower panels). **Figure 5.9 (a)** shows one representative depalmitoylation experiment with corresponding western blot analysis. Similar results were obtained for three separate experiments.

Autoradiographs as in the upper panels of **a** were scanned and signals quantitated **(b)** in the area of the film shown. Open circles = absence, filled circles = presence of 8-OH-DPAT. Results for three separate experiments were quantified and data is shown as mean +/- S.E.M., n= 3. In order to compare remaining levels of [^{3}H] palmitate from separate experiments it was necessary to express the remaining levels of [^{3}H] palmitate for each sample as a percentage of the maximal level observed (0 min chase time).

Figure 5.9

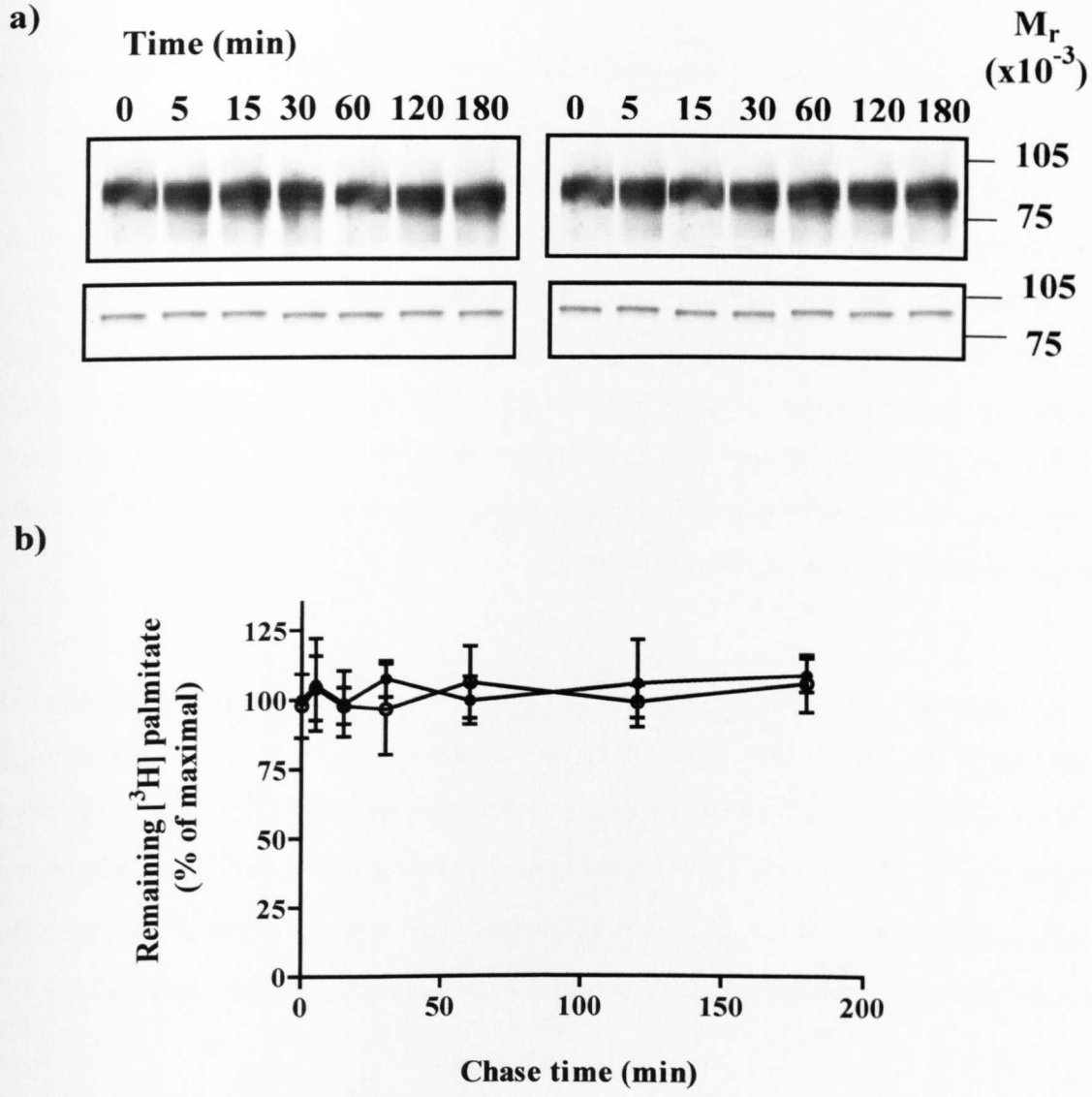

a)
Time (min)
0 5 15 30 60 120 180
0 5 15 30 60 120 180
M_r ($x10^{-3}$)
105
75
105
75
b)
Remaining [^{3}H] palmitate (% of maximal)
125
100
75
50
25
0
0
50
100
150
200
Chase time (min)

Figure 5.10

Lack of basal and 8-OH-DPAT-stimulated de-palmitoylation of a 5-$HT_{1A}Cys^{420}Ser$-receptor-$G_{o1}\alpha Cys^{3}Ser,Cys^{351}Ile$ ($C^{420}S$, $C^{3}S$) fusion protein

HEK293T cells were transfected to express a 5-$HT_{1A}Cys^{420}Ser$-receptor-$G_{o1}\alpha Cys^{3}Ser,Cys^{351}Ile$ (**$C^{420}S$, $C^{3}S$**) fusion protein. Cells were incubated with [^{3}H] palmitate for 30 min, washed and then excess non-radioactive palmitate was added in the absence (**a**, left panels) or presence (**a**, right panels) of 8-OH-DPAT (100μM). Samples were harvested at varying times and cell lysates produced that were either immunoprecipitated with antiserum ON1 prior to SDS-PAGE and autoradiography for 1 month (**a**, upper panels) or resolved directly by SDS-PAGE and immunoblotted with antiserum ON1 (**a**, lower panels). **Figure 5.10** (**a**) shows one representative depalmitoylation experiment with corresponding western blot analysis. Similar results were obtained for three separate experiments.

Autoradiographs as in the upper panels of **a** were scanned and signals quantitated (**b**) in the area of the film shown. Open circles = absence, filled circles = presence of 8-OH-DPAT. Results for three separate experiments were quantified and data is shown as mean +/- S.E.M., n= 3. In order to compare remaining levels of [^{3}H] palmitate from separate experiments it was necessary to express the remaining levels of [^{3}H] palmitate for each sample as a percentage of the maximal level observed (0 min chase time).

Figure 5.10

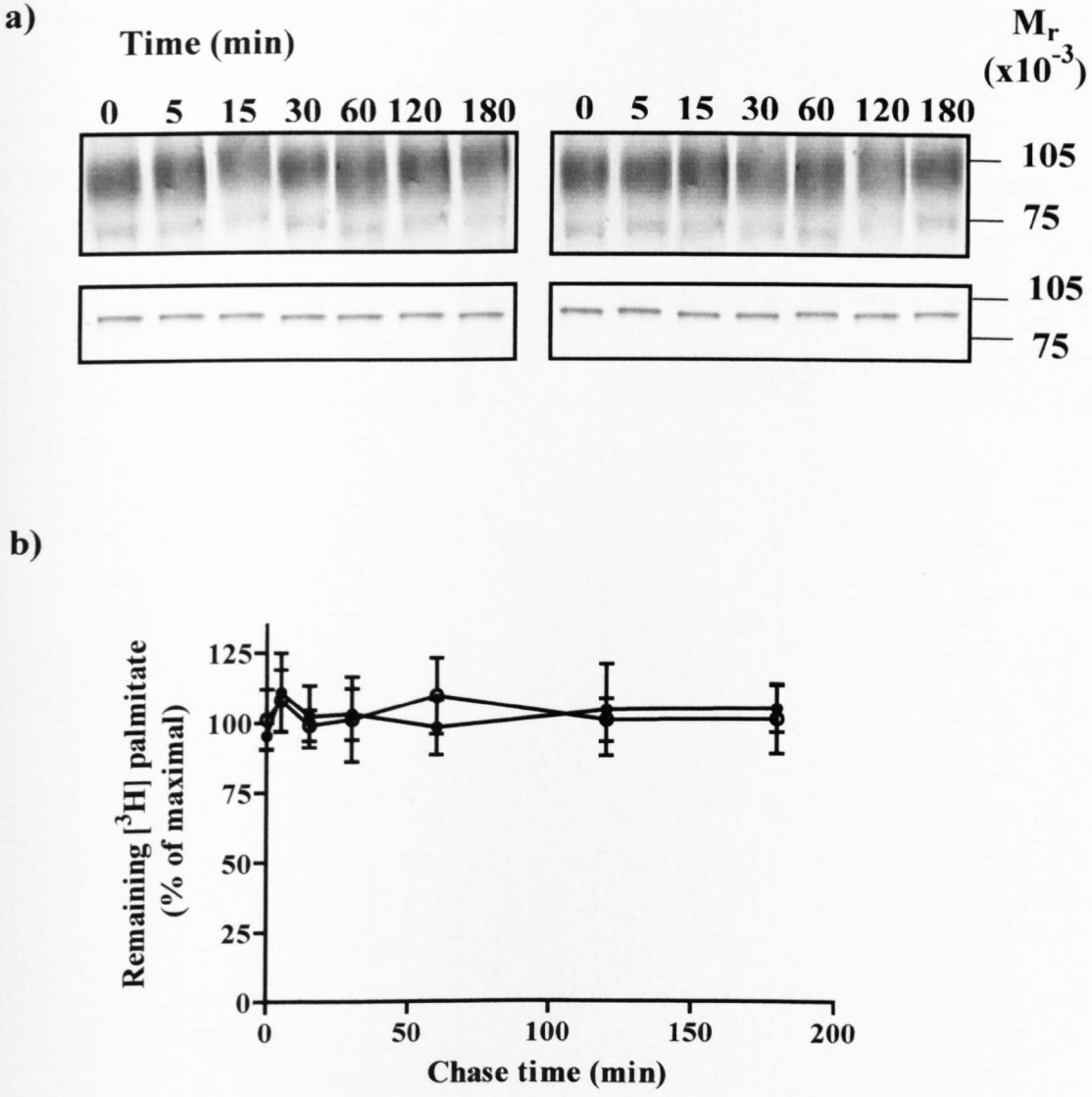

a)
Time (min)
0 5 15 30 60 120 180
0 5 15 30 60 120 180
M_r ($x10^{-3}$)
105
75
105
75
b)
Remaining [^{3}H] palmitate (% of maximal)
125
100
75
50
25
0
0
50
100
150
200
Chase time (min)

Table 5.3

Comparison of the depalmitoylation results from Figure 5.7-5.10 for the four selected palmitoylation-variant fusion proteins (Figure 5.7: WT, Figure 5.8: $C^{417}S$, $C^{420}S$, Figure 5.9: $C^{3}S$, and Figure 5.10: $C^{420}S$, $C^{3}S$) in the presence and absence of 8-OH-DPAT

The results from **Figures 5.7-5.10** were presented in a tabular form for clarity of comparison between the constructs. Non-linear regression analysis was used to determine the $t_{1/2}$ (min) for depalmitoylation of 5-HT_{1A}-receptor-$G_{o1}\alpha Cys^{351}$Ile fusion proteins (where depalmitoylation occurred) in the presence and absence of 8-OH-DPAT.

Table 5.3

Construct	Potential Site of [^{3}H] Palmitate Incorporation	$t_{1/2}$ (min) Depalmitoylation (- 8-OH-DPAT)	$t_{1/2}$ (min) Depalmitoylation (+ 8-OH-DPAT)
WT	GPCR $Cys^{417}$and Cys^{420} residues and G protein Cys^3 residue	45.5 +/- 15.6	48.4 +/- 23.5
$C^{417}S$, $C^{420}S$	G protein Cys^3 residue	15.3 +/- 6.5	6.3 +/- 2.4
C^3S	GPCR $Cys^{417}$and Cys^{420} residues	No depalmitoylation	No depalmitoylation
$C^{420}S$, C^3S	GPCR Cys^{417} residue	No depalmitoylation	No depalmitoylation

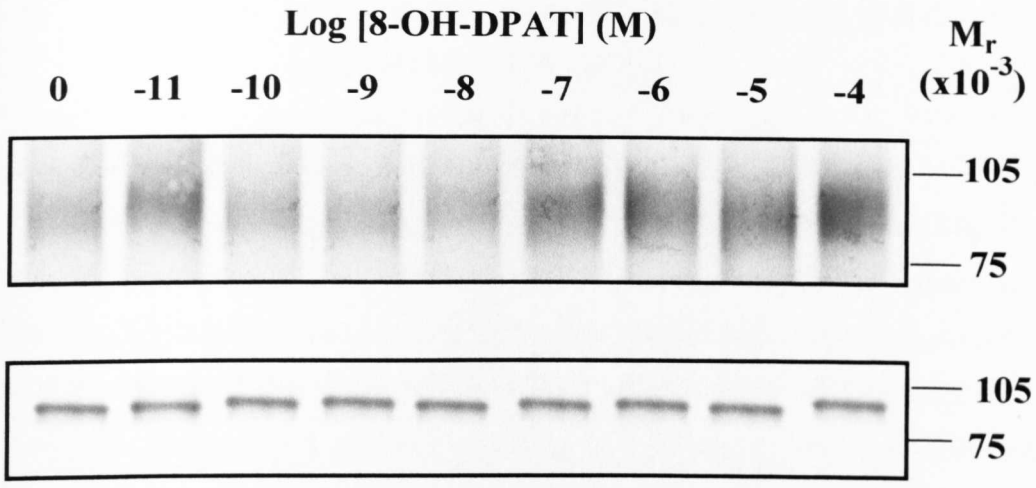
Figure 5.11

Concentration-response of 8-OH-DPAT regulated incorporation of [^{3}H] palmitate into the 5-HT$_{1A}$-receptor-G$_{o1}\alpha$Cys^{351}Ile (WT) fusion protein

A 5-HT$_{1A}$-receptor-G$_{o1}\alpha$Cys^{351}Ile (**WT**) fusion protein was expressed in HEK293T cells. Cells were incubated with [^{3}H] palmitate for 30 min in the presence of varying concentrations of 8-OH-DPAT. Samples were harvested and cell lysates produced. These were either immunoprecipitated with antiserum ON1 prior to SDS-PAGE and autoradiography for 1 month (**a**, upper panel) or resolved directly by SDS-PAGE and immunoblotted with antiserum ON1 (**a**, lower panel). **Figure 5.11 (a)** shows one representative concentration-response palmitoylation experiment with corresponding western blot analysis. Similar results were obtained for three separate experiments.

Autoradiographs as in the upper panel of **a** were scanned and signals quantitated **(b)** in the area of the film shown. The effect of 8-OH-DPAT was quantified for three separate experiments and data is shown as mean +/- S.E.M., n= 3. In order to compare levels of incorporation from separate experiments it was necessary to express the levels of incorporation for each sample as a percentage of the incorporation level observed for the unstimulated construct.

Figure 5.11


a)
Log [8-OH-DPAT] (M)
0
-11
-10
-9
-8
-7
-6
-5
-4
M_r ($x10^{-3}$)
105
75
105
75

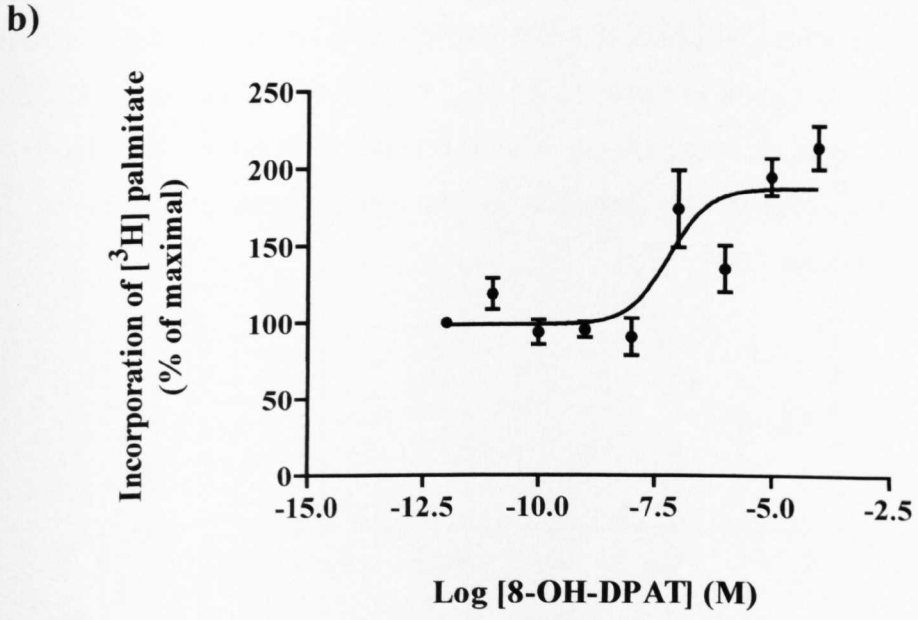

b)
Incorporation of [^{3}H] palmitate (% of maximal)
250
200
150
100
50
0
-15.0
-12.5
-10.0
-7.5
-5.0
-2.5
Log [8-OH-DPAT] (M)

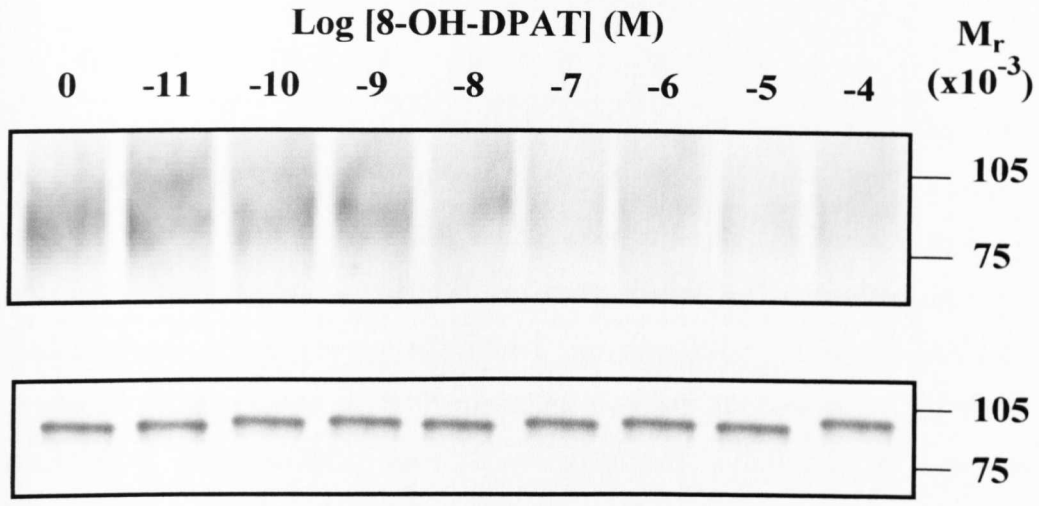
Figure 5.12

Concentration-response of 8-OH-DPAT regulated incorporation of [^{3}H] palmitate into the 5-HT$_{1A}$Cys^{417}Ser, Cys^{420}Ser-receptor-G$_{o1}\alpha$Cys^{351}Ile (C^{417}S, C^{420}S) fusion protein

A 5-HT$_{1A}$Cys^{417}Ser, Cys^{420}Ser-receptor-G$_{o1}\alpha$Cys^{351}Ile (**C^{417}S, C^{420}S**) fusion protein was expressed in HEK293T cells. Cells were incubated with [^{3}H] palmitate for 30 min in the presence of varying concentrations of 8-OH-DPAT. Samples were harvested and cell lysates produced. These were either immunoprecipitated with antiserum ON1 prior to SDS-PAGE and autoradiography for 1 month (**a**, upper panel) or resolved directly by SDS-PAGE and immunoblotted with antiserum ON1 (**a**, lower panel). **Figure 5.12 (a)** shows one representative concentration-response palmitoylation experiment with corresponding western blot analysis. Similar results were obtained for three separate experiments.

Autoradiographs as in the upper panel of **a** were scanned and signals quantitated **(b)** in the area of the film shown. The effect of 8-OH-DPAT was quantified for three separate experiments and data is shown as mean +/- S.E.M., n= 3. In order to compare levels of incorporation from separate experiments it was necessary to express the levels of incorporation for each sample as a percentage of the incorporation level observed for the unstimulated construct.

Figure 5.12


a)
Log [8-OH-DPAT] (M)
0
-11
-10
-9
-8
-7
-6
-5
-4
M_r ($x10^{-3}$)
105
75
105
75

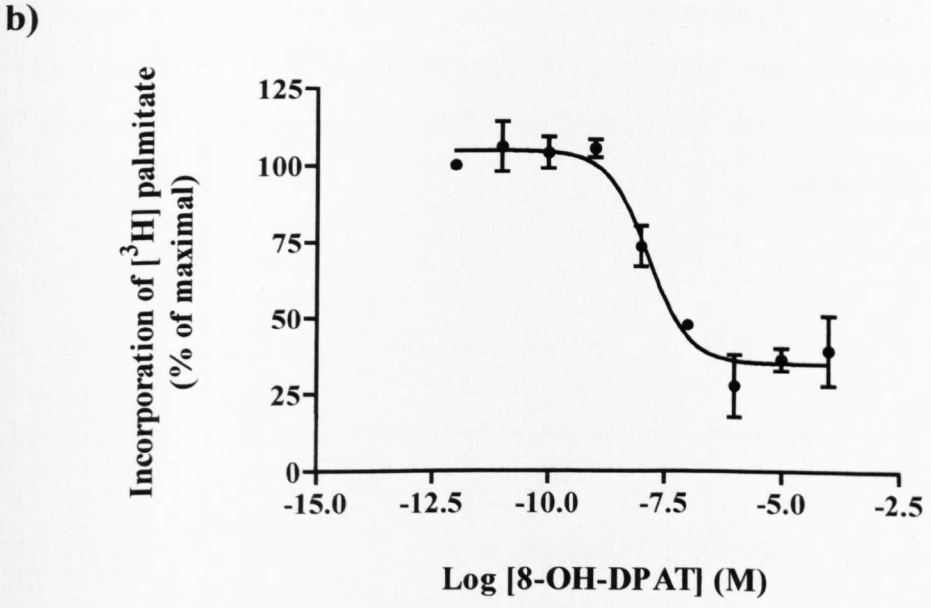

b)
Incorporation of [^{3}H] palmitate (% of maximal)
125
100
75
50
25
0
-15.0
-12.5
-10.0
-7.5
-5.0
-2.5
Log [8-OH-DPAT] (M)

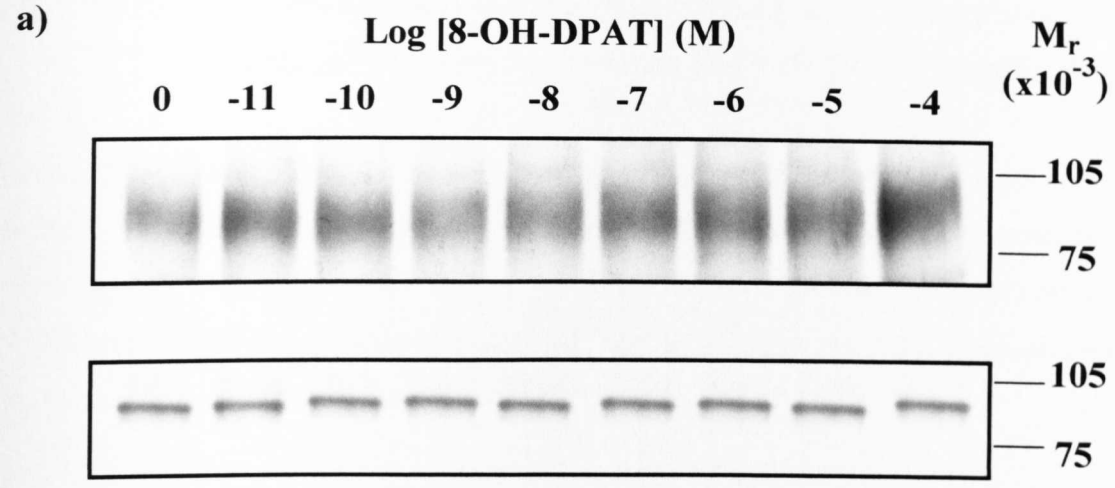
Figure 5.13

Concentration-response of 8-OH-DPAT regulated incorporation of [^{3}H] palmitate into the 5-HT$_{1A}$Cys^{420}Ser-receptor-G$_{o1}\alpha$Cys^{3}Ser, Cys^{351}Ile (C^{420}S, C^{3}S) fusion protein

A 5-HT$_{1A}$Cys^{420}Ser-receptor-G$_{o1}\alpha$Cys^{3}Ser, Cys^{351}Ile (**C^{420}S, C^{3}S**) fusion protein was expressed in HEK293T cells. Cells were incubated with [^{3}H] palmitate for 30 min in the presence of varying concentrations of 8-OH-DPAT. Samples were harvested and cell lysates produced. These were either immunoprecipitated with antiserum ON1 prior to SDS-PAGE and autoradiography for 1 month (**a**, upper panel) or resolved directly by SDS-PAGE and immunoblotted with antiserum ON1 (**a**, lower panel). **Figure 5.13 (a)** shows one representative concentration-response palmitoylation experiment with corresponding western blot analysis. Similar results were obtained for three separate experiments.

Autoradiographs as in the upper panel of **a** were scanned and signals quantitated (**b**) in the area of the film shown. The effect of 8-OH-DPAT was quantified for three separate experiments and data is shown as mean +/- S.E.M., n= 3. In order to compare levels of incorporation from separate experiments it was necessary to express the levels of incorporation for each sample as a percentage of the incorporation level observed for the unstimulated construct.

Figure 5.13


a)
Log [8-OH-DPAT] (M)
0
-11
-10
-9
-8
-7
-6
-5
-4
M_r ($x10^{-3}$)
105
75
105
75

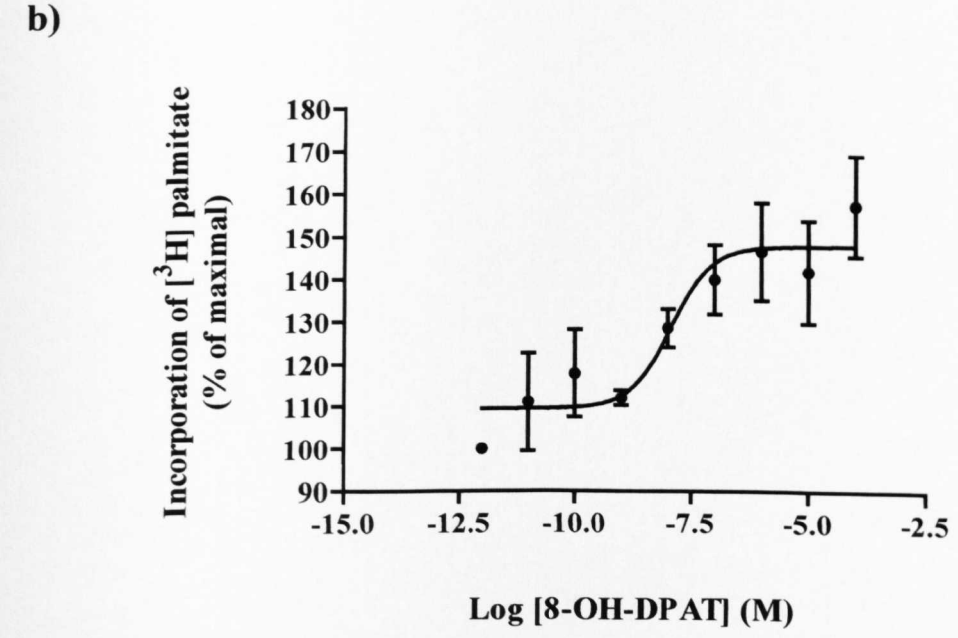

b)
Incorporation of [^{3}H] palmitate (% of maximal)
180
170
160
150
140
130
120
110
100
90
-15.0
-12.5
-10.0
-7.5
-5.0
-2.5
Log [8-OH-DPAT] (M)

Table 5.4

Comparison of 8-OH-DPAT concentration-response regulated incorporation of [^{3}H] palmitate into three palmitoylation-variant fusion proteins (for Figure 5.11: WT, for Figure 5.12: $C^{417}S$, $C^{420}S$ and for Figure 5.13: $C^{420}S$, $C^{3}S$)

The results from **Figures 5.11-5.13** were presented in a tabular form for clarity of comparison between the constructs. Non-linear regression analysis was used to determine the EC_{50} for 8-OH-DPAT regulated incorporation of [^{3}H] palmitate into the three selected 5-HT_{1A}-receptor -$G_{o1}\alpha Cys^{351}$Ile fusion proteins (mean +/- SEM, n=3).

Table 5.4

Construct	EC_{50} (nM) for 8-OH-DPAT-stimulated [^{3}H] palmitate incorporation
5-HT_{1A}-receptor-$G_{o1}\alpha Cys^{351}Ile$ (**WT**)	63 +/- 48
5-HT_{1A}-receptor $Cys^{417}Ser$, $Cys^{420}Ser$-$G_{o1}\alpha Cys^{351}Ile$ (**$C^{417}S$, $C^{420}S$**)	14 +/- 8
5-HT_{1A}-receptor $Cys^{420}Ser$-$G_{o1}\alpha Cys^{3}Ser$, $Cys^{351}Ile$ (**$C^{420}S$, $C^{3}S$**)	12 +/- 7

Figure 5.14

Incorporation of [^{3}H] palmitate into endogenously expressed $G_{o1}\alpha$ protein in the presence and absence of 8-OH-DPAT

A 5-HT_{1A}-receptor-$G_{o1}\alpha$Cys^{351}Ile (**WT**) fusion protein was expressed in HEK293T cells. Cells were incubated with [^{3}H] palmitate for the indicated times in the absence (-) or presence (+) of 100μM 8-OH-DPAT. Samples were harvested and cell lysates produced. These were either immunoprecipitated with antiserum ON1 prior to SDS-PAGE and autoradiography for 1 month (upper panel) or resolved directly by SDS-PAGE and immunoblotted with antiserum ON1 (lower panel). Rather than the fusion protein, labelling and expression of endogenous $G_{o1}\alpha$ is shown. **Figure 5.14** shows one representative palmitoylation experiment with corresponding western blot analysis. Similar results were obtained for three separate experiments.

Figure 5.14

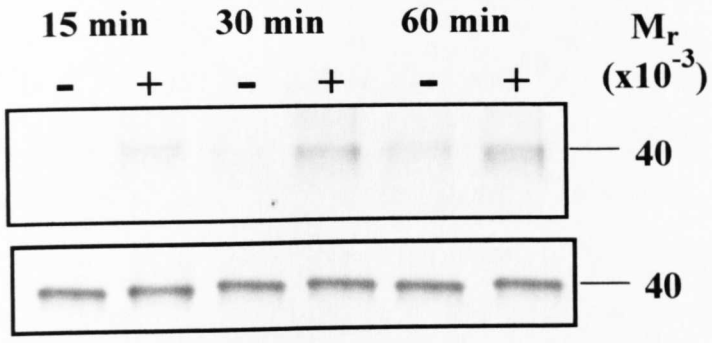


Figure 5.15

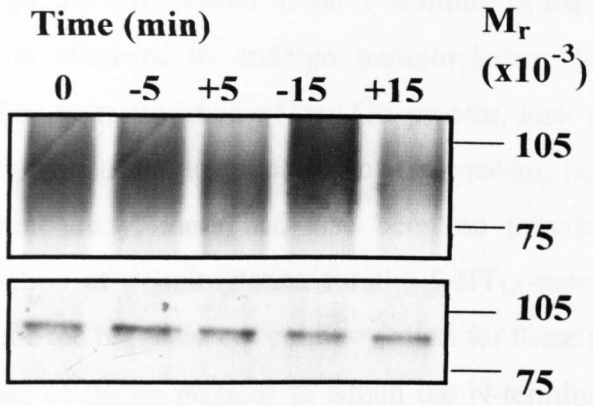
Repalmitoylation of the 5-$HT_{1A}$$Cys^{417}$Ser, Cys^{420}Ser-receptor-$G_{o1}\alpha Cys^{351}$Ile ($C^{417}S$, $C^{420}S$) fusion protein

A 5-$HT_{1A}$$Cys^{417}$Ser, Cys^{420}Ser-receptor-$G_{o1}\alpha Cys^{351}$Ile (**$C^{417}S$, $C^{420}S$**) fusion protein was expressed in HEK293T cells. Cells were incubated with [^{3}H] palmitate for 30 min in the absence of agonist. After the 30 min incubation, cells were incubated with [^{3}H] palmitate for the indicated times in the absence (-) or presence (+) of 100μM 8-OH-DPAT. Samples were harvested and cell lysates produced. These were either immunoprecipitated with antiserum ON1 prior to SDS-PAGE and autoradiography for 1 month (**a**, upper panel) or resolved directly by SDS-PAGE and immunoblotted with antiserum ON1 (**a**, lower panel). **Figure 5.15** (**a**) shows one representative repalmitoylation experiment with corresponding western blot analysis. Similar results were obtained for three separate experiments.

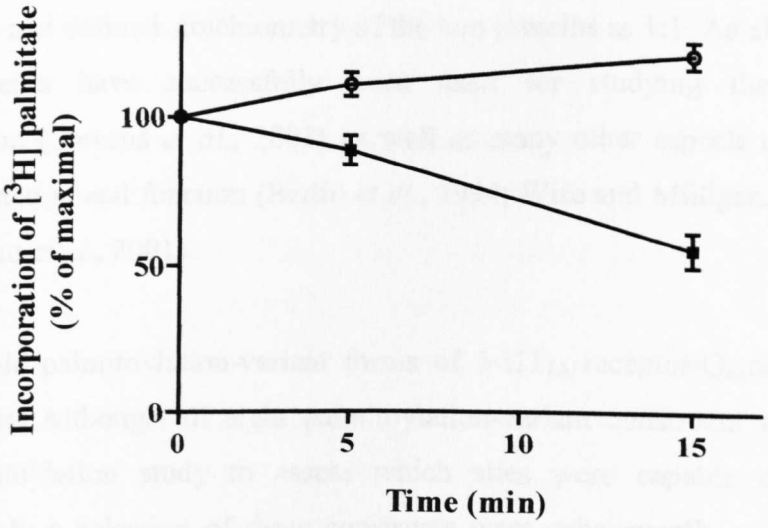
Autoradiographs as in the upper panel of **a** were scanned and signals quantitated **(b)** in the area of the film shown. Open circles = absence, filled squares = presence of 8-OH-DPAT. Results for three separate experiments were quantified and data is shown as mean +/- S.E.M., n= 3. In order to compare levels of incorporated [^{3}H] palmitate from separate experiments it was necessary to express the levels of [^{3}H] palmitate for each sample as a percentage of the level observed for the 0 min (post-steady-state incubation) sample.

Figure 5.15

a)



b)



5.3 Discussion

Around ten years ago, the Cys^3 residue at the N-terminus of the $G_{o1}\alpha$ protein (Grassie *et al.*, 1994) was first observed to undergo palmitoylation. Since that time, further information regarding palmitoylation of this Gα protein, how it may be regulated and how that regulation may affect the function of the protein, has been limited. At the outset of the current study, there had also been no previous publications on the regulation and function of palmitoylation for the 5-HT_{1A}-receptor. Consequently, we decided to investigate the regulation of palmitoylation for these two proteins. In order to do this we made use of fusion proteins in which the N-terminus of the $G_{o1}\alpha Cys^{351}Ile$ protein was linked in-frame to the C-terminal tail of the 5-HT_{1A}-receptor.

Once again, the reasons for employing fusion proteins in the current study were in order to benefit from some of the advantageous features imparted by the nature of the fusion: features such as close proximity of GPCR and G protein (allowing effective interactions) and defined stoichiometry of the two proteins as 1:1. As already discussed, fusion proteins have successfully been used for studying the regulation of palmitoylation (Stevens *et al.*, 2001) as well as many other aspects of GPCR and G protein interaction and function (Bertin *et al.*, 1994; Wise and Milligan, 1997; Seifert *et al.*, 1998; Guo *et al.*, 2001).

Eight possible palmitoylation-variant forms of 5-HT_{1A}-receptor-$G_{o1}\alpha Cys^{351}Ile$ fusion proteins exist. Although all eight palmitoylation-variant constructs were used in an initial palmitoylation study to assess which sites were capable of incorporating palmitate, only a selection of these constructs were subsequently used to assess the kinetics of palmitoylation and depalmitoylation reactions for the GPCR and the G protein. This is largely because it is more useful to study each potential palmitoylation site in isolation, rather than in combination with other sites.

During the progress of the current study a publication appeared documenting regulation of the 5-HT_{1A}-receptor palmitoylation and its functional role (Papoucheva *et al.*, 2004). The findings from the Papoucheva *et al.* study showed some similarities to the results obtained herein for the G protein-fused 5-HT_{1A}-receptor. However, a number of very

different observations were also made in the two studies. The results observed by Papoucheva *et al.* will be described in full and compared to the results of the current study after the regulation of palmitoylation is discussed for the $G_{o1}\alpha$ protein portion of the 5-HT_{1A}-receptor-$G_{o1}\alpha Cys^{351}$Ile fusions.

The incorporation of [^{3}H] palmitate into the $G_{o1}\alpha$ protein segment of the 5-HT_{1A}-receptor-$G_{o1}\alpha Cys^{351}$Ile fusion proteins was initially confirmed in a palmitoylation experiment comparing levels of incorporation into all eight palmitoylation variant fusions. Upon subsequent analysis of the 5-$HT_{1A}Cys^{417}$Ser, Cys^{420}Ser-receptor-$G_{o1}\alpha Cys^{351}$Ile (**$C^{417}S$, $C^{420}S$**) fusion protein (capable of incorporating palmitate only into the G protein site) by pulse-labelling time-course experiments, 8-OH-DPAT-stimulation was found to lead to decreased levels of [^{3}H] palmitate incorporation into the fused $G_{o1}\alpha$ protein. A similar effect had already been observed in the current study by the use of α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}$Ile fusions (**Chapter 3**). For the $G_{o1}\alpha Cys^{351}$Ile protein fused to the α_{2A}-adrenoceptor, approximately 60% less incorporation of [^{3}H] palmitate was observed in the presence of adrenaline compared with basal levels. The current results, for the $G_{o1}\alpha Cys^{351}$Ile protein fused to the 5-HT_{1A}-receptor, appear to demonstrate even less incorporation of [^{3}H] palmitate into the G protein in response to agonist (approximately 80% less for 8-OH-DPAT-stimulated fusions than for unstimulated). There was also another similarity from pulse-labelling studies of the two sets of fusion proteins. The $t_{1/2}$ for incorporation of palmitate into the fused G protein was, in both cases, unaltered by agonist-stimulation. In addition, the actual $t_{1/2}$ values for incorporation of [^{3}H] palmitate into the 5-HT_{1A}-receptor-$G_{o1}\alpha Cys^{351}$Ile fusions (unstimulated = 11.3 +/- 4.6 min, stimulated = 4.4 +/- 5.6 min) were very similar to the values for the α_{2A}-adrenoceptor-$G_{o1}\alpha$ Cys^{351}Ile fusions (unstimulated = 8.2 +/- 1.3 min, stimulated = 8.3 +/- 2.0 min). An agonist stimulated decrease in incorporation level of [^{3}H] palmitate had also previously been observed for a β_2-adrenoceptor-$G_s\alpha$ fusion protein (Loisel *et al.*, 1999). In contrast, a distinct effect of agonist was observed for an α_{1b}-adrenoceptor-$G_{11}\alpha$ fusion protein. In the latter case, agonist enhanced the levels of palmitate incorporated into the fusion (Stevens *et al.*, 2001).

As well as dynamic palmitoylation, the $G_{o1}\alpha$ protein portion of the 5-HT_{1A}-receptor-$G_{o1}\alpha Cys^{351}$Ile fusion proteins was also found to undergo dynamic depalmitoylation. In pulse-chase format assays the $G_{o1}\alpha$ protein incorporated [^{3}H] palmitate during the pulse period, then rapidly lost the palmitate during the chase period with $t_{1/2}$ for depalmitoylation assessed to be 15.3 +/- 6.5 min in the absence of 8-OH-DPAT and 6.3 +/- 2.4 min in the presence of 8-OH-DPAT. Upon comparison with the results for the α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}$Ile fusions ($t_{1/2}$ unstimulated = 37.0 +/- 5.0 min, $t_{1/2}$ stimulated = 17.0 +/- 2.0 min), some similarities and also some differences were observed. Firstly, depalmitoylation of the 5-HT_{1A}-receptor-fused $G_{o1}\alpha$ Cys^{351}Ile protein (basal and stimulated levels) appeared to be more rapid than for the α_{2A}-adrenoceptor-fused $G_{o1}\alpha$ Cys^{351}Ile protein. Secondly, the rate of depalmitoylation of α_{2A}-adrenoceptor-fused $G_{o1}\alpha$ Cys^{351}Ile protein was significantly increased in the presence of the agonist adrenaline. In contrast, statistical analysis of the basal and 8-OH-DPAT-stimulated depalmitoylation rate for the 5-HT_{1A}-receptor-fused $G_{o1}\alpha Cys^{351}$Ile protein did not confirm such an increase. I expect this observation is a result of the relatively large experimental errors for this assay and that upon further careful repeats, the errors could be reduced and thus reveal the statistically significant effect of agonist that is implied from the quantitation of **Figure 5.4**. A similar agonist-stimulated rate of $G\alpha$ protein depalmitoylation has been observed in a study with $G_i\alpha$ (Chen and Manning, 2000). In this study, stimulation of the 5-HT_{1A}-receptor by the agonist 8-OH-DPAT increased the depalmitoylation rate of the $G_i\alpha$ protein.

The ability of various concentrations of 8-OH-DPAT to regulate 5-HT_{1A}-receptor-fused $G_{o1}\alpha Cys^{351}$Ile protein palmitoylation was then assessed. 8-OH-DPAT caused a concentration-dependent reduction in levels of [^{3}H] palmitate incorporation into the fused $G_{o1}\alpha Cys^{351}$Ile protein with an EC_{50} of 14 +/- 8 nM. A very similar EC_{50} (~10 nM) was also observed for 8-OH-DPAT regulated incorporation of [^{3}H] palmitate into the Giα protein (Chen and Manning, 2000). However, in the latter case the agonist resulted in increased levels of [^{3}H] palmitate incorporation upon agonist stimulation.

Given that my results, observed for 5-HT_{1A}-receptor fused $G_{o1}\alpha Cys^{351}$Ile proteins, displayed a seemingly opposite pattern of agonist-regulated incorporation of [^{3}H]

palmitate than for a number of other non-fused Gα proteins, including $G_i\alpha$ (Chen and Manning, 2000; Bhamre et al.1998) and $G_s\alpha$ (Wedegaertner and Bourne, 1995) we decided to investigate whether constraint of the Gα protein in a fusion could have affected the observed levels of [^{3}H] palmitate incorporation. This was assessed by the ability of the 5-HT_{1A}Cys^{417}Ser, Cys^{420}Ser-receptor-$G_{o1}\alpha$Cys^{351}Ile (**C^{417}S, C^{420}S**) fusion protein to stimulate the endogenous $G_{o1}\alpha$ protein. In this experiment, agonist regulation of G protein palmitoylation was once again observed. However, in contrast to our results with the fused $G_{o1}\alpha$Cys^{351}Ile protein, 8-OH-DPAT led to an increase in incorporation of [^{3}H] palmitate into the endogenous G protein, thus indicating that some limitation of the fusion protein was responsible for the observation of an opposite pattern of regulated palmityolation. Previously it had been reported that the ability of agonist-stimulated fusion proteins to become repalmitoylated might be limited (Loisel *et al.*, 1999). Therefore, this was tested in a repalmitoylation assay using the 5-HT_{1A}Cys^{417}Ser, Cys^{420}Ser-receptor-$G_{o1}\alpha$Cys^{351}Ile (**C^{417}S, C^{420}S**) fusion protein. Akin to the results observed for the β_2-adrenoceptor-$G_s\alpha$ fusion protein (Loisel *et al.*, 1999) and the α_{2A}Cys^{442}Ala-adrenoceptor-$G_{o1}\alpha$Cys^{351}Ile (**Cys^{442}Ala**) fusion protein (**Chapter 3**), repalmitoylation of the 5-HT_{1A}Cys^{417}Ser, Cys^{420}Ser-receptor-$G_{o1}\alpha$Cys^{351}Ile fusion protein was reduced in response to agonist. Therefore, the apparently opposite pattern of agonist-regulated incorporation of [^{3}H] palmitate into fused and non-fused $G_{o1}\alpha$ protein, may reflect some restriction of the fused protein to become repalmitoylated after depalmitoylation events.

The fact that the fused G proteins exhibit opposite patterns of [^{3}H] palmitate incorporation to non-fused G proteins in pulse-labelling studies does not necessarily indicate that the fusion system is unsuitable for palmitoylation studies. As already discussed, the results from pulse-labelling studies do not give us definitive descriptions of changes in palmitoylation, since the results of such an assay represent both the palmitoylation and depalmitoylation reactions. As a result, pulse-labelling assays only tell us whether agonist is capable of producing an effect without detailed insight into potential mechanisms. The same observation can still be made in the fused system, meaning that even in light of some of the observations made in the current study, fusions are still considered to be useful tools for the study of the regulation of palmitoylation.

Now that the regulation of $G_{o1}\alpha Cys^{351}Ile$ protein palmitoylation has been discussed in full the regulation of 5-HT_{1A}-receptor palmitoylation can be considered. In the study by Papoucheva *et al.* (2004), the regulation of 5-HT_{1A}-receptor palmitoylation and the associated functional role of this modification were reported. These authors observed incorporation of palmitate into both the Cys^{417} and the Cys^{420} residues of the 5-HT_{1A}-receptor. However, rather than being a dynamically regulated post-translational modification, they reported that palmitate was incorporated into this GPCR early after receptor synthesis and once attached, was essentially irreversible. These authors also reported that palmitoylation efficiency at this GPCR was not modulated by receptor stimulation with agonists. Some of these results were similar to those obtained in the current study by use of 5-HT_{1A}-receptor-$G_{o1}\alpha Cys^{351}Ile$ fusion proteins but there were also a number of contrasting results between the two studies. Herein, the results of the current study will be addressed point-by-point for comparison with the results of the Papoucheva *et al.* (2004) study.

Before performing a variety of experiments to assess the regulation of palmitoylation in the GPCR and G protein parts of 5-HT_{1A}-receptor-$G_{o1}\alpha Cys^{351}Ile$ fusion proteins, it was necessary to confirm which sites in the fusion were responsible for incorporation of palmitate. Surprisingly, when the ability of all eight palmitoylation-variant 5-HT_{1A}-receptor-$G_{o1}\alpha Cys^{351}Ile$ constructs to incorporate [^{3}H] palmitic acid were assessed in parallel, palmitate was found to incorporate into the first receptor palmitoylation site (Cys^{417}) but not into the second (Cys^{420}) site of the 5-HT_{1A}-receptor. The lack of incorporation into the Cys^{420} site of the 5-HT_{1A}-receptor observed in these studies is in direct contrast to the findings of Papoucheva *et al.* (2004). These authors convincingly demonstrated the incorporation of palmitate into both sites of the 5-HT_{1A}-receptor in their study using a non-fused receptor. The reason for such a difference is difficult to ascertain. Given that in the current study the identity of all mutant constructs were checked by sequencing, one can only assume the contrasting results reflect some difference(s) in the experimental system used.

It was also apparent from this study that the presence of the 5-HT_{1A}-receptor agonist 8-OH-DPAT led to altered levels of palmitate incorporation into the 5-HT_{1A}-receptor.

This was observed both in pulse-labelling studies and in 8-OH-DPAT-stimulated concentration-response experiments.

In pulse labelling studies with the **$C^{420}S$, $C^{3}S$** fusion (reflecting only C^{417} palmitoylation), 8-OH-DPAT led to approximately twice the basal levels (374.1 +/- 79.9 % versus 179.5 +/- 22.5 %) of [^{3}H] palmitate incorporation into the first receptor palmitoylation site. From these results we were also able to determine that such an effect was not attributable to an increased rate of palmitoylation, since the observed $t_{1/2}$ for palmitate incorporation was statistically the same ($p>0.05$) in the presence (67.8 +/- 29.2 min) and the absence (50.9 +/- 14.1 min) of 8-OH-DPAT. Furthermore we were able to show that none of these results were significantly different ($p>0.05$) if the **$C^{3}S$** fusion (which reflects the palmitoylation of both potential receptor sites) was used. Thus providing further support that dynamic palmitoylation of the C^{420} site on the fused 5-HT_{1A}-receptor did not occur. When considering this result one must also bear in mind the distinct possibility that non-radiolabelled palmitate may already be irreversibly incorporated at this site and thus [^{3}H] palmitate in this assay cannot replace it.

Concentration-response experiments performed on the **$C^{420}S$, $C^{3}S$** fusion further demonstrated that 8-OH-DPAT stimulated an increase in [^{3}H] palmitate incorporation into the first receptor palmitoylation site. Furthermore such regulation of receptor palmitoylation was found to occur with essentially the same ($p>0.05$) EC_{50} (12 +/- 7 nM) as for 8-OH-DPAT-stimulated incorporation of [^{3}H] palmitate into the fused $G_{o1}\alpha Cys^{351}$Ile protein (14 +/- 8nM).

In contrast, Papoucheva *et al.* (2004) did not observe agonist-regulation of palmitoylation in response to the 5-HT_{1A}-receptor agonist serotonin (100pM-10μM). This is another baffling difference observed for the two palmitoylation studies, which is very difficult to explain. Even more difficult given that the agonist effects have been convincingly shown for both studies. Once again, some difference(s) in the experimental system used must be responsible for the contrasting observations. Such lack of agonist-regulation of GPCR palmitoylation, observed by Papoucheva *et al.* (2004), is not only in contrast to our findings but it is also different to observations made for other GPCRs such as the 5-HT_{4A}-receptor (Ponimaskin *et al.*, 2002) and the

β_2-adrenoceptor (Loisel *et al.*, 1996), therefore I find the lack of agonist-regulation reported in the Papoucheva *et al.* (2004) paper rather surprising.

In the current study there was one observation made which was in agreement to the findings of Papoucheva *et al.* (2004). Neither the **$C^{420}S$, $C^{3}S$** nor the **$C^{3}S$** fusion proteins were found to undergo depalmitoylation in pulse-chase palmitoylation assays. In these assays palmitate was incorporated into the fusions but remained stably attached for the entire three-hour chase period analysed. This result was in keeping with the results of similar pulse-chase assays performed for the non-fused 5-HT_{1A}-receptor.

By taking all of my observations for the receptor together I have interpreted my results as follows:

1 Palmitate, once attached to the 5-HT_{1A}-receptor Cys^{417} site, is stable and cannot be removed (at least not within a 3 hour timescale).

2 Not all receptor palmitoylation sites already have palmitate attached after expression of the 5-HT_{1A}-receptor-$G_{o1}\alpha Cys^{351}Ile$ fusion proteins, hence allowing us to incorporate [^{3}H] palmitate into our fusions during the palmitoylation assays

3 8-OH-DPAT stimulates the incorporation of [^{3}H] palmitate into our fusions without affecting the rate of palmitoylation. It perhaps promotes some fusion conformation more favourable for the incorporation of palmitate.

However, one further observation made in the Papoucheva *et al.* (2004) study would seem to be in disagreement with this hypothesis. These authors seemed to demonstrate that palmitolyation of the 5-HT_{1A}-receptor happens immediately after protein synthesis. They did this by monitoring in parallel, 5-HT_{1A} receptor incorporation of [^{35}S] methionine or [^{3}H] palmitate in the presence of cycloheximide (an inhibitor of protein synthesis). They observed that blocking synthesis of protein (confirmed by no labelling with [^{35}S] methionine) also blocked the incorporation of [^{3}H] palmitate into the 5-HT_{1A}-receptor. The authors took these results in combination with their other findings to mean that palmitoylation of the 5-HT_{1A}-receptor occurs early after synthesis of this GPCR and that once attached the palmitate is stable and not subject to dynamic regulation. The implication of this result for the cycloheximide experiments was somewhat surprising to

me. Given that Papoucheva *et al.* (2004) claim that all incorporation of [^{3}H] palmitate represents incorporation into newly synthesised receptor, remarkably high levels of new receptor synthesis must be occurring with their expression system. In their studies, over a labelling time of only 30 minutes, there is substantial incorporation of [^{3}H] palmitate (corresponding to substantial levels of new protein synthesis). I found this observation quite surprising. I wouldn't have expected such high levels of new protein synthesis to occur over such a short time. In my studies Western blot analysis, performed in parallel with palmitoylation assays, confirmed that no detectable difference in expression levels of my fusion proteins occurred over the time-course of a typical assay. However, since I have not performed any experiments on protein synthesis/degradation I cannot really compare my results to these findings. It would be useful therefore, to perform such assays and also to assess palmitoylation of the 5-HT_{1A}-receptor-$G_{o1}\alpha Cys^{351}Ile$ fusion proteins in the presence of cycloheximide for comparison with the studies of Papoucheva *et al.* (2004).

In conclusion, it is very difficult to ascertain the reason(s) for the differences reported between the study by Papoucheva *et al.* (2004) and the results of the current study. However some aspect of the different experimental systems must be accountable. The work of Papoucheva *et al.* (2004) was performed using the murine form of the non-fused 5-HT_{1A}-receptor expressed in Sf.9 insect cells whereas the current study used the human form of the 5-HT_{1A}-receptor fused to the $G_{o1}\alpha Cys^{351}Ile$ protein and expressed in HEK293T cells. I am very sceptical that such a variety of differences could be due to the fusion context of my study, however, this possibility cannot be excluded. Such differences confirm the need for a number of comparative experiments (e.g. analysis of palmitoylation for the non-fused human 5-HT_{1A}-receptor in HEK293T cells) before a clear understanding of the regulation of 5-HT_{1A}-receptor palmitoylation can be obtained.

In summary, the data from this chapter provides similar conclusions for the regulation of $G_{o1}\alpha Cys^{351}Ile$ protein palmitoylation fused to the 5-HT_{1A}-receptor than previously observed in **Chapter 3** for the regulation of this same Gα protein fused to the α_{2A}-adrenoceptor. This is perhaps indicative of a more general role for Gα protein palmitoylation. In contrast the results of the current chapter highlight very different

patterns of regulation of GPCR palmitoylation for the 5-HT_{1A}-receptor and the α_{2A}-adrenoceptor. This difference in regulation of palmitoylation may result in different observations for the functional significance of palmitate attached to these two receptors. Given that a functional analysis of the significance of palmitoylation has been performed for the α_{2A}-adrenoceptor-$G_{o1}\alpha$ fusions in **Chapter 4**, I have also performed a similar analysis for the palmitoylation-variant 5-HT_{1A}-receptor-$G_{o1}\alpha Cys^{351}$Ile fusion proteins in the last results chapter of this thesis (**Chapter 6**).

Chapter 6

Functional consequences of palmitoylation in 5-HT_{1A}-receptor-$G_{o1}\alpha Cys^{351}Ile$ fusion proteins

6.1 Introduction

Reversible attachment of palmitate to some GPCRs and G proteins via repeated cycles of palmitoylation and depalmitoylation has been implicated in the modulation of a number of signalling processes (Ross, 1995; Mumby 1997). For several GPCRs (Hayashi and Haga, 1997; Hawtin *et al.*, 2001; Ponimaskin *et al.*, 2001) palmitoylation can be modulated by agonist stimulation. In addition, agonist-stimulation of several GPCRs has been shown to modulate palmitoylation of receptor-coupled G proteins (Gurdal *et al.*, 1997; Mumby and Muntz, 1995; Chen and Manning, 2000; Stevens *et al.*, 2001). As a result, a number of studies of GPCR and G protein palmitoylation have been performed (Ponimaskin *et al.*, 2001; Chen and Manning, 2000; Papoucheva *et al.*, 2004) and a wide variety of functional consequences have been reported (Qanbar and Bouvier, 2003; Hawtin *et al.*, 2001: Ponimaskin *et al.*, 2002; Miggin *et al.*, 2003).

As already outlined in **Chapter 4**, roles for Gα protein palmitoylation have been suggested in RGS protein-Gα protein interactions (Tu *et al.*, 1997; Ross and Wilkie, 2000) and membrane localisation/targeting of the Gα protein (Wedegaertner, 1998; Dunphy and Linder, 1998; Mumby, 1997; Song *et al.*, 1997). GPCR palmitoylation (also discussed in **Chapter 4**) has been implicated in the modulation of a number of functional properties such as GPCR-G protein interactions (Hayashi and Haga, 1997), GPCR phosphorylation and desensitisation (Moffett *et al.*, 1993) and GPCR downregulation (Kawate *et al.*, 1997; Munshi *et al.*, 2001).

In this study the functional consequences of palmitoylation of the 5-HT_{1A}-receptor and the $G_{o1}\alpha Cys^{351}Ile$ protein are studied for the same palmitoylation-variant 5-HT_{1A}-receptor-$G_{o1}\alpha Cys^{351}Ile$ fusion proteins used in **Chapter 5**. At present there is a lack of data concerning the functional consequences of $G_{o1}\alpha Cys^{351}Ile$ protein palmitoylation. In addition, at the time of commencement of the current study there were no publications on 5-HT_{1A}-receptor palmitoylation. More recently however, one study (Papoucheva *et al.*, 2004) has contained a number of findings on the functional consequences of palmitoylation for this GPCR. These authors found palmitoylation of the 5-HT_{1A}-receptor to be important for coupling to $G_i\alpha$ and $G\beta\gamma$ as well as for the inhibition of forskolin-stimulated cAMP formation. In terms of the current study, it was decided to

use the palmitoylation-variant 5-HT_{1A}-receptor-$G_{o1}\alpha Cys^{351}Ile$ fusion proteins (already studied for regulation of palmitoylation in **Chapter 5**) to assess the functional importance of palmitoylation in the two fused proteins. This was largely due to the previously reported success of GPCR-G protein fusions as a model system to study various functional properties (Wise and Milligan, 1997; Cavalli *et al.*, 2000; Stevens *et al.*, 2001; Ugur *et al.*, 2003; Bertaso *et al.*, 2003). Herein, the importance of GPCR and G protein palmitoylation of the 5-HT_{1A}-receptor-$G_{o1}\alpha Cys^{351}Ile$ fusion proteins were assessed in terms of radioligand binding and GTPγS binding.

6.2 Results

Expression of 5-HT_{1A}-receptor-$G_{o1}\alpha Cys^{351}Ile$ fusion proteins and determination of their affinity for the agonist 8-OH-DPAT and the antagonist [^{3}H]-WAY100635

The palmitoylation variant 5-HT_{1A}-receptor-$G_{o1}\alpha Cys^{351}Ile$ fusion proteins already created and studied in terms of regulation of palmitoylation (**Chapter 5**) were transiently transfected into HEK293T cells. After transfection, cells were harvested and cell membranes prepared for analysis. 5-HT_{1A}-receptor-$G_{o1}\alpha Cys^{351}Ile$ fusion protein expression levels were investigated by Western blot analysis. Immunodetection of the $G_{o1}\alpha Cys^{351}Ile$ protein N terminus (via ON1 antiserum) or the C terminus (via OC2 antiserum) confirmed expression of the fusion proteins post-transfection (**Figure 6.1**). Immunoblotting of transiently transfected membranes with both the ON1 and OC2 antisera detected immuno-reactive bands of molecular mass ~75-100kDa (predicted molecular weight 85kDa) which were not present in mock transfected membranes. In addition, the immunodetected levels of the 5-HT_{1A}-receptor-$G_{o1}\alpha Cys^{3}Ser$, $Cys^{351}Ile$ (**$C^{3}S$**), the 5-HT_{1A}-receptor$Cys^{417}Ser$-$G_{o1}\alpha Cys^{3}Ser$, $Cys^{351}Ile$ (**$C^{417}S$, $C^{3}S$**), the 5-HT_{1A}-receptor$Cys^{420}Ser$-$G_{o1}\alpha Cys^{3}Ser$, $Cys^{351}Ile$ (**$C^{420}S$, $C^{3}S$**) and the 5-HT_{1A}-receptor$Cys^{417}Ser$, $Cys^{420}Ser$-$G_{o1}\alpha Cys^{3}Ser$, $Cys^{351}Ile$ (**$C^{417}S$, $C^{420}S$, $C^{3}S$**) fusion proteins were similar to each other when immunoblotting was carried out with either the ON1 or the OC2 antisera. This confirmed that the presence of the $Cys^{3}Ser$ mutation on the $G_{o1}\alpha Cys^{351}Ile$ protein did not affect the ability of the ON1 antiserum to recognise this region of the $G_{o1}\alpha$ protein.

The expression of the 5-HT_{1A}-receptor-$G_{o1}\alpha Cys^{351}Ile$ fusion proteins was further assessed by the binding of a near saturating concentration (~5nM) of the tritiated antagonist [^{3}H]-WAY100635 (Fletcher *et al.*, 1996). From these experiments the transient membrane expression of each construct was estimated to be in the region of 10 pmol/mg (**Figure 6.2, Table 6.1**).

In order to obtain a more accurate measurement of each 5-HT_{1A}-receptor-$G_{o1}\alpha Cys^{351}Ile$ fusion protein expression level, saturation binding assays were performed using various

concentrations of [^{3}H]-WAY100635 (ranging from 0.05 to 5nM). From these experiments the expression level and the affinity for the antagonist (K_d) for each construct was obtained (**Figure 6.3, Table 6.2**). The expression levels of all 5-HT_{1A}-receptor-$G_{o1}\alpha Cys^{351}Ile$ fusion protein constructs were in the region of 12 pmol/mg. The affinity for [^{3}H]-WAY100635 for all eight palmitoylation variant fusion proteins was approximately 0.33nM. Neither the values for expression level ($p>0.05$) nor K_d ($p>0.05$) were significantly different for each of the 5-HT_{1A}-receptor-$G_{o1}\alpha Cys^{351}Ile$ fusion protein constructs.

Assessment of the ability of the 5-HT_{1A}-receptor-$G_{o1}\alpha Cys^{351}Ile$ fusion proteins to activate signalling

The ability of the 5-HT_{1A}-receptor-$G_{o1}\alpha Cys^{351}Ile$ fusion proteins to activate signalling was assessed in terms of the ability to bind GTP (assessed via a GTPγS binding assay). In order to compare the GTPγS binding of various palmitoylation variant 5-HT_{1A}-receptor-$G_{o1}\alpha Cys^{351}Ile$ fusion proteins within one assay, a number of control experiments had to be performed in order to establish appropriate experimental conditions. These control experiments were not performed on all palmitoylation variant constructs but instead were carried out for the 5-HT_{1A}-receptor-$G_{o1}\alpha Cys^{351}Ile$ (**WT**) and the 5-HT_{1A}-receptor $Cys^{417}Ser$, $Cys^{420}Ser$-$G_{o1}\alpha Cys^{3}Ser$, $Cys^{351}Ile$ (**$C^{417}S$, $C^{420}S$, $C^{3}S$**) fusion proteins only. The effects of increasing incubation times and increasing the amount of fusion protein used in the assay were assessed in **Figure 6.4** and **Figure 6.5** respectively.

From **Figure 6.4** increased [^{35}S] GTPγS binding was observed with increasing quantities of fusion protein. A non-linear rate of [^{35}S] GTPγS binding started to be observed with quantities of fusion protein over ~25 fmol. For this reason it was decided to use 10fmol fusion protein/tube in subsequent assays.

From **Figure 6.5** increased [^{35}S] GTPγS binding was observed with increasing lengths of incubation. Saturation of [^{35}S] GTPγS binding was observed after ~5 min. For this reason it was decided to use a 2.5 min incubation length in subsequent assays. Therefore

in all subsequent assays an incubation time of 2.5 min was used to assess GTPγS binding to 10 fmol/tube of each 5-HT_{1A}-receptor-$G_{o1}\alpha Cys^{351}$Ile fusion protein. One further point noted from **Figures 6.4** and **6.5** was that the 5-HT_{1A}-receptor Cys^{417}Ser, Cys^{420}Ser-$G_{o1}\alpha Cys^{3}$Ser, Cys^{351}Ile (**$C^{417}S$, $C^{420}S$, $C^{3}S**) fusion protein bound more GTPγS than the 5-HT_{1A}-receptor-$G_{o1}\alpha Cys^{351}$Ile (**WT**) fusion protein, perhaps indicating a role for either GPCR or G protein palmitoylation (or both) in determining levels of GTPγS binding.

This was then investigated for four of the palmitoylation-variant 5-HT_{1A}-receptor-$G_{o1}\alpha Cys^{351}$Ile fusion proteins within one assay (**Figure 6.6, Table 6.3**). The four selected constructs were the 5-HT_{1A}-receptor-$G_{o1}\alpha Cys^{351}$Ile (**WT**), the 5-HT_{1A}-receptor Cys^{417}Ser, Cys^{420}Ser-$G_{o1}\alpha Cys^{351}$Ile (**$C^{417}S$, $C^{420}S$**), the 5-HT_{1A}-receptor-$G_{o1}\alpha Cys^{3}$Ser, Cys^{351}Ile (**$C^{3}S$**) and the 5-HT_{1A}-receptor Cys^{417}Ser, Cys^{420}Ser-$G_{o1}\alpha Cys^{3}$Ser, Cys^{351}Ile (**$C^{417}S$, $C^{420}S$, $C^{3}S$**) fusion proteins.

In the presence of 10^{-4}M 8-OH-DPAT, GTPγS binding was significantly stimulated approximately 2-fold over basal levels ($p<0.05$) for all 5-HT_{1A}-receptor-$G_{o1}\alpha Cys^{351}$Ile fusion proteins tested. Interestingly, the levels of basal ($p<0.05$) and 8-OH-DPAT-stimulated ($p<0.05$) GTPγS binding for the four fusions were not all equal.

Instead, the basal ($p>0.05$) and agonist-stimulated ($p>0.05$) GTPγS binding levels for the 5-HT_{1A}-receptor-$G_{o1}\alpha Cys^{351}$Ile (**WT**) and the 5-HT_{1A}-receptor-$G_{o1}\alpha Cys^{3}$Ser, Cys^{351}Ile (**$C^{3}S$**) constructs were found to be essentially the same.

Similarly the basal ($p>0.05$) and agonist-stimulated ($p>0.05$) GTPγS binding levels for the 5-HT_{1A}-receptor Cys^{417}Ser, Cys^{420}Ser-$G_{o1}\alpha Cys^{351}$Ile (**$C^{417}S$, $C^{420}S$**) and the 5-HT_{1A}-receptor Cys^{417}Ser, Cys^{420}Ser-$G_{o1}\alpha Cys^{3}$Ser, Cys^{351}Ile (**$C^{417}S$, $C^{420}S$, $C^{3}S$**) constructs were also found to be the same.

Basal and 8-OH-DPAT-stimulated GTPγS binding levels were found to be ~1.5 times higher for both the 5-HT_{1A}-receptor Cys^{417}Ser, Cys^{420}Ser-$G_{o1}\alpha Cys^{351}$Ile (**$C^{417}S$, $C^{420}S$**) and the 5-HT_{1A}-receptor Cys^{417}Ser, Cys^{420}Ser-$G_{o1}\alpha Cys^{3}$Ser, Cys^{351}Ile (**$C^{417}S$, $C^{420}S$,**

C^{3}S) constructs than for the 5-HT$_{1A}$-receptor-G$_{o1}$αCys^{351}Ile (**WT**) and the 5-HT$_{1A}$-receptor-G$_{o1}$αCys^{3}Ser, Cys^{351}Ile (**C^{3}S**) constructs. Taken together, these results seem to indicate a requirement for palmitoylation of the Cys^{417} and Cys^{420} residues of the 5-HT$_{1A}$-receptor portion of the 5-HT$_{1A}$-receptor-G$_{o1}$αCys^{351}Ile fusion proteins in levels of constitutive activity.

The last experiment performed in the current study (**Figure 6.7, Table 6.4**) explored the high levels of basal GTPγS binding observed for the 5-HT$_{1A}$-receptor-G$_{o1}$αCys^{351}Ile fusion proteins. The inverse agonist spiperone (100μM) was assessed in terms of its ability to decrease the basal level of GTPγS binding to the 5-HT$_{1A}$-receptor-G$_{o1}$αCys^{351}Ile (**WT**) fusion protein. As expected, a reduction in the level of basal GTPγS binding from approximately one half (in the absence of spiperone) to approximately one tenth (in the presence of spiperone) of the 8-OH-DPAT-stimulated levels was observed.

Figure 6.1

Western blot analysis of membranes transiently transfected with the 5-HT_{1A}-receptor-$G_{o1}\alpha Cys^{351}Ile$ fusion proteins

3μg of membrane preparations from HEK293T cells transiently transfected with the empty vector (**pcDNA3**, lane **1**), 5-HT_{1A}-receptor-$G_{o1}\alpha Cys^{351}Ile$ (**WT**, lane **2**), 5-$HT_{1A}Cys^{417}Ser$-receptor-$G_{o1}\alpha Cys^{351}Ile$ (**$C^{417}S$**, lane **3**), 5-$HT_{1A}Cys^{417}Ser$-receptor-$G_{o1}\alpha Cys^{3}Ser$, $Cys^{351}Ile$ (**$C^{417}S$, $C^{3}S$**, lane **4**), 5-$HT_{1A}Cys^{420}Ser$-receptor-$G_{o1}\alpha Cys^{351}Ile$ (**$C^{420}S$**, lane **5**), 5-$HT_{1A}Cys^{420}Ser$-receptor-$G_{o1}\alpha Cys^{3}Ser$, $Cys^{351}Ile$ (**$C^{420}S$, $C^{3}S$, lane 6**), 5-$HT_{1A}Cys^{417}Ser$, $Cys^{420}Ser$-receptor-$G_{o1}\alpha Cys^{351}Ile$ (**$C^{417}S$, $C^{420}S$**, lane **7**), 5-HT_{1A}-receptor-$G_{o1}\alpha Cys^{3}Ser$, $Cys^{351}Ile$ (**$C^{3}S$**, lane **8**), and 5-$HT_{1A}Cys^{417}Ser$, $Cys^{420}Ser$-receptor-$G_{o1}\alpha Cys^{3}Ser$, $Cys^{351}Ile$ (**$C^{417}S$, $C^{420}S$, $C^{3}S$**, lane **9**) fusion proteins were resolved on SDS-PAGE gels then transferred onto nitrocellulose membranes. Immunoblotting was carried out with a) ON1 and b) OC2 antisera. Two further experiments produced similar results.

Figure 6.1

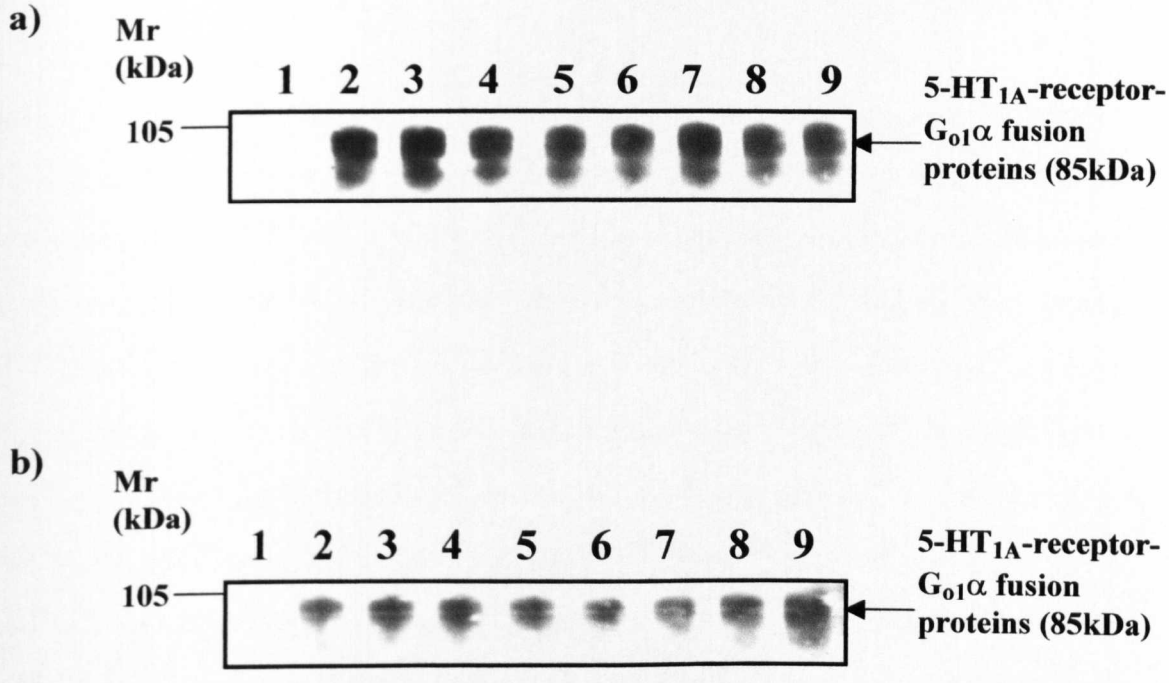

a)
Mr
(kDa)
1 2 3 4 5 6 7 8 9
105
5-HT1A-receptor-
Go1α fusion
proteins (85kDa)
b)
Mr
(kDa)
1 2 3 4 5 6 7 8 9
105
5-HT1A-receptor-
Go1α fusion
proteins (85kDa)

Figure 6.2

Analysis of expression levels of 5-HT_{1A}-receptor-$G_{o1}\alpha Cys^{351}$Ile fusion proteins: determined from binding of a near saturating (~5nM) concentration of [^{3}H]-WAY100635 to membranes expressing the fusion proteins

HEK293T cells were transfected to express the 5-HT_{1A}-receptor-$G_{o1}\alpha Cys^{351}$Ile fusion proteins. Membranes expressing the fusion proteins were prepared and binding of a near-saturating concentration (~5nM) of [^{3}H]-WAY100635 to 0.5μg of each membrane sample was assessed. The expression levels are shown for the 5-HT_{1A}-receptor-$G_{o1}\alpha Cys^{351}$Ile (**WT,** turquoise bars), 5-$HT_{1A}Cys^{417}$Ser-receptor-$G_{o1}\alpha Cys^{351}$Ile (**$C^{417}S$,** green bars), 5-$HT_{1A}Cys^{417}$Ser-receptor-$G_{o1}\alpha Cys^{3}$Ser, Cys^{351}Ile (**$C^{417}S$, $C^{3}S$,** purple bars), 5-$HT_{1A}Cys^{420}$Ser-receptor-$G_{o1}\alpha Cys^{351}$Ile (**$C^{420}S$,** brown bars), 5-$HT_{1A}Cys^{420}$Ser-receptor-$G_{o1}\alpha Cys^{3}$Ser, Cys^{351}Ile (**$C^{420}S$, $C^{3}S$,** red bars), 5-$HT_{1A}Cys^{417}$Ser, Cys^{420}Ser-receptor-$G_{o1}\alpha Cys^{351}$Ile (**$C^{417}S$, $C^{420}S$,** yellow bars), 5-HT_{1A}-receptor-$G_{o1}\alpha Cys^{3}$Ser, Cys^{351}Ile (**$C^{3}S$,** blue bars), and 5-$HT_{1A}Cys^{417}$Ser, Cys^{420}Ser-receptor-$G_{o1}\alpha Cys^{3}$Ser, Cys^{351}Ile (**$C^{417}S$, $C^{420}S$, $C^{3}S$,** pink bars) fusion proteins. Results are from triplicate determinations. Analysis is representative of three similar experiments.

Table 6.1

Comparison of expression levels of 5-HT_{1A}-receptor-$G_{o1}\alpha Cys^{351}$Ile fusion proteins determined from binding of near saturating (~5nM) concentration of [^{3}H]-WAY100635 to membranes expressing the fusion proteins

The results from **Figure 6.2** were presented in a tabular form for clarity of comparison between the constructs. The expression level (pmol/mg) of each fusion protein is given as mean +/- SEM (n=3).

Figure 6.2

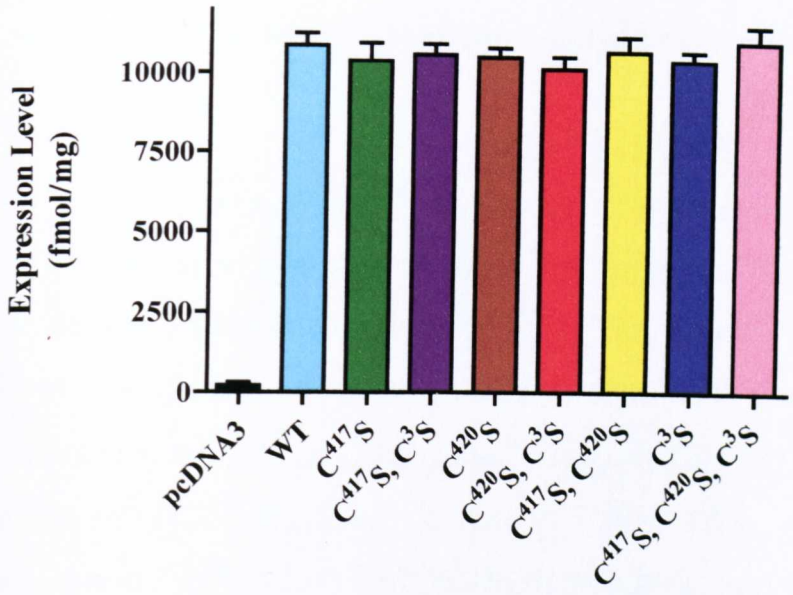


Table 6.1

Construct	Expression Level (fmol/mg)
5-HT_{1A}-receptor-$G_{o1}\alpha Cys^{351}Ile$ (**WT**)	10877 +/- 375
5-HT_{1A} $Cys^{417}Ser$-receptor-$G_{o1}\alpha Cys^{351}Ile$ (**$C^{417}S$**)	10398 +/- 562
5-HT_{1A} $Cys^{417}Ser$-receptor-$G_{o1}\alpha Cys^{3}Ser$, $Cys^{351}Ile$ (**$C^{417}S$, $C^{3}S$**)	10611 +/- 330
5-HT_{1A} $Cys^{420}Ser$-receptor-$G_{o1}\alpha Cys^{351}Ile$ (**$C^{420}S$**)	10545 +/- 289
5-HT_{1A} $Cys^{420}Ser$-receptor-$G_{o1}\alpha Cys^{3}Ser$, $Cys^{351}Ile$ (**$C^{420}S$, $C^{3}S$**)	10162 +/- 405
5-HT_{1A} $Cys^{417}Ser$, $Cys^{420}Ser$-receptor-$G_{o1}\alpha Cys^{351}Ile$ (**$C^{417}S$, $C^{420}S$**)	10721 +/- 467
5-HT_{1A}-receptor-$G_{o1}\alpha Cys^{3}Ser$, $Cys^{351}Ile$ (**$C^{3}S$**)	10437 +/- 299
5-HT_{1A} $Cys^{417}Ser$, $Cys^{420}Ser$-receptor-$G_{o1}\alpha Cys^{3}Ser$, $Cys^{351}Ile$ (**$C^{417}S$, $C^{420}S$ $C^{3}S$**)	11045 +/- 489

Figure 6.3

Analysis of expression levels of 5-HT_{1A}-receptor-$G_{o1}\alpha Cys^{351}$Ile fusion proteins by [^{3}H]-WAY100635 saturation binding analysis to membranes expressing the fusion proteins

HEK293T cells were transfected to express the 5-HT_{1A}-receptor-$G_{o1}\alpha Cys^{351}$Ile fusion proteins. Membranes expressing the fusion proteins were prepared and saturation binding of [^{3}H]-WAY100635 (using 0.05–5nM radioligand) to 0.5μg of each membrane sample was assessed. The saturation binding analyses are shown for the 5-HT_{1A}-receptor-$G_{o1}\alpha Cys^{351}$Ile (**WT,** turquoise line), 5-$HT_{1A}Cys^{417}$Ser-receptor-$G_{o1}\alpha Cys^{351}$Ile (**$C^{417}S$,** green line), 5-$HT_{1A}Cys^{417}$Ser-receptor-$G_{o1}\alpha Cys^{3}$Ser, Cys^{351}Ile (**$C^{417}S$, $C^{3}S$,** purple line), 5-$HT_{1A}Cys^{420}$Ser-receptor-$G_{o1}\alpha Cys^{351}$Ile (**$C^{420}S$,** brown line), 5-$HT_{1A}Cys^{420}$Ser-receptor-$G_{o1}\alpha Cys^{3}$Ser, Cys^{351}Ile (**$C^{420}S$, $C^{3}S$,** red line), 5-$HT_{1A}Cys^{417}$Ser, Cys^{420}Ser-receptor-$G_{o1}\alpha Cys^{351}$Ile (**$C^{417}S$, $C^{420}S$,** yellow line), 5-HT_{1A}-receptor-$G_{o1}\alpha Cys^{3}$Ser, Cys^{351}Ile (**$C^{3}S$,** blue line), and 5-$HT_{1A}Cys^{417}$Ser, Cys^{420}Ser-receptor-$G_{o1}\alpha Cys^{3}$Ser, Cys^{351}Ile (**$C^{417}S$, $C^{420}S$, $C^{3}S$,** pink line) fusion proteins. Results are from triplicate determinations. Analysis is representative of three similar experiments.

Table 6.2

Comparison of 5-HT_{1A}-receptor-$G_{o1}\alpha Cys^{351}$Ile fusion protein expression levels and K_d for [^{3}H]-WAY100635 binding to 5-HT_{1A}-receptor-$G_{o1}\alpha Cys^{351}$Ile fusion proteins, determined by saturation binding analysis to membranes expressing the fusion proteins

The results from **Figure 6.3** were presented in a tabular form for clarity of comparison between the constructs. Non-linear regression analysis was used to determine the expression level (fmol/mg) and the equilibrium dissociation constant, K_d (nM) for each 5-HT_{1A}-receptor-$G_{o1}\alpha Cys^{351}$Ile fusion protein.

Figure 6.3

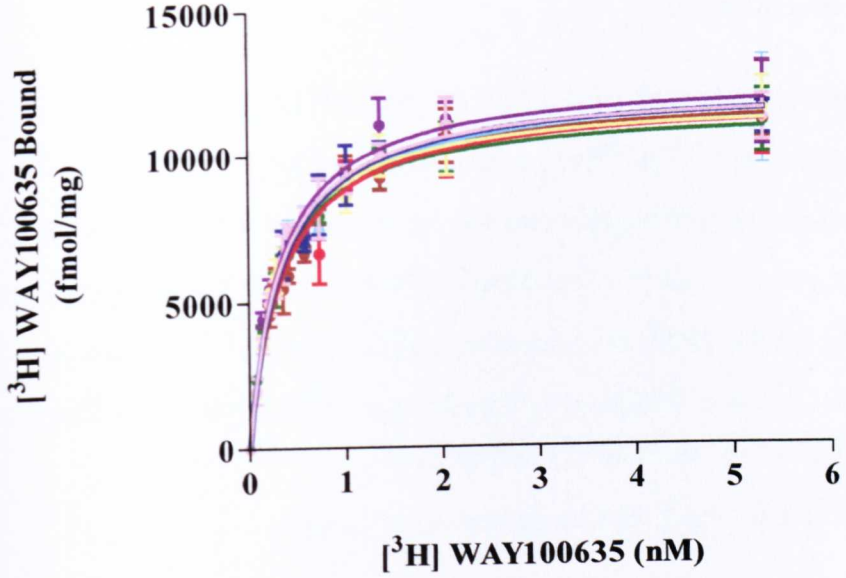


Table 6.2

Construct	Expression Level (fmol/mg)	K_d (nM) for [^{3}H]WAY100635
5-HT$_{1A}$-receptor-$G_{o1}\alpha$Cys^{351}Ile (**WT**)	11985 +/- 352	0.30 +/- 0.03
5-HT$_{1A}$ Cys^{417}Ser-receptor-$G_{o1}\alpha$Cys^{351}Ile (**C^{417}S**)	11561 +/- 179	0.30 +/- 0.02
5-HT$_{1A}$ Cys^{417}Ser-receptor-$G_{o1}\alpha$Cys^{3}Ser, Cys^{351}Ile (**C^{417}S, C^{3}S**)	12661 +/- 391	0.32 +/- 0.03
5-HT$_{1A}$ Cys^{420}Ser-receptor-$G_{o1}\alpha$Cys^{351}Ile (**C^{420}S**)	12186 +/- 393	0.38 +/- 0.04
5-HT$_{1A}$ Cys^{420}Ser-receptor-$G_{o1}\alpha$Cys^{3}Ser, Cys^{351}Ile (**C^{420}S, C^{3}S**)	11931 +/- 639	0.35 +/- 0.06
5-HT$_{1A}$ Cys^{417}Ser, Cys^{420}Ser-receptor-$G_{o1}\alpha$Cys^{351}Ile (**C^{417}S, C^{420}S**)	11863 +/- 329	0.31 +/- 0.03
5-HT$_{1A}$-receptor-$G_{o1}\alpha$Cys^{3}Ser, Cys^{351}Ile (**C^{3}S**)	12335 +/- 294	0.34 +/- 0.03
5-HT$_{1A}$ Cys^{417}Ser, Cys^{420}Ser-receptor-$G_{o1}\alpha$Cys^{3}Ser, Cys^{351}Ile (**C^{417}S, C^{420}S, C^{3}S**)	12310 +/- 251	0.33 +/- 0.02

Figure 6.4

Analysing levels of [^{35}S] GTPγS binding to various quantities of 5-HT$_{1A}$-receptor-G$_{o1}$αCys^{351}Ile fusion proteins

HEK293T cells were transfected with 5-HT$_{1A}$-receptor-G$_{o1}$αCys^{351}Ile (**WT**, turquoise line) or 5-HT$_{1A}$Cys^{417}Ser, Cys^{420}Ser-receptor-G$_{o1}$αCys^{3}Ser, Cys^{351}Ile (**C^{417}S, C^{420}S, C^{3}S**, pink line) fusion proteins. Membranes were prepared from these cells and samples containing various quantities (5-100 fmol) of [^{3}H]-WAY100635 binding sites were used to measure 100μM 8-OH-DPAT-stimulated binding of [^{35}S] GTPγS to the two fusion proteins during a 2.5 min incubation. Results are from triplicate determinations. Analysis is representative of three similar experiments.

Figure 6.4

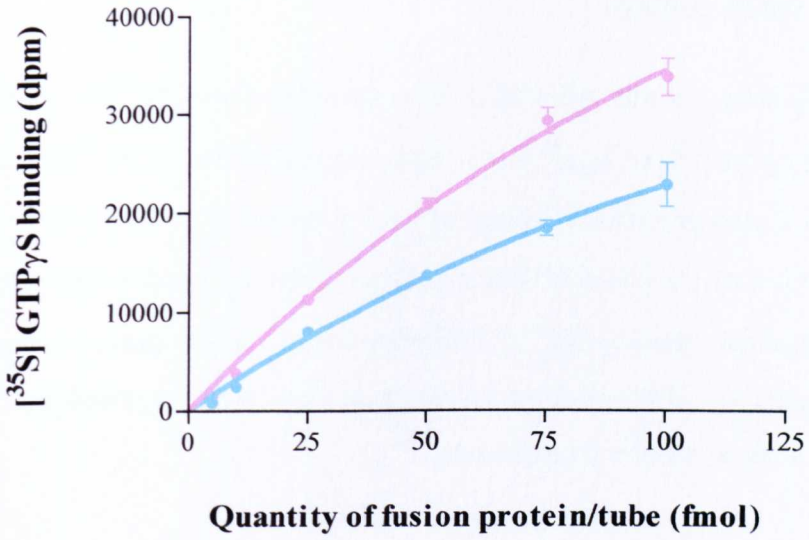

40000
30000
20000
10000
0
[35S] GTPγS binding (dpm)
0
25
50
75
100
125
Quantity of fusion protein/tube (fmol)

Figure 6.5

Analysing the time course of [^{35}S] GTPγS binding to 5-HT$_{1A}$-receptor-G$_{o1}$αCys^{351}Ile fusion proteins

HEK293T cells were transfected with 5-HT$_{1A}$-receptor-G$_{o1}$αCys^{351}Ile (**WT**, turquoise line) or 5-HT$_{1A}$Cys^{417}Ser, Cys^{420}Ser-receptor-G$_{o1}$αCys^{3}Ser, Cys^{351}Ile (**C^{417}S, C^{420}S, C^{3}S**, pink line) fusion proteins. Membranes were prepared from these cells and samples containing 10 fmol of [^{3}H]-WAY100635 binding sites were used to measure 100μM 8-OH-DPAT-stimulated binding of [^{35}S] GTPγS to the two fusion proteins over a range of incubation lengths (0.5-20 min). Results are from triplicate determinations. Analysis is representative of three similar experiments.

Figure 6.5

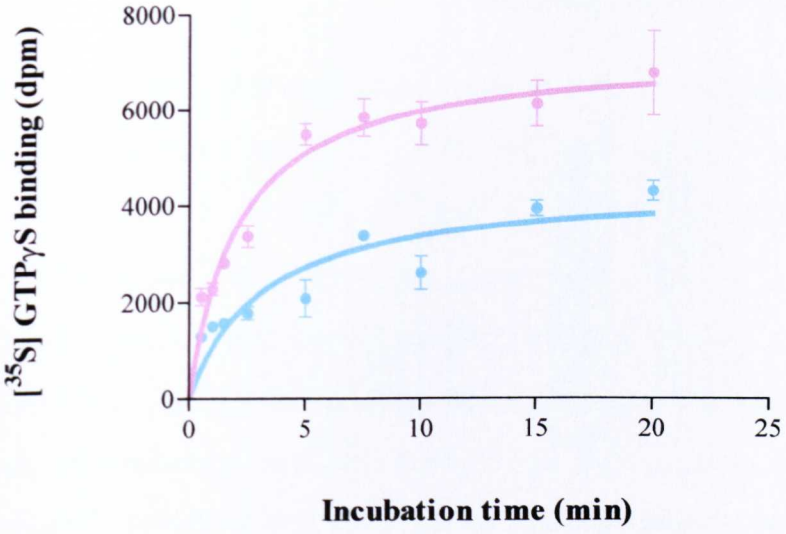

8000
6000
4000
2000
0
0
5
10
15
20
25
[35S] GTPγS binding (dpm)
Incubation time (min)

Figure 6.6

Analysis of the [^{35}S] GTPγS binding of four palmitoylation-variant 5-HT_{1A}-receptor-$G_{o1}\alpha Cys^{351}$Ile fusion proteins

HEK293T cells were transfected with empty vector (**pcDNA3,** black bars) or to express either 5-HT_{1A}-receptor-$G_{o1}\alpha Cys^{351}$Ile (**WT,** turquoise bars), 5-$HT_{1A}Cys^{417}$Ser, Cys^{420}Ser-receptor-$G_{o1}\alpha Cys^{351}$Ile (**$C^{417}S$, $C^{420}S$,** yellow bars), 5-HT_{1A}-receptor-$G_{o1}\alpha Cys^{3}$Ser, Cys^{351}Ile (**$C^{3}S$,** blue bars), and 5-$HT_{1A}Cys^{417}$Ser, Cys^{420}Ser-receptor-$G_{o1}\alpha Cys^{3}$Ser, Cys^{351}Ile (**$C^{417}S$, $C^{420}S$, $C^{3}S$,** pink bars) fusion proteins. Membranes were prepared from these cells and samples containing 10fmol of [^{3}H]-WAY100635 binding sites were used to measure basal or 100μM 8-OH-DPAT-stimulated binding of [^{35}S] GTPγS to the various fusion proteins during a 2.5 min incubation. Results are from triplicate determinations. Analysis is representative of three similar experiments.

Table 6.3

Comparison of basal and 8-OH-DPAT-stimulated [^{35}S] GTPγS binding of four palmitoylation-variant 5-HT_{1A}-receptor-$G_{o1}\alpha Cys^{351}$Ile fusion proteins

The results from **Figure 6.6** were presented in a tabular form for clarity of comparison between the constructs. The basal and 8-OH-DPAT-stimulated [^{35}S] GTPγS binding (dpm) of each fusion protein is given as mean +/- SEM (n=3).

Figure 6.6

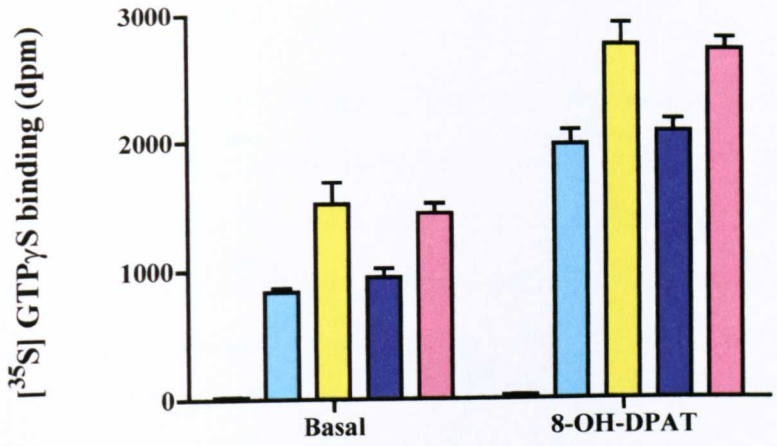


Table 6.3

Construct	Basal [^{35}S] GTPγS binding (dpm)	Adrenaline-stimulated [^{35}S] GTPγS binding (dpm)
5-HT$_{1A}$-receptor-G$_{o1}$αCys^{351}Ile (**WT**)	839 +/- 71	1979 +/- 115
5-HT$_{1A}$ Cys^{417}Ser, Cys^{420}Ser-receptor-G$_{o1}$αCys^{351}Ile (**C^{417}S, C^{420}S**)	1519 +/- 167	2765 +/- 171
5-HT$_{1A}$-receptor-G$_{o1}$αCys^{3}Ser, Cys^{351}Ile (**C^{3}S**)	949 +/- 67	2083 +/- 95
5-HT$_{1A}$ Cys^{417}Ser, Cys^{420}Ser-receptor-G$_{o1}$αCys^{3}Ser, Cys^{351}Ile (**C^{417}S, C^{420}S, C^{3}S**)	1445 +/- 155	2730 +/- 91

Figure 6.7

Analysing the effect of spiperone on basal [^{35}S] GTPγS binding to the 5-HT$_{1A}$-receptor-G$_{o1}$αCys^{351}Ile (WT) fusion protein

HEK293T cells were transfected with 5-HT$_{1A}$-receptor-G$_{o1}$αCys^{351}Ile (**WT**) fusion protein. Membranes were prepared from these cells and samples containing 10 fmol of [^{3}H]-WAY100635 binding sites were used to measure levels of basal (blue bar) or basal in the presence of 100μM spiperone (black bar) or 100μM 8-OH-DPAT-stimulated (red bar) binding of [^{35}S] GTPγS to the fusion protein. Results are from triplicate determinations. Analysis is representative of three similar experiments.

Table 6.4

Comparison of basal, basal in the presence of spiperone and 100μM 8-OH-DPAT-stimulated [^{35}S] GTPγS binding to the 5-HT$_{1A}$-receptor-G$_{o1}$αCys^{351}Ile (WT) fusion protein

The results from **Figure 6.7** were presented in a tabular form for clarity of comparison between the constructs. The binding of [^{35}S] GTPγS (dpm) to the fusion protein in all three conditions is given as mean +/- SEM (n=3).

Figure 6.7

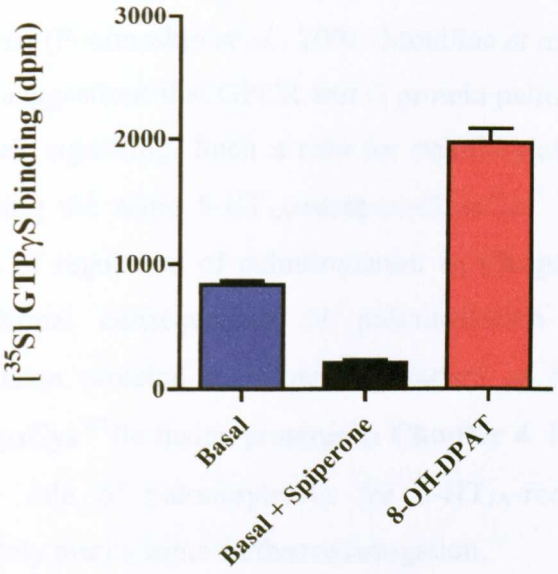


Table 6.4

Sample	GTPγS binding (dpm)
Basal	862 +/- 41
Basal + Spiperone	211 +/- 24
8-OH-DPAT	2012 +/- 103

6.3 Discussion

The observation of dynamic regulation of GPCR and G protein palmitoylation in response to agonist (Ponimaskin *et al.*, 2001; Mouillac *et al.*, 1992; Chen and Manning, 2000) has led to suggestions that GPCR and G protein palmitoylation may play a role in the control of cell signalling. Such a role for palmitoylation was investigated in the current work using the same 5-HT_{1A}-receptor-$G_{o1}\alpha Cys^{351}Ile$ fusion proteins already studied in terms of regulation of palmitoylation in **Chapter 5**. Due to limitations of time, the functional consequences of palmitoylation for the 5-HT_{1A}-receptor-$G_{o1}\alpha Cys^{351}Ile$ fusion proteins could not be studied as extensively as for the α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ fusion proteins in **Chapter 4**. However, it is clear from my results that the role of palmitoylation for 5-HT_{1A}-receptor-$G_{o1}\alpha Cys^{351}Ile$ fusion constructs definitely merits some further investigation.

All eight palmitoylation-variant 5-HT_{1A}-receptor-$G_{o1}\alpha Cys^{351}Ile$ fusion proteins were assessed in terms of expression levels and antagonist binding affinity, however, just four were selected to study activation of signalling via GTPγS binding studies. The four selected constructs were the 5-HT_{1A}-receptor-$G_{o1}\alpha Cys^{351}Ile$ (**WT**) construct (in which all three potential palmitoylation sites; two sites in the GPCR and one site in the G protein, are unaltered), the 5-HT_{1A}-receptor $Cys^{417}Ser$, $Cys^{420}Ser$-$G_{o1}\alpha Cys^{351}Ile$ (**$C^{417}S$, $C^{420}S$**) construct (which has the ability to undergo palmitoylation only in the G protein), the 5-HT_{1A}-receptor-$G_{o1}\alpha Cys^{3}Ser$, $Cys^{351}Ile$ (**$C^{3}S$**) construct (which has the potential to undergo palmitoylation only in the GPCR) and the 5-HT_{1A}-receptor $Cys^{417}Ser$, $Cys^{420}Ser$-$G_{o1}\alpha Cys^{3}Ser$, $Cys^{351}Ile$ (**$C^{417}S$, $C^{420}S$, $C^{3}S$**) construct (which has no potential palmitoylation sites).

The first functional property to be studied was the expression levels of the eight palmitoylation-variant fusion proteins. Fusion protein expression levels were assessed both by Western blot analysis and antagonist binding studies of membranes expressing the fusion proteins. Western blot analysis by use of either ON1 antiserum (against residues 1-16 of $G_{o1}\alpha$) or OC2 antiserum (against residues 345-354 of $G_{o1}\alpha$) revealed similar expression levels for all eight palmitoylation-variant 5-HT_{1A}-receptor-$G_{o1}\alpha Cys^{351}Ile$ fusion proteins. This observation was supported by the results from the

binding of a near-saturating (~5nM) concentration of [^{3}H]-WAY100635, as well as by saturation binding analysis (of 0.05-5nM) of the same radioligand to the fusions. From the antagonist binding studies, the expression levels of all eight palmitoylation-variant fusion proteins were found to be in the region of 12 pmol/mg. Similar results, where palmitoylation does not affect protein expression levels, have previously been observed with the α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ fusions (**Chapter 4**) and for the TRH receptor (Tanaka *et al.*, 1998). There have also been a number of contrasting observations, where lack of palmitoylation resulted in reduced expression levels. Such observations were made for the LH/hCG receptor (Zhu *et al.*, 1995), the vasopressin V_2 receptor (Schulein *et al.*, 1996) and the CCR_5 receptor (Percherancier *et al.*, 2001).

The next functional property to be studied for the eight palmitoylation-variant 5-HT_{1A}-receptor-$G_{o1}\alpha Cys^{351}Ile$ fusion proteins was the affinity for the antagonist [^{3}H]-WAY100635. Once again, similar to the observations for the α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ fusions (**Chapter 4**), the ability of neither the GPCR nor the Gα protein to be palmitoylated had any affect on the affinity of antagonist binding to the fusion proteins. The K_d for binding to all eight fusions was calculated to be approximately 0.33nM. Some similar studies have been performed for a number of GPCRs (Karnik *et al.*, 1993; Tanaka *et al.*, 1998; Hawtin *et al.*, 2001; Schulein *et al.*, 1996; Jin *et al.*, 1997), although most have assessed affinity for agonist molecules rather than antagonists. In general, it seems that replacement of palmitoylated cysteines does not change this pharmacological property of GPCRs.

Next, the importance of GPCR and G protein palmitylation was assessed for the activation of signalling. In the work of Papoucheva *et al.* (2004), the functional consequences of palmitoylation for the non-fused 5-HT_{1A}-receptor were explored. These authors found that replacement of either Cys^{417} or Cys^{420} of the GPCR led to a significantly reduced coupling of this GPCR to the $G_{i3}\alpha$ protein and that GPCR-G protein communication was completely abolished in the double mutant receptor (assessed by 5-HT-stimulated GTPγS binding studies). Furthermore, they found that palmitoylation-deficient mutants were unable to inhibit forskolin-stimulated cAMP formation and also reduced the receptor-dependent activation of extracellular signal-regulated kinase. Taken together, their results suggested roles for 5-HT_{1A}-receptor

palmitoylation in signalling through the Gβγ pathway as well as signalling through the $G_{i3}\alpha$ protein.

In the current study, after determination of appropriate experimental conditions of 10fmol fusion/assay and a 2.5 min incubation time, GTPγS binding was assessed in the presence and absence of 10^{-4}M 8-OH-DPAT for the four selected 5-HT_{1A}-receptor-$G_{o1}\alpha Cys^{351}Ile$ fusion proteins. In the presence of 10^{-4}M 8-OH-DPAT, GTPγS binding was stimulated approximately 2-fold over the high basal levels for all 5-HT_{1A}-receptor-$G_{o1}\alpha Cys^{351}Ile$ fusion proteins tested. However, approximately ~1.5 times higher levels of basal and 8-OH-DPAT-stimulated GTPγS binding were observed for the two fusions with mutated $5HT_{1A}$-receptors (the **$C^{417}S$, $C^{420}S$** and the **$C^{417}S$, $C^{420}S$, $C^{3}S$** fusions) than for either the wild type fusion or the fusion in which only the $G_{o1}\alpha$ protein has been mutated. This surprising result was in marked contrast to the results from the study by Papoucheva *et al.* (2004). The reasons for such a difference are difficult to ascertain. Perhaps one of the many differences in the experimental systems used for the two studies can be held in account. In the current study, which suggests that loss of palmitoylation of the 5-HT_{1A}-receptor may lead to enhanced signalling, experiments were performed with the 5-HT_{1A}-receptor-$G_{o1}\alpha Cys^{351}Ile$ fusion protein, whereas Papoucheva *et al.* performed their studies on the non-fused 5-HT_{1A}-receptor signalling through $G_{i3}\alpha$ (not $G_{o1}\alpha$). In addition there are a number of other experimental differences such as their use of Sf.9 insect cells whilst we have used HEK293T cells and their use of the murine receptor where we have used the human receptor. It is clear from these results that it will now be important to assess the role of palmitoylation of the non-fused human 5-HT_{1A}-receptor in HEK293T cells (still signalling through $G_{o1}\alpha Cys^{351}Ile$), in order to ensure that the fusion protein itself is not producing results differing from those obtained in a non-fused system.

In light of the high levels of basal GTPγS binding observed for all the 5-HT_{1A}-receptor-$G_{o1}\alpha Cys^{351}Ile$ fusion proteins used in the current study, we decided to perform one final experiment. Constitutive activity had previously been reported for 5-HT_{1A}-receptor-$G_{o}\alpha$ (Welsby *et al.*, 2002) and 5-HT_{1A}-receptor-$G_{i}\alpha$ (Kellett *et al.*, 1999) fusion proteins containing the $Cys^{351}Ile$ mutation in the Gα protein. It had also previously been reported that the inverse agonist spiperone (Newman-Tancredi *et al.*, 1997) could be used to

decrease the levels of constitutive activity for these fusions (Welsby *et al.*, 2002). In this study, the presence of 100μM spiperone was found to reduce basal levels of GTPγS binding to the 5-HT_{1A}-receptor-$G_{o1}\alpha Cys^{351}Ile$ (**WT**) fusion protein to approximately one tenth of the 8-OH-DPAT-stimulated levels (from approximately one half in the absence of spiperone). Thus the current study provided further support for the role of spiperone as an inverse agonist capable of reducing the constitutive activity of the 5-HT_{1A}-receptor-$G_{o1}\alpha Cys^{351}Ile$ fusion proteins.

If all the results obtained in this chapter are taken together we can see that palmitoylation of the $G_{o1}\alpha Cys^{351}Ile$ protein part of the 5-HT_{1A}-receptor-$G_{o1}\alpha Cys^{351}Ile$ fusion protein does not appear to be required for determination of the fusion protein expression levels, their affinity for the antagonist WAY100635, or their ability to bind GTP. Similarly, palmitoylation of the 5-HT_{1A}-receptor part of the 5-HT_{1A}-receptor-$G_{o1}\alpha Cys^{351}Ile$ fusion proteins, was not required for determination of fusion protein expression levels or their affinity for the antagonist [^{3}H]-WAY100635. In contrast, lack of palmitoylation in the 5-HT_{1A}-receptor part of the fusions did affect activation of signalling. In specific, the 5-HT_{1A}-receptor-$G_{o1}\alpha Cys^{351}Ile$ fusions, which were palmitoylation-deficient in the receptor portions, displayed enhanced levels of constitutive GTPγS binding.

Chapter 7

Final Discussion

7.0 Final Discussion

The G protein-coupled receptor (GPCR) family is involved in transduction of a wide variety of cell signals to intracellular enzymes and ion channels via guanine nucleotide binding proteins (G proteins). GPCR signalling is initiated by the binding of a specific ligand to the extracellular side of the receptor. Two such ligands for GPCRs are adrenaline: a catecholamine hormone released into the plasma at times of stress or increased energy need and serotonin: an important neurotransmitter and local hormone in the CNS and intestine.

In the current study, use was made of GPCR-G protein fusion proteins between either the α_{2A}-adrenoceptor or the 5-HT_{1A} serotonin receptor and the $G_{o1}\alpha Cys^{351}Ile$ G protein. The $Cys^{351}Ile$ mutation in the C-terminal tail of the fused $G_{o1}\alpha$ protein was desirable in order to perform functional studies without interference from endogenous G proteins (Jones and Reed, 1987; Lochrie and Simon, 1988; Burt *et al.*, 1998). Mutation of the Cys^{351} residue, renders the G protein resistant to ADP-ribosylation by pertussis toxin. Practically, this allows the endogenous $G_{o1}\alpha$ proteins to be inactivated by the toxin such that any remaining $G_{o1}\alpha$ protein signalling will be through the fused $G_{o1}\alpha Cys^{351}Ile$ protein only. Such fusion constructs have been useful tools to study the enzymatic capacity of G proteins, to measure ligand efficacy, and to evaluate receptor-coupling specificities of related G proteins (Seifert *at al.*, 1999; Milligan, 2000). In addition, these fusions have recently been used successfully to study the characteristics of GPCR and G protein palmitoylation (Stevens *et al.*, 2001).

Palmitate is a 16-carbon saturated fatty acid modification, attached to some (but not all) GPCRs and Gα proteins via a labile thioester linkage (Magee *et al.*, 1987). The lability of the thioester linkage means that palmitate attached via this bond can be readily removed and thus the reaction has the potential to be regulated (Mumby, 1997; Qanbar and Bouvier, 2003). Such a property may co-ordinate regulation of signalling, by determining either the location of proteins or their propensity to interact with other molecules. In addition, dynamic regulation of palmitoylation of some GPCRs and G proteins has been observed in response to stimulation by agonist (Ponimaskin *et al.*,

2001; Ng *et al.*, 1994; Mouillac *et al.*, 1992; Wedegartner and Bourne, 1994; Chen and Manning, 2000).

The observation of such agonist-regulated alterations in palmitoylation led to suggestions that GPCR and G protein palmitoylation may play important functional roles. Roles for Gα protein palmitoylation have been suggested in RGS protein-Gα protein interactions (Tu *et al.*, 1997; Ross and Wilkie, 2000) and membrane localisation/targeting of the Gα protein (Wedegaertner, 1998; Dunphy and Linder, 1998; Mumby, 1997; Song *et al.*, 1997), whereas roles for GPCR palmitoylation have mainly been implicated in the modulation of a number of functional properties such as GPCR-G protein interactions (Hayashi and Haga, 1997), GPCR phosphorylation and desensitisation (Moffett *et al.*, 1993) and GPCR downregulation (Kawate *et al.*, 1997; Munshi *et al.*, 2001).

For the purposes of this discussion, it is useful to summarise the regulation of palmitoylation for the $G_{o1}\alpha Cys^{351}Ile$ protein (from both the α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ and the 5-HT_{1A}-receptor-$G_{o1}\alpha Cys^{351}Ile$ fusion studies) separately from the results obtained for each GPCR.

When $G_{o1}\alpha Cys^{351}Ile$ was fused to either the α_{2A}-adrenoceptor or the 5-HT_{1A}-receptor, essentially the same patterns were observed for the regulation of its palmitoylation. In both cases the G protein dynamically incorporated [^{3}H] palmitate in a time-dependent manner and agonist-stimulation resulted in reduced levels of [^{3}H] palmitate incorporation. These results were similar to those previously obtained for a β_2-adrenoceptor-$G_s\alpha$ fusion protein (Loisel *et al.*, 1999).

The $G_{o1}\alpha Cys^{351}Ile$ part of both fusions proteins also displayed dynamic depalmitoylation and in the case with the α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ fusion protein, the rate of this depalmitoylation was significantly stimulated by agonist. In the study using the 5-HT_{1A}-receptor-$G_{o1}\alpha Cys^{351}Ile$ fusions, a similar effect was also observed. However, the experimental errors observed in that study meant that the implicated effect of agonist was not statistically significant. An 8-OH-DPAT-stimulated

rate of Gα protein depalmitoylation has been observed in a study with $G_i\alpha$ (Chen and Manning, 2000).

Agonist regulation of $G_{o1}\alpha Cys^{351}Ile$ protein palmitoylation was also observed to be concentration-dependent in both studies. For the α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ fusion, such regulation correlated with agonist occupancy of the receptor. Unfortunately due to time constraints, competition-binding analyses for the 5-HT_{1A}-receptor-$G_{o1}\alpha Cys^{351}Ile$ fusion proteins could not be performed. As a result, the degree of correlation between agonist occupancy of the 5-HT_{1A}-receptor and agonist regulation of palmitoylation could not be determined in the current study. Interestingly though, a very similar EC_{50} (~10 nM) was observed for 8-OH-DPAT regulated incorporation of [^{3}H] palmitate into the $G_i\alpha$ protein (Chen and Manning, 2000) as was observed in the current study with the 5-HT_{1A}-receptor fused $G_{o1}\alpha Cys^{351}Ile$ protein.

Another interesting observation made in the current study was that agonist regulation of palmitoylation required activation of the G protein. This was directly demonstrated in the current study by use of a $Gly^{204}Ala$ mutant of the $G_{o1}\alpha$ protein (which is incapable of binding GTP). Palmitoylation studies were performed for α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ proteins incorporating this mutation and although dynamic in that this form of the G protein did incorporate [^{3}H] palmitate, acylation of this form of the G protein was not regulated by agonist.

When the current regulation of palmitoylation results were compared with the results from previously published studies for other non-fused Gα proteins, opposite patterns of palmitate incorporation were observed for fused and non-fused Gα proteins. For a number of non-fused Gα proteins, including $G_i\alpha$ (Chen and Manning, 2000; Bhamre *et al.*1998) and $G_s\alpha$ (Wedegaertner and Bourne, 1995), agonist-stimulation led to increases in palmitate incorporation. This was in contrast to the decreases observed herein with the fusion proteins. Given the similar observations made upon comparison of [^{3}H] palmitate incorporation into fused and non-fused β_2-adrenoceptor and $G_s\alpha$ proteins (Loisel *et al.*, 1996, 1999), we decided to investigate whether constraint of the Gα protein in a fusion could affect the observed levels of [^{3}H] palmitate incorporation. This was assessed by the ability of the fusion protein to stimulate the endogenous

$G_{o1}\alpha$ protein. Agonist regulation of endogenous G protein palmitoylation was once again observed (both with adrenaline and with 8-OH-DPAT). However, in contrast to the results with the fused $G_{o1}\alpha Cys^{351}Ile$ protein, agonist led to an increase in incorporation of [^{3}H] palmitate into the endogenous G protein, thus indicating that some limitation of the fusion protein was responsible for the alternative pattern of regulated palmitoylation. In the study by Loisel *et al.* (1999) it had been reported that the ability of agonist-stimulated β_2-adrenoceptor-$G_s\alpha$ fusion proteins to become repalmitoylated was limited. In similar assays performed in the current study, the repalmitoylation of α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ and 5-HT_{1A}-receptor-$G_{o1}\alpha Cys^{351}Ile$ fusion proteins were also reduced in response to agonist. Therefore, the apparently opposite pattern of agonist-regulated incorporation of [^{3}H] palmitate into fused and non-fused $G_{o1}\alpha$ protein, may reflect some restriction of the fused protein to become repalmitoylated after depalmitoylation events. As already discussed, such a limitation does not necessarily mean that fusion proteins are unsuitable for palmitoylation studies; since results from pulse-labelling studies do not give us definitive descriptions of changes in palmitoylation anyway. Pulse-labelling studies represent a combination of both the palmitoylation and depalmitoylation reactions, meaning the only real conclusion to be drawn from such studies is whether agonist can regulate the palmitoylation status. The same conclusion is therefore still obtained by use of a fusion protein. Nevertheless it would be useful to explore the characteristics of the non-fused G protein further, to assess how well the findings of the current fusion study correlate with the regulation of palmitoylation in the endogenous G protein.

The current study also produced a number of different observations with regard to the regulation of GPCR palmitoylation. Firstly, the α_{2A}-adrenoceptor had previously been shown to incorporate palmitate into its Cys^{442} residue (Kennedy and Limbird, 1993, 1994), although it was reported that the half-life of [^{3}H] palmitate on the GPCR was in the region of 10 hours and was similar to the half-life of the protein. In addition, these authors reported only a very slight agonist stimulation of de-palmitoylation rate for the α_{2A}-adrenoceptor. In the current study however, a much shorter ($t_{1/2}$~30 min) half-life of [^{3}H] palmitate on the GPCR was observed in addition to no effects of the agonist adrenaline. To address these differences, I attempted a palmitoylation study with the non-fused α_{2A}-adrenoceptor. In the assay, a HA-tag present on the N-terminus of the

protein was used for immunoprecipitation. Unfortunately, the extent of immunoprecipitation achieved using the anti-HA antiserum was relatively poor in comparison with the ON1 antiserum used to immunoprecipitate the fusions. This resulted in no detectable signal for incorporation of [^{3}H] palmitate into the receptor with exposure times of up to one month. Consequently, differences in regulation of palmitoylation for fused and non-fused α_{2A}-adrenoceptor could not be addressed in the present study.

The observation of stable attachment of palmitate to the α_{2A}-adrenoceptor (Kennedy and Limbird, 1993, 1994) is similar to the scenario observed in a recent study of palmitoylation of the 5-HT$_{1A}$ receptor (Papoucheva *et al.*, 2004). These authors reported incorporation of palmitate into both the Cys^{417} and the Cys^{420} residues of the 5-HT$_{1A}$-receptor. However, rather than being a dynamically regulated post-translational modification, they reported that palmitate was incorporated into this GPCR early after receptor synthesis and once attached, was essentially irreversible. These authors also reported that palmitoylation efficiency at this GPCR was not modulated by receptor stimulation with agonists. In the current study, when the ability of palmitoylation-variant 5-HT$_{1A}$-receptor-G$_{o1}\alpha$Cys^{351}Ile constructs to incorporate [^{3}H] palmitic acid was assessed, palmitate was found to incorporate into the first receptor palmitoylation site (Cys^{417}) but not into the second (Cys^{420}) site of the 5-HT$_{1A}$-receptor. The lack of dynamic palmitate incorporation into the Cys^{420} site of the 5-HT$_{1A}$-receptor observed in these studies is therefore in direct contrast to the findings of Papoucheva *et al.* (2004).

It was also apparent from my study that the presence of the 5-HT$_{1A}$-receptor agonist 8-OH-DPAT led to altered levels of palmitate incorporation into the 5-HT$_{1A}$-receptor. This was observed both in pulse-labelling studies and in 8-OH-DPAT-stimulated concentration-response experiments, where 8-OH-DPAT stimulated incorporation of palmitate into the receptor with an EC$_{50}$ of ~14nM. From my pulse labelling studies I was able to confirm that whilst agonist enhanced the incorporation of palmitate, it did so without affecting the rate of palmitoylation.

Finally, upon analysis of 5-HT$_{1A}$-receptor-G$_{o1}\alpha$Cys^{351}Ile pulse-chase palmitoylation assays, a lack of 5-HT$_{1A}$-receptor depalmitoylation was observed over a three-hour

chase period. These depalmitoylation results were in agreement with the findings of Papoucheva *et al.* (2004), who suggested that palmitate was stably attached to the 5-HT_{1A}-receptor.

Taking my results from **Chapters 3** and **5** together I can conclude that the regulation of palmitoylation for the fused α_{2A}-adrenoceptor, the fused 5-HT_{1A}-receptor and the fused $G_{o1}\alpha$ protein are all slightly different. The fused α_{2A}-adrenoceptor is capable of dynamic palmitoylation as well as dynamic depalmitoylation at its Cys^{442} residue and neither processes are regulated by the adrenoceptor agonist adrenaline. In contrast, the 5-HT_{1A}-receptor is capable only of dynamic palmitoylation, not dynamic depalmitoylation. In addition, such dynamic palmitoylation only occurs at the Cys^{417} residue of this GPCR and can be regulated by the 5-HT_{1A}-receptor agonist 8-OH-DPAT. The Cys^{420} residue of the 5-HT_{1A}-receptor either does not incorporate palmitate or already has non-radiolabelled palmitate irreversibly attached to it prior to palmitoylation assays. Lastly, the fused $G_{o1}\alpha Cys^{351}Ile$ protein like the α_{2A}-adrenoceptor, can undergo dynamic palmitoylation as well as dynamic depalmitoylation at its Cys^{3} residue. However, unlike the α_{2A}-adrenoceptor and more like the 5-HT_{1A}-receptor, palmitoylation of the $G_{o1}\alpha Cys^{351}Ile$ protein can be regulated by the adrenoceptor agonist adrenaline or the 5-HT_{1A}-receptor agonist 8-OH-DPAT.

In **Chapters 4** and **6**, the requirements for GPCR and G protein palmitoylation of the α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ and 5-HT_{1A}-receptor-$G_{o1}\alpha Cys^{351}Ile$ fusion proteins were assessed for various functional properties. Palmitoylation of neither parts of the fusions were required to determine fusion protein expression levels or the affinity for antagonist molecules. Similar observations have previously been made for the TRH receptor (Tanaka *et al.*, 1998) and the dopamine D_1 receptor (Jin *et al.*, 1997). In addition for the fused α_{2A}-adrenoceptor or the $G_{o1}\alpha Cys^{351}Ile$ protein constrained to either GPCR, palmitoylation was not required for the ability to bind or to hydrolyse GTP or the ability to influence the efficiency of RGS16 protein to accelerate the GTPase reaction (latter two observations were only investigated for α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ fusions). These results were in support of the previous observation regarding the functional role of α_{2A}-adrenoceptor palmitoylation (Kennedy and Limbird, 1993) whereby mutation of palmitoylation sites did not alter GPCR function.

Therefore the role of palmitate attached to this GPCR must be for some other purpose. Similarly, the observation that agonist can regulate the palmitoylation of the $G_{o1}\alpha Cys^{351}Ile$ protein would seem to suggest some important role for palmitoylation in the functioning of this protein. Given that no such role has been determined in the current study, this modification must play an alternative role. One possible alternative role may be in membrane localisation/association. Agonist-promoted depalmitoylation of G proteins has been observed by many groups (Iiri *et al.*, 1996). It is thought that this phenomenon may allow deacylated protein to leave the caveolar membrane (Li *et al.*, 1995) and localise to either the cytoplasm (Wedegaertner *et al.*, 1996) or non-caveolar membrane (Huang *et al.*, 1999). It would therefore be interesting to test an unstimulated, an agonist-stimulated and a palmitoylation-deficient $G_{o1}\alpha$ protein, in an experiment such as a sucrose density gradient separation, to see if they localise to different regions within the cell.

In contrast to the observation that α_{2A}-adrenoceptor palmitoylation was not important for signalling, 5-HT_{1A}-receptor palmitoylation was found to be important for determining levels of constitutive activity of the 5-HT_{1A}-receptor-$G_{o1}\alpha Cys^{351}Ile$ fusion proteins. In the current study, mutation of the receptor palmitoylation sites led to enhanced levels of [^{35}S] GTPγS binding to the fusions. This observation was in contrast to the loss of GPCR-G protein communication reported upon replacement of Cys^{417} and Cys^{420} of the non-fused 5-HT_{1A}-receptor (Papoucheva *et al.*, 2004). Once again, the reasons for such a difference are tricky to explain and will require some further investigation. Due to the time constraints of the PhD, only a limited set of these functional experiments could be performed for the 5-HT_{1A}-receptor-$G_{o1}\alpha Cys^{351}Ile$ fusions. However, the demonstration of basal constitutive activity of the 5-HT_{1A}-receptor mutants is very interesting indeed and will undoubtedly be investigated further by the Molecular Pharmacology group at Glasgow University.

By taking all the results from the functional studies for α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ and 5-HT_{1A}-receptor-$G_{o1}\alpha Cys^{351}Ile$ fusion proteins together we can make a number of conclusions. Firstly, we can exclude any importance of α_{2A}-adrenoceptor and $G_{o1}\alpha Cys^{351}Ile$ protein palmitoylation in determining protein expression levels, the affinity for agonist or antagonist molecules, the ability to bind or to hydrolyse GTP or

the ability affect the efficiency of RGS proteins to accelerate the GTPase reaction. Similarly, we can exclude any importance of palmitate for 5-HT_{1A}-receptor protein expression levels or the affinity for the antagonist [^{3}H] WAY100635. We have however, identified a role for 5-HT_{1A}-receptor palmitoylation in determining levels of constitutive activity of the 5-HT_{1A}-receptor-$G_{o1}\alpha$Cys^{351}Ile fusion proteins and hope to continue this work in further studies.

During this study, the observation of different experimental results from studies using non-fused α_{2A}-adrenoceptor, 5-HT_{1A}-receptor and G_o has led to careful consideration of the use of fusion proteins for palmitoylation studies. I am very sceptical that such a variety of differences could be due solely to the fusion context of my study; given the large number of other experimental differences between the fusion-based and the non-fusion-based studies. However, this possibility cannot be excluded and will have to be addressed by a number of comparative experiments. Firstly, pulse labelling and pulse chase experiments with the non-fused receptors and $G_{o1}\alpha$ protein, would be very useful. Such experiments would allow the direct determination of whether it is the fusion protein which is responsible for the different results. Secondly, it may be beneficial to determine the extent of palmitate labelling and [^{35}S] methionine labelling in the presence of cycloheximide for comparison with the Papoucheva *et al.* (2004) studies.

In summary, the data from this thesis shows that a great deal of useful information can be obtained by the use of GPCR-G protein fusion proteins. They have been successfully used in this study to investigate a number of aspects of GPCR and G protein palmitoylation. As always, the results obtained from any model system must always be taken in context and if possible an attempt must be made to assess the relevance of those results to the situation *in vivo*. Although fusion proteins may have their limitations for palmitoylation studies one must also bear in mind some of the limitations which can be encountered studying palmitoylation in a non-fusion context. Perhaps in future investigations, a co-ordinated study in which the characteristics of palmitoylation are studied both in fused and non-fused proteins will be of use.

Chapter 8

Appendix

8.0 Appendix

8.1 cDNA sequences of α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ constructs

The cDNA sequence of each α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ fusion construct is given below. The GPCR portion is shown in red font. The G protein portion is shown in blue font. Potential palmitoylation sites are shown in underlined black font.

α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ **(WT)**

GGTACCATGT ATCCTTACGA CGTTCCTGAC TACGCACTAG TTCCTCGTAT GGGCTCCCTG
CAGCCGGAAG CGGGCAACGC GAGCTGGAAT GGGACAGAGG CGCCGGGGGG CGGCGCCCGG
GCCACCCCCT ACTCCCTGCA GGTGACACTG ACGCTGGTGT GCCTGGCCGG CCTGCTCATG
CTGTTCACCG TGTTCGGCAA CGTGCTTGTC ATCATTGCCG TGTTCACAAG CCGCGCGCTC
AAGGCGCCCC AGAACCTCTT CCTGGTGTCT CTGGCCTCGG CTGACATCCT AGTGGCCACG
CTTGTCATCC CTTTCTCGCT GGCCAACGAG GTCATGGGCT ACTGGTACTT CGGCAAGGCG
TGGTGTGAGA TCTACCTGGC GCTCGACGTG CTCTTCTGCA CGTCGTCCAT CGTGCACCTG
TGTGCCATCA GCTTGGATCG TTACTGGTCC ATCACCCAGG CCATAGAGTA CAACCTGAAG
CGCACGCCAC GCCGCATCAA AGCAATCATC GTCACCGTGT GGGTCATCTC GGCCGTCATC
TCCTTCCCGC CGCTCATCTC CATCGAGAAG AAGGCAGGCG GCGGTGGCCA GCAGCCGGCC
GAACCGCGCT GCGAGATCAA CGACCAGAAG TGGTACGTCA TCTCGTCTTG CATCGGCTCC
TTCTTCGCGC CCTGCCTCAT CATGATCCTG GTCTATGTGC GCATCTATCA GATAGCCAAG
CGCCGCACCC GCGTGCCGCC CAGCCGCCGG GGTCCTGATG CGGCCGCCGC GCTGCCGGGG
GGCGCCGAGC GCAGGCCCAA TGGCCTAGGC CCCGAGCGCG GCGTGGGTCG CGTGGGCGCC
GAGGCCGAGC CGCTACCCGT CCAGCTCAAC GGTGCCCCGG GGGAGCCCGC GCCCGCTGGG
CCCCGCGACG CTGACGGGCT GGACCTCGAG GAGAGCTCCT CGTCTGAGCA CGCCGAGCGG
CCCCCTGGGC CCCGCAGGTC CGAGCGCGGC CCTCGGGCCA AGAGCAAGGC TCGGGCGAGC
CAGGTAAAGC CCGGGGACAG CCTGCCACGG CGCGGGCCGG GGGCGCCCGG GCCGGGGGCG
CCCGCGACTG GGGCCGGGGA GGAGCGCGGC GGGGTCGCCA AGGCGTCGCG CTGGCGCGGA
AGGCAGAACC GCGAGAAGCG CTTCACTTTC GTGCTGGCGG TGGTCATAGG CGTGTTCGTG
GTCTGTTGGT TCCCCTTCTT CTTCACCTAT ACGCTCACGG CCGTAGGCTG CTCGGTGCCG
CCCACTCTCT TCAAGTTCTT CTTCTGGTTC GGCTACTGCA ACAGCTCGCT GAATCCGGTT
ATCTACACCA TCTTCAATCA CGACTTCCGC CGCGCCTTCA AGAAGATCCT CTGCCGTGGG
GACAGGAAAC GGATCGCCAT GGGATGTACT CTGAGCGCAG AGGAGAGAGC CGCCCTCGAG
CGGAGCAAGG CGATTGAGAA AAATCTCAAA GAAGATGGCA TCAGCGCCGC CAAAGACGTG
AAATTACTCC TGCTGGGGGC TGGAGAATCA GGAAAAAGCA CCATTGTGAA GCAGATGAAG
ATCATCCATG AAGATGGCTT CTCTGGAGAA GACGTAAAGC AGTACAAGCC TGTCGTCTAC
AGCAACACCA TCCAGTCTCT GGCAGCCATT GTGCGGGCCA TGGATACTCT GGGCGTGGAG
TATGGTGACA AGGAGAGGAA GGCAGACTCC AAGATGGTGT GTGACGTGGT GAGTCGCATG
GAGGACACTG AACCATTCTC TGCAGAACTG CTTTCTGCCA TGATGCGACT CTGGGGCGAC
TCGGGGATCC AGGAGTGCTT CAACCGATCT CGGGAGTATC AGCTCAACGA CTCTGCCAAA
TACTACCTGG ACAGCTTGGA TCGGATTGGA GCCGCTGACT ACCAGCCCAC CGAGCAGGAC
ATCCTCCGAA CCAGGGTCAA AACAACTGGC ATCGTAGAAA CCCACTTCAC CTTCAAGAAC
CTCCACTTCA GGCTGTTTGA CGTTGGGGGC CAGCGATCTG AACGTAAGAA GTGGATCCAC
TGCTTCGAGG ATGTCACGGC CATCATCTTC TGTGTCGCAC TCAGCGGCTA TGACCAGGTG
CTCCACGAGG ACGAAACCAC GAACCGCATG CACGAGTCTC TCATGCTCTT CGACTCCATC
TGTAACAACA AGTTTTTCAT CGATACCTCC ATCATTCTCT TCCTCAACAA GAAAGACCTC
TTTGGCGAGA AGATTAAGAA GTCACCCTTG ACCATCTGCT TTCCTGAATA CCCAGGCTCC
AACACCTATG AAGACGCAGC TGCCTACATC CAAACACAGT TTGAAAGCAA AAACCGCTCA
CCCAACAAAG AAATTTACTG TCACATGACT TGTGCCACAG ACACGAATAA TATCCAGGTG
GTATTCGACG CCGTCACCGA CATCATCATT GCCAACAATC TCCGGGGCTG TGGCTTGTAC
TGACCTCTTG TCCTGTATAG CAACCTATTT GACTGCTTCA TGGACTCTTT GCTGTTGATG
TTGATCTCCT GGTAGCATGA CCTTTGGCCT TTGTAAGACA CACAGCCTTT CTGTACCAAG
CCCCTGTCTA ACCTACGACC CCAGAGTGAC TGACGGCTGT GTATTTCTGT AGAATGCTGT
AGAATACGGT TTTAGTTGAG TCTTTACATT TAGAACTTGA AAGGATTTAA AAAAAAAAAA
ACATTTCTCA TGTGCTTTGT AGCTTTAAAA AGGAAAACTC ACCATTTCAT CCATATTTC

α_{2A}Cys^{442}Ala-adrenoceptor-$G_{o1}\alpha$Cys^{351}Ile ($C^{442}A$)

GGTACCATGT ATCCTTACGA CGTTCCTGAC TACGCACTAG TTCCTCGTAT GGGCTCCCTG
CAGCCGGAAG CGGGCAACGC GAGCTGGAAT GGGACAGAGG CGCCGGGGGG CGGCGCCCGG
GCCACCCCCT ACTCCCTGCA GGTGACACTG ACGCTGGTGT GCCTGGCCGG CCTGCTCATG
CTGTTCACCG TGTTCGGCAA CGTGCTTGTC ATCATTGCCG TGTTCACAAG CCGCGCGCTC
AAGGCGCCCC AGAACCTCTT CCTGGTGTCT CTGGCCTCGG CTGACATCCT AGTGGCCACG
CTTGTCATCC CTTTCTCGCT GGCCAACGAG GTCATGGGCT ACTGGTACTT CGGCAAGGCG
TGGTGTGAGA TCTACCTGGC GCTCGACGTG CTCTTCTGCA CGTCGTCCAT CGTGCACCTG
TGTGCCATCA GCTTGGATCG TTACTGGTCC ATCACCCAGG CCATAGAGTA CAACCTGAAG
CGCACGCCAC GCCGCATCAA AGCAATCATC GTCACCGTGT GGGTCATCTC GGCCGTCATC
TCCTTCCCGC CGCTCATCTC CATCGAGAAG AAGGCAGGCG GCGGTGGCCA GCAGCCGGCC
GAACCGCGCT GCGAGATCAA CGACCAGAAG TGGTACGTCA TCTCGTCTTG CATCGGCTCC
TTCTTCGCGC CCTGCCTCAT CATGATCCTG GTCTATGTGC GCATCTATCA GATAGCCAAG
CGCCGCACCC GCGTGCCGCC CAGCCGCCGG GGTCCTGATG CGGCCGCCGC GCTGCCGGGG
GGCGCCGAGC GCAGGCCCAA TGGCCTAGGC CCCGAGCGCG GCGTGGGTCG CGTGGGCGCC
GAGGCCGAGC CGCTACCCGT CCAGCTCAAC GGTGCCCCGG GGGAGCCCGC GCCCGCTGGG
CCCCGCGACG CTGACGGGCT GGACCTCGAG GAGAGCTCCT CGTCTGAGCA CGCCGAGCGG
CCCCCTGGGC CCCGCAGGTC CGAGCGCGGC CCTCGGGCCA AGAGCAAGGC TCGGGCGAGC
CAGGTAAAGC CCGGGGACAG CCTGCCACGG CGCGGGCCGG GGGCGCCCGG GCCGGGGGCG
CCCGCGACTG GGGCCGGGGA GGAGCGCGGC GGGGTCGCCA AGGCGTCGCG CTGGCGCGGA
AGGCAGAACC GCGAGAAGCG CTTCACTTTC GTGCTGGCGG TGGTCATAGG CGTGTTCGTG
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CCCACTCTCT TCAAGTTCTT CTTCTGGTTC GGCTACTGCA ACAGCTCGCT GAATCCGGTT
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CGGAGCAAGG CGATTGAGAA AAATCTCAAA GAAGATGGCA TCAGCGCCGC CAAAGACGTG
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GAGGACACTG AACCATTCTC TGCAGAACTG CTTTCTGCCA TGATGCGACT CTGGGGCGAC
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CTCCACGAGG ACGAAACCAC GAACCGCATG CACGAGTCTC TCATGCTCTT CGACTCCATC
TGTAACAACA AGTTTTTCAT CGATACCTCC ATCATTCTCT TCCTCAACAA GAAAGACCTC
TTTGGCGAGA AGATTAAGAA GTCACCCTTG ACCATCTGCT TTCCTGAATA CCCAGGCTCC
AACACCTATG AAGACGCAGC TGCCTACATC CAAACACAGT TTGAAAGCAA AAACCGCTCA
CCCAACAAAG AAATTTACTG TCACATGACT TGTGCCACAG ACACGAATAA TATCCAGGTG
GTATTCGACG CCGTCACCGA CATCATCATT GCCAACAATC TCCGGGGCTG TGGCTTGTAC
TGACCTCTTG TCCTGTATAG CAACCTATTT GACTGCTTCA TGGACTCTTT GCTGTTGATG
TTGATCTCCT GGTAGCATGA CCTTTGGCCT TTGTAAGACA CACAGCCTTT CTGTACCAAG
CCCCTGTCTA ACCTACGACC CCAGAGTGAC TGACGGCTGT GTATTTCTGT AGAATGCTGT
AGAATACGGT TTTAGTTGAG TCTTTACATT TAGAACTTGA AAGGATTTAA AAAAAAAAAA
ACATTTCTCA TGTGCTTTGT AGCTTTAAAA AGGAAAACTC ACCATTTCAT CCATATTTC

α_{2A}-adrenoceptor-$G_{o1}\alpha$Cys^{3}Ser,Cys^{351}Ile ($C^{3}S$)

GGTACCATGT ATCCTTACGA CGTTCCTGAC TACGCACTAG TTCCTCGTAT GGGCTCCCTG
CAGCCGGAAG CGGGCAACGC GAGCTGGAAT GGGACAGAGG CGCCGGGGGG CGGCGCCCGG
GCCACCCCCT ACTCCCTGCA GGTGACACTG ACGCTGGTGT GCCTGGCCGG CCTGCTCATG
CTGTTCACCG TGTTCGGCAA CGTGCTTGTC ATCATTGCCG TGTTCACAAG CCGCGCGCTC
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CTTGTCATCC CTTTCTCGCT GGCCAACGAG GTCATGGGCT ACTGGTACTT CGGCAAGGCG
TGGTGTGAGA TCTACCTGGC GCTCGACGTG CTCTTCTGCA CGTCGTCCAT CGTGCACCTG
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CGCACGCCAC GCCGCATCAA AGCAATCATC GTCACCGTGT GGGTCATCTC GGCCGTCATC
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GAACCGCGCT GCGAGATCAA CGACCAGAAG TGGTACGTCA TCTCGTCTTG CATCGGCTCC
TTCTTCGCGC CCTGCCTCAT CATGATCCTG GTCTATGTGC GCATCTATCA GATAGCCAAG
CGCCGCACCC GCGTGCCGCC CAGCCGCCGG GGTCCTGATG CGGCCGCCGC GCTGCCGGGG
GGCGCCGAGC GCAGGCCCAA TGGCCTAGGC CCCGAGCGCG GCGTGGGTCG CGTGGGCGCC
GAGGCCGAGC CGCTACCCGT CCAGCTCAAC GGTGCCCCGG GGGAGCCCGC GCCCGCTGGG
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GAGGACACTG AACCATTCTC TGCAGAACTG CTTTCTGCCA TGATGCGACT CTGGGGCGAC
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AGAATACGGT TTTAGTTGAG TCTTTACATT TAGAACTTGA AAGGATTTAA AAAAAAAAAA
ACATTTCTCA TGTGCTTTGT AGCTTTAAAA AGGAAAACTC ACCATTTCAT CCATATTTC

α_{2A}Cys^{442}Ala-adrenoceptor-$G_{o1}\alpha$Cys^{3}Ser, Cys^{351}Ile (C^{442}A, C^{3}S)

GGTACCATGT ATCCTTACGA CGTTCCTGAC TACGCACTAG TTCCTCGTAT GGGCTCCCTG
CAGCCGGAAG CGGGCAACGC GAGCTGGAAT GGGACAGAGG CGCCGGGGGG CGGCGCCCGG
GCCACCCCCT ACTCCCTGCA GGTGACACTG ACGCTGGTGT GCCTGGCCGG CCTGCTCATG
CTGTTCACCG TGTTCGGCAA CGTGCTTGTC ATCATTGCCG TGTTCACAAG CCGCGCGCTC
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CTTGTCATCC CTTTCTCGCT GGCCAACGAG GTCATGGGCT ACTGGTACTT CGGCAAGGCG
TGGTGTGAGA TCTACCTGGC GCTCGACGTG CTCTTCTGCA CGTCGTCCAT CGTGCACCTG
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CGCACGCCAC GCCGCATCAA AGCAATCATC GTCACCGTGT GGGTCATCTC GGCCGTCATC
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GAACCGCGCT GCGAGATCAA CGACCAGAAG TGGTACGTCA TCTCGTCTTG CATCGGCTCC
TTCTTCGCGC CCTGCCTCAT CATGATCCTG GTCTATGTGC GCATCTATCA GATAGCCAAG
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GAGGCCGAGC CGCTACCCGT CCAGCTCAAC GGTGCCCCGG GGGAGCCCGC GCCCGCTGGG
CCCCGCGACG CTGACGGGCT GGACCTCGAG GAGAGCTCCT CGTCTGAGCA CGCCGAGCGG
CCCCCTGGGC CCCGCAGGTC CGAGCGCGGC CCTCGGGCCA AGAGCAAGGC TCGGGCGAGC
CAGGTAAAGC CCGGGGACAG CCTGCCACGG CGCGGGCCGG GGGCGCCCGG GCCGGGGGCG
CCCGCGACTG GGGCCGGGGA GGAGCGCGGC GGGGTCGCCA AGGCGTCGCG CTGGCGCGGA
AGGCAGAACC GCGAGAAGCG CTTCACTTTC GTGCTGGCGG TGGTCATAGG CGTGTTCGTG
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CCCACTCTCT TCAAGTTCTT CTTCTGGTTC GGCTACTGCA ACAGCTCGCT GAATCCGGTT
ATCTACACCA TCTTCAATCA CGACTTCCGC CGCGCCTTCA AGAAGATCCT CGCACGTGGG
GACAGGAAAC GGATCGCCAT GGGAAGTACT CTGAGCGCAG AGGAGAGAGC CGCCCTCGAG
CGGAGCAAGG CGATTGAGAA AAATCTCAAA GAAGATGGCA TCAGCGCCGC CAAAGACGTG
AAATTACTCC TGCTGGGGGC TGGAGAATCA GGAAAAAGCA CCATTGTGAA GCAGATGAAG
ATCATCCATG AAGATGGCTT CTCTGGAGAA GACGTAAAGC AGTACAAGCC TGTCGTCTAC
AGCAACACCA TCCAGTCTCT GGCAGCCATT GTGCGGGCCA TGGATACTCT GGGCGTGGAG
TATGGTGACA AGGAGAGGAA GGCAGACTCC AAGATGGTGT GTGACGTGGT GAGTCGCATG
GAGGACACTG AACCATTCTC TGCAGAACTG CTTTCTGCCA TGATGCGACT CTGGGGCGAC
TCGGGGATCC AGGAGTGCTT CAACCGATCT CGGGAGTATC AGCTCAACGA CTCTGCCAAA
TACTACCTGG ACAGCTTGGA TCGGATTGGA GCCGCTGACT ACCAGCCCAC CGAGCAGGAC
ATCCTCCGAA CCAGGGTCAA AACAACTGGC ATCGTAGAAA CCCACTTCAC CTTCAAGAAC
CTCCACTTCA GGCTGTTTGA CGTTGGGGGC CAGCGATCTG AACGTAAGAA GTGGATCCAC
TGCTTCGAGG ATGTCACGGC CATCATCTTC TGTGTCGCAC TCAGCGGCTA TGACCAGGTG
CTCCACGAGG ACGAAACCAC GAACCGCATG CACGAGTCTC TCATGCTCTT CGACTCCATC
TGTAACAACA AGTTTTTCAT CGATACCTCC ATCATTCTCT TCCTCAACAA GAAAGACCTC
TTTGGCGAGA AGATTAAGAA GTCACCCTTG ACCATCTGCT TTCCTGAATA CCCAGGCTCC
AACACCTATG AAGACGCAGC TGCCTACATC CAAACACAGT TTGAAAGCAA AAACCGCTCA
CCCAACAAAG AAATTTACTG TCACATGACT TGTGCCACAG ACACGAATAA TATCCAGGTG
GTATTCGACG CCGTCACCGA CATCATCATT GCCAACAATC TCCGGGGCTG TGGCTTGTAC
TGACCTCTTG TCCTGTATAG CAACCTATTT GACTGCTTCA TGGACTCTTT GCTGTTGATG
TTGATCTCCT GGTAGCATGA CCTTTGGCCT TTGTAAGACA CACAGCCTTT CTGTACCAAG
CCCCTGTCTA ACCTACGACC CCAGAGTGAC TGACGGCTGT GTATTTCTGT AGAATGCTGT
AGAATACGGT TTTAGTTGAG TCTTTACATT TAGAACTTGA AAGGATTTAA AAAAAAAAAA
ACATTTCTCA TGTGCTTTGT AGCTTTAAAA AGGAAAACTC ACCATTTCAT CCATATTTC

8.2 cDNA sequences of 5-HT_{1A}-receptor-$G_{o1}\alpha Cys^{351}$Ile constructs

The cDNA sequence of each $5HT_{1A}$-receptor-$G_{o1}\alpha Cys^{351}$Ile fusion construct is given below. The GPCR portion is shown in red font. The G protein portion is shown in blue font. Linker region is shown in green font. Potential palmitoylation sites are shown in underlined black font.

5-HT_{1A}-receptor-$G_{o1}\alpha Cys^{351}$Ile **(WT)**

ATGGATGTGC TCAGCCCTGG TCAGGGCAAC AACACCACAT CACCACCGGC TCCCTTTGAG
ACCGGCGGCA ACACTACTGG TATCTCCGAC GTGACCGTCA GCTACCAAGT GATCACCTCT
CTGCTGCTGG GCACGCTCAT CTTCTGCGCG GTGCTGGGCA ATGCGTGCGT GGTGGCTGCC
ATCGCCTTGG AGCGCTCCCT GCAGAACGTG GCCAATTATC TTATTGGCTC TTTGGCGGTC
ACCGACCTCA TGGTGTCGGT GTTGGTGCTG CCCATGGCCG CGCTGTATCA GGTGCTCAAC
AAGTGGACAC TGGGCCAGGT AACCTGCGAC CTGTTCATCG CCCTCGACGT GCTGTGCTGC
ACCTCATCCA TCTTGCACCT GTGCGCCATC GCGCTGGACA GGTACTGGGC CATCACGGAC
CCCATCGACT ACGTGAACAA GAGGACGCCC CGGCGCGCCG CTGCGCTCAT CTCGCTCACT
TGGCTTATTG GCTTCCTCAT CTCTATCCCG CCCATGCTGG GCTGGCGCAC CCCGGAAGAC
CGCTCGGACC CCGACGCATG CACCATTAGC AAGGATCATG GCTACACTAT CTATTCCACC
TTTGGAGCTT TCTACATCCC GCTGCTGCTC ATGCTGGTTC TCTATGGGCG CATATTCCGA
GCTGCGCGCT TCCGCATCCG CAAGACGGTC AAAAAGGTGG AGAAGACCGG AGCGGACACC
CGCCATGGAG CATCTCCCGC CCCGCAGCCC AAGAAGAGTG TGAATGGAGA GTCGGGGAGC
AGGAACTGGA GGCTGGGCGT GGAGAGCAAG GCTGGGGGTG CTCTGTGCGC CAATGGCGCG
GTGAGGCAAG GTGACGATGG CGCCGCCCTG GAGGTGATCG AGGTGCACCG AGTGGGCAAC
TCCAAAGAGC ACTTGCCTCT GCCCAGCGAG GCTGGTCCTA CCCCTTGTGC CCCCGCCTCT
TTCGAGAGGA AAAATGAGCG CAACGCCGAG GCGAAGCGCA AGATGGCCCT GGCCCGAGAG
AGGAAGACAG TGAAGACGCT GGGCATCATC ATGGGCACCT TCATCCTCTG CTGGCTGCCC
TTCTTCATCG TGGCTCTTGT TCTGCCCTTC TGCGAGAGCA GCTGCCACAT GCCCACCCTG
TTGGGCGCCA TAATCAATTG GCTGGGCTAC TCCAACTCTC TGCTTAACCC CGTCATTTAC
GCATACTTCA ACAAGGACTT TCAAAACGCG TTTAAGAAGA TCATTAAGTG TAAGTTCTGC
CGCCAGGGAT CTATGGGATG TACTCTGAGC GCAGAGGAGA GAGCCGCCCT CGAGCGGAGC
AAGGCGATTG AGAAAAATCT CAAAGAAGAT GGCATCAGCG CCGCCAAAGA CGTGAAATTA
CTCCTGCTGG GGGCTGGAGA ATCAGGAAAA AGCACCATTG TGAAGCAGAT GAAGATCATC
CATGAAGATG GCTTCTCTGG AGAAGACGTA AAGCAGTACA AGCCTGTCGT CTACAGCAAC
ACCATCCAGT CTCTGGCAGC CATTGTGCGG GCCATGGATA CTCTGGGCGT GGAGTATGGT
GACAAGGAGA GGAAGGCAGA CTCCAAGATG GTGTGTGACG TGGTGAGTCG CATGGAGGAC
ACTGAACCAT TCTCTGCAGA ACTGCTTTCT GCCATGATGC GACTCTGGGG CGACTCGGGG
ATCCAGGAGT GCTTCAACCG ATCTCGGGAG TATCAGCTCA ACGACTCTGC CAAATACTAC
CTGGACAGCT TGGATCGGAT TGGAGCCGCT GACTACCAGC CCACCGAGCA GGACATCCTC
CGAACCAGGG TCAAAACAAC TGGCATCGTA GAAACCCACT TCACCTTCAA GAACCTCCAC
TTCAGGCTGT TTGACGTTGG GGGCCAGCGA TCTGAACGTA AGAAGTGGAT CCACTGCTTC
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GAGGACGAAA CCACGAACCG CATGCACGAG TCTCTCATGC TCTTCGACTC CATCTGTAAC
AACAAGTTTT TCATCGATAC CTCCATCATT CTCTTCCTCA ACAAGAAAGA CCTCTTTGGC
GAGAAGATTA AGAAGTCACC CTTGACCATC TGCTTTCCTG AATACCCAGG CTCCAACACC
TATGAAGACG CAGCTGCCTA CATCCAAACA CAGTTTGAAA GCAAAAACCG CTCACCCAAC
AAAGAAATTT ACTGTCACAT GACTTGTGCC ACAGACACGA ATAATATCCA GGTGGTATTC
GACGCCGTCA CCGACATCAT CATTGCCAAC AATCTCCGGG GCTGTGGCTT GTACTGA

5-HT_{1A}Cys^{417}Ser-receptor-$G_{o1}\alpha$Cys^{351}Ile ($C^{417}S$)

ATGGATGTGC TCAGCCCTGG TCAGGGCAAC AACACCACAT CACCACCGGC TCCCTTTGAG
ACCGGCGGCA ACACTACTGG TATCTCCGAC GTGACCGTCA GCTACCAAGT GATCACCTCT
CTGCTGCTGG GCACGCTCAT CTTCTGCGCG GTGCTGGGCA ATGCGTGCGT GGTGGCTGCC
ATCGCCTTGG AGCGCTCCCT GCAGAACGTG GCCAATTATC TTATTGGCTC TTTGGCGGTC
ACCGACCTCA TGGTGTCGGT GTTGGTGCTG CCCATGGCCG CGCTGTATCA GGTGCTCAAC
AAGTGGACAC TGGGCCAGGT AACCTGCGAC CTGTTCATCG CCCTCGACGT GCTGTGCTGC
ACCTCATCCA TCTTGCACCT GTGCGCCATC GCGCTGGACA GGTACTGGGC CATCACGGAC
CCCATCGACT ACGTGAACAA GAGGACGCCC CGGCGCGCCG CTGCGCTCAT CTCGCTCACT
TGGCTTATTG GCTTCCTCAT CTCTATCCCG CCCATGCTGG GCTGGCGCAC CCCGGAAGAC
CGCTCGGACC CCGACGCATG CACCATTAGC AAGGATCATG GCTACACTAT CTATTCCACC
TTTGGAGCTT TCTACATCCC GCTGCTGCTC ATGCTGGTTC TCTATGGGCG CATATTCCGA
GCTGCGCGCT TCCGCATCCG CAAGACGGTC AAAAAGGTGG AGAAGACCGG AGCGGACACC
CGCCATGGAG CATCTCCCGC CCCGCAGCCC AAGAAGAGTG TGAATGGAGA GTCGGGGAGC
AGGAACTGGA GGCTGGGCGT GGAGAGCAAG GCTGGGGGTG CTCTGTGCGC CAATGGCGCG
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TTCTTCATCG TGGCTCTTGT TCTGCCCTTC TGCGAGAGCA GCTGCCACAT GCCCACCCTG
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GAGGACGAAA CCACGAACCG CATGCACGAG TCTCTCATGC TCTTCGACTC CATCTGTAAC
AACAAGTTTT TCATCGATAC CTCCATCATT CTCTTCCTCA ACAAGAAAGA CCTCTTTGGC
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TATGAAGACG CAGCTGCCTA CATCCAAACA CAGTTTGAAA GCAAAAACCG CTCACCCAAC
AAAGAAATTT ACTGTCACAT GACTTGTGCC ACAGACACGA ATAATATCCA GGTGGTATTC
GACGCCGTCA CCGACATCAT CATTGCCAAC AATCTCCGGG GCTGTGGCTT GTACTGA

5-$HT_{1A}$$Cys^{417}$Ser-receptor-$G_{o1}\alpha$$Cys^{3}$Ser, Cys^{351}Ile ($C^{417}S$, $C^{3}S$)

ATGGATGTGC TCAGCCCTGG TCAGGGCAAC AACACCACAT CACCACCGGC TCCCTTTGAG
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ATCGCCTTGG AGCGCTCCCT GCAGAACGTG GCCAATTATC TTATTGGCTC TTTGGCGGTC
ACCGACCTCA TGGTGTCGGT GTTGGTGCTG CCCATGGCCG CGCTGTATCA GGTGCTCAAC
AAGTGGACAC TGGGCCAGGT AACCTGCGAC CTGTTCATCG CCCTCGACGT GCTGTGCTGC
ACCTCATCCA TCTTGCACCT GTGCGCCATC GCGCTGGACA GGTACTGGGC CATCACGGAC
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TGGCTTATTG GCTTCCTCAT CTCTATCCCG CCCATGCTGG GCTGGCGCAC CCCGGAAGAC
CGCTCGGACC CCGACGCATG CACCATTAGC AAGGATCATG GCTACACTAT CTATTCCACC
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GCTGCGCGCT TCCGCATCCG CAAGACGGTC AAAAAGGTGG AGAAGACCGG AGCGGACACC
CGCCATGGAG CATCTCCCGC CCCGCAGCCC AAGAAGAGTG TGAATGGAGA GTCGGGGAGC
AGGAACTGGA GGCTGGGCGT GGAGAGCAAG GCTGGGGGTG CTCTGTGCGC CAATGGCGCG
GTGAGGCAAG GTGACGATGG CGCCGCCCTG GAGGTGATCG AGGTGCACCG AGTGGGCAAC
TCCAAAGAGC ACTTGCCTCT GCCCAGCGAG GCTGGTCCTA CCCCTTGTGC CCCCGCCTCT
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AGGAAGACAG TGAAGACGCT GGGCATCATC ATGGGCACCT TCATCCTCTG CTGGCTGCCC
TTCTTCATCG TGGCTCTTGT TCTGCCCTTC TGCGAGAGCA GCTGCCACAT GCCCACCCTG
TTGGGCGCCA TAATCAATTG GCTGGGCTAC TCCAACTCTC TGCTTAACCC CGTCATTTAC
GCATACTTCA ACAAGGACTT TCAAAACGCG TTTAAGAAGA TCATTAAGTC TAAGTTCTGC
CGCCAGGGAT CTATGGGAAG TACTCTGAGC GCAGAGGAGA GAGCCGCCCT CGAGCGGAGC
AAGGCGATTG AGAAAAATCT CAAAGAAGAT GGCATCAGCG CCGCCAAAGA CGTGAAATTA
CTCCTGCTGG GGGCTGGAGA ATCAGGAAAA AGCACCATTG TGAAGCAGAT GAAGATCATC
CATGAAGATG GCTTCTCTGG AGAAGACGTA AAGCAGTACA AGCCTGTCGT CTACAGCAAC
ACCATCCAGT CTCTGGCAGC CATTGTGCGG GCCATGGATA CTCTGGGCGT GGAGTATGGT
GACAAGGAGA GGAAGGCAGA CTCCAAGATG GTGTGTGACG TGGTGAGTCG CATGGAGGAC
ACTGAACCAT TCTCTGCAGA ACTGCTTTCT GCCATGATGC GACTCTGGGG CGACTCGGGG
ATCCAGGAGT GCTTCAACCG ATCTCGGGAG TATCAGCTCA ACGACTCTGC CAAATACTAC
CTGGACAGCT TGGATCGGAT TGGAGCCGCT GACTACCAGC CCACCGAGCA GGACATCCTC
CGAACCAGGG TCAAAACAAC TGGCATCGTA GAAACCCACT TCACCTTCAA GAACCTCCAC
TTCAGGCTGT TTGACGTTGG GGGCCAGCGA TCTGAACGTA AGAAGTGGAT CCACTGCTTC
GAGGATGTCA CGGCCATCAT CTTCTGTGTC GCACTCAGCG GCTATGACCA GGTGCTCCAC
GAGGACGAAA CCACGAACCG CATGCACGAG TCTCTCATGC TCTTCGACTC CATCTGTAAC
AACAAGTTTT TCATCGATAC CTCCATCATT CTCTTCCTCA ACAAGAAAGA CCTCTTTGGC
GAGAAGATTA AGAAGTCACC CTTGACCATC TGCTTTCCTG AATACCCAGG CTCCAACACC
TATGAAGACG CAGCTGCCTA CATCCAAACA CAGTTTGAAA GCAAAAACCG CTCACCCAAC
AAAGAAATTT ACTGTCACAT GACTTGTGCC ACAGACACGA ATAATATCCA GGTGGTATTC
GACGCCGTCA CCGACATCAT CATTGCCAAC AATCTCCGGG GCTGTGGCTT GTACTGA

5-HT_{1A}Cys^{420}Ser-receptor-$G_{o1}\alpha$Cys^{351}Ile ($C^{420}S$)

ATGGATGTGC TCAGCCCTGG TCAGGGCAAC AACACCACAT CACCACCGGC TCCCTTTGAG
ACCGGCGGCA ACACTACTGG TATCTCCGAC GTGACCGTCA GCTACCAAGT GATCACCTCT
CTGCTGCTGG GCACGCTCAT CTTCTGCGCG GTGCTGGGCA ATGCGTGCGT GGTGGCTGCC
ATCGCCTTGG AGCGCTCCCT GCAGAACGTG GCCAATTATC TTATTGGCTC TTTGGCGGTC
ACCGACCTCA TGGTGTCGGT GTTGGTGCTG CCCATGGCCG CGCTGTATCA GGTGCTCAAC
AAGTGGACAC TGGGCCAGGT AACCTGCGAC CTGTTCATCG CCCTCGACGT GCTGTGCTGC
ACCTCATCCA TCTTGCACCT GTGCGCCATC GCGCTGGACA GGTACTGGGC CATCACGGAC
CCCATCGACT ACGTGAACAA GAGGACGCCC CGGCGCGCCG CTGCGCTCAT CTCGCTCACT
TGGCTTATTG GCTTCCTCAT CTCTATCCCG CCCATGCTGG GCTGGCGCAC CCCGGAAGAC
CGCTCGGACC CCGACGCATG CACCATTAGC AAGGATCATG GCTACACTAT CTATTCCACC
TTTGGAGCTT TCTACATCCC GCTGCTGCTC ATGCTGGTTC TCTATGGGCG CATATTCCGA
GCTGCGCGCT TCCGCATCCG CAAGACGGTC AAAAAGGTGG AGAAGACCGG AGCGGACACC
CGCCATGGAG CATCTCCCGC CCCGCAGCCC AAGAAGAGTG TGAATGGAGA GTCGGGGAGC
AGGAACTGGA GGCTGGGCGT GGAGAGCAAG GCTGGGGGTG CTCTGTGCGC CAATGGCGCG
GTGAGGCAAG GTGACGATGG CGCCGCCCTG GAGGTGATCG AGGTGCACCG AGTGGGCAAC
TCCAAAGAGC ACTTGCCTCT GCCCAGCGAG GCTGGTCCTA CCCCTTGTGC CCCCGCCTCT
TTCGAGAGGA AAAATGAGCG CAACGCCGAG GCGAAGCGCA AGATGGCCCT GGCCCGAGAG
AGGAAGACAG TGAAGACGCT GGGCATCATC ATGGGCACCT TCATCCTCTG CTGGCTGCCC
TTCTTCATCG TGGCTCTTGT TCTGCCCTTC TGCGAGAGCA GCTGCCACAT GCCCACCCTG
TTGGGCGCCA TAATCAATTG GCTGGGCTAC TCCAACTCTC TGCTTAACCC CGTCATTTAC
GCATACTTCA ACAAGGACTT TCAAAACGCG TTTAAGAAGA TCATTAAGTG TAAGTTCTCC
CGCCAGGGAT CTATGGGATG TACTCTGAGC GCAGAGGAGA GAGCCGCCCT CGAGCGGAGC
AAGGCGATTG AGAAAAATCT CAAAGAAGAT GGCATCAGCG CCGCCAAAGA CGTGAAATTA
CTCCTGCTGG GGGCTGGAGA ATCAGGAAAA AGCACCATTG TGAAGCAGAT GAAGATCATC
CATGAAGATG GCTTCTCTGG AGAAGACGTA AAGCAGTACA AGCCTGTCGT CTACAGCAAC
ACCATCCAGT CTCTGGCAGC CATTGTGCGG GCCATGGATA CTCTGGGCGT GGAGTATGGT
GACAAGGAGA GGAAGGCAGA CTCCAAGATG GTGTGTGACG TGGTGAGTCG CATGGAGGAC
ACTGAACCAT TCTCTGCAGA ACTGCTTTCT GCCATGATGC GACTCTGGGG CGACTCGGGG
ATCCAGGAGT GCTTCAACCG ATCTCGGGAG TATCAGCTCA ACGACTCTGC CAAATACTAC
CTGGACAGCT TGGATCGGAT TGGAGCCGCT GACTACCAGC CCACCGAGCA GGACATCCTC
CGAACCAGGG TCAAAACAAC TGGCATCGTA GAAACCCACT TCACCTTCAA GAACCTCCAC
TTCAGGCTGT TTGACGTTGG GGGCCAGCGA TCTGAACGTA AGAAGTGGAT CCACTGCTTC
GAGGATGTCA CGGCCATCAT CTTCTGTGTC GCACTCAGCG GCTATGACCA GGTGCTCCAC
GAGGACGAAA CCACGAACCG CATGCACGAG TCTCTCATGC TCTTCGACTC CATCTGTAAC
AACAAGTTTT TCATCGATAC CTCCATCATT CTCTTCCTCA ACAAGAAAGA CCTCTTTGGC
GAGAAGATTA AGAAGTCACC CTTGACCATC TGCTTTCCTG AATACCCAGG CTCCAACACC
TATGAAGACG CAGCTGCCTA CATCCAAACA CAGTTTGAAA GCAAAAACCG CTCACCCAAC
AAAGAAATTT ACTGTCACAT GACTTGTGCC ACAGACACGA ATAATATCCA GGTGGTATTC
GACGCCGTCA CCGACATCAT CATTGCCAAC AATCTCCGGG GCTGTGGCTT GTACTGA

5-$HT_{1A}$$Cys^{420}$Ser-receptor-$G_{o1}\alpha$$Cys^{3}$Ser, Cys^{351}Ile ($C^{420}S$, $C^{3}S$)

ATGGATGTGC TCAGCCCTGG TCAGGGCAAC AACACCACAT CACCACCGGC TCCCTTTGAG
ACCGGCGGCA ACACTACTGG TATCTCCGAC GTGACCGTCA GCTACCAAGT GATCACCTCT
CTGCTGCTGG GCACGCTCAT CTTCTGCGCG GTGCTGGGCA ATGCGTGCGT GGTGGCTGCC
ATCGCCTTGG AGCGCTCCCT GCAGAACGTG GCCAATTATC TTATTGGCTC TTTGGCGGTC
ACCGACCTCA TGGTGTCGGT GTTGGTGCTG CCCATGGCCG CGCTGTATCA GGTGCTCAAC
AAGTGGACAC TGGGCCAGGT AACCTGCGAC CTGTTCATCG CCCTCGACGT GCTGTGCTGC
ACCTCATCCA TCTTGCACCT GTGCGCCATC GCGCTGGACA GGTACTGGGC CATCACGGAC
CCCATCGACT ACGTGAACAA GAGGACGCCC CGGCGCGCCG CTGCGCTCAT CTCGCTCACT
TGGCTTATTG GCTTCCTCAT CTCTATCCCG CCCATGCTGG GCTGGCGCAC CCCGGAAGAC
CGCTCGGACC CCGACGCATG CACCATTAGC AAGGATCATG GCTACACTAT CTATTCCACC
TTTGGAGCTT TCTACATCCC GCTGCTGCTC ATGCTGGTTC TCTATGGGCG CATATTCCGA
GCTGCGCGCT TCCGCATCCG CAAGACGGTC AAAAAGGTGG AGAAGACCGG AGCGGACACC
CGCCATGGAG CATCTCCCGC CCCGCAGCCC AAGAAGAGTG TGAATGGAGA GTCGGGGAGC
AGGAACTGGA GGCTGGGCGT GGAGAGCAAG GCTGGGGGTG CTCTGTGCGC CAATGGCGCG
GTGAGGCAAG GTGACGATGG CGCCGCCCTG GAGGTGATCG AGGTGCACCG AGTGGGCAAC
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AGGAAGACAG TGAAGACGCT GGGCATCATC ATGGGCACCT TCATCCTCTG CTGGCTGCCC
TTCTTCATCG TGGCTCTTGT TCTGCCCTTC TGCGAGAGCA GCTGCCACAT GCCCACCCTG
TTGGGCGCCA TAATCAATTG GCTGGGCTAC TCCAACTCTC TGCTTAACCC CGTCATTTAC
GCATACTTCA ACAAGGACTT TCAAAACGCG TTTAAGAAGA TCATTAAGTG TAAGTTCTCC
CGCCAGGGAT CTATGGGAAG TACTCTGAGC GCAGAGGAGA GAGCCGCCCT CGAGCGGAGC
AAGGCGATTG AGAAAAATCT CAAAGAAGAT GGCATCAGCG CCGCCAAAGA CGTGAAATTA
CTCCTGCTGG GGGCTGGAGA ATCAGGAAAA AGCACCATTG TGAAGCAGAT GAAGATCATC
CATGAAGATG GCTTCTCTGG AGAAGACGTA AAGCAGTACA AGCCTGTCGT CTACAGCAAC
ACCATCCAGT CTCTGGCAGC CATTGTGCGG GCCATGGATA CTCTGGGCGT GGAGTATGGT
GACAAGGAGA GGAAGGCAGA CTCCAAGATG GTGTGTGACG TGGTGAGTCG CATGGAGGAC
ACTGAACCAT TCTCTGCAGA ACTGCTTTCT GCCATGATGC GACTCTGGGG CGACTCGGGG
ATCCAGGAGT GCTTCAACCG ATCTCGGGAG TATCAGCTCA ACGACTCTGC CAAATACTAC
CTGGACAGCT TGGATCGGAT TGGAGCCGCT GACTACCAGC CCACCGAGCA GGACATCCTC
CGAACCAGGG TCAAAACAAC TGGCATCGTA GAAACCCACT TCACCTTCAA GAACCTCCAC
TTCAGGCTGT TTGACGTTGG GGGCCAGCGA TCTGAACGTA AGAAGTGGAT CCACTGCTTC
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GAGGACGAAA CCACGAACCG CATGCACGAG TCTCTCATGC TCTTCGACTC CATCTGTAAC
AACAAGTTTT TCATCGATAC CTCCATCATT CTCTTCCTCA ACAAGAAAGA CCTCTTTGGC
GAGAAGATTA AGAAGTCACC CTTGACCATC TGCTTTCCTG AATACCCAGG CTCCAACACC
TATGAAGACG CAGCTGCCTA CATCCAAACA CAGTTTGAAA GCAAAAACCG CTCACCCAAC
AAAGAAATTT ACTGTCACAT GACTTGTGCC ACAGACACGA ATAATATCCA GGTGGTATTC
GACGCCGTCA CCGACATCAT CATTGCCAAC AATCTCCGGG GCTGTGGCTT GTACTGA

5-HT_{1A}Cys^{417}Ser, Cys^{420}Ser-receptor-$G_{o1}\alpha$Cys^{351}Ile ($C^{417}S$, $C^{420}S$)

ATGGATGTGC TCAGCCCTGG TCAGGGCAAC AACACCACAT CACCACCGGC TCCCTTTGAG
ACCGGCGGCA ACACTACTGG TATCTCCGAC GTGACCGTCA GCTACCAAGT GATCACCTCT
CTGCTGCTGG GCACGCTCAT CTTCTGCGCG GTGCTGGGCA ATGCGTGCGT GGTGGCTGCC
ATCGCCTTGG AGCGCTCCCT GCAGAACGTG GCCAATTATC TTATTGGCTC TTTGGCGGTC
ACCGACCTCA TGGTGTCGGT GTTGGTGCTG CCCATGGCCG CGCTGTATCA GGTGCTCAAC
AAGTGGACAC TGGGCCAGGT AACCTGCGAC CTGTTCATCG CCCTCGACGT GCTGTGCTGC
ACCTCATCCA TCTTGCACCT GTGCGCCATC GCGCTGGACA GGTACTGGGC CATCACGGAC
CCCATCGACT ACGTGAACAA GAGGACGCCC CGGCGCGCCG CTGCGCTCAT CTCGCTCACT
TGGCTTATTG GCTTCCTCAT CTCTATCCCG CCCATGCTGG GCTGGCGCAC CCCGGAAGAC
CGCTCGGACC CCGACGCATG CACCATTAGC AAGGATCATG GCTACACTAT CTATTCCACC
TTTGGAGCTT TCTACATCCC GCTGCTGCTC ATGCTGGTTC TCTATGGGCG CATATTCCGA
GCTGCGCGCT TCCGCATCCG CAAGACGGTC AAAAAGGTGG AGAAGACCGG AGCGGACACC
CGCCATGGAG CATCTCCCGC CCCGCAGCCC AAGAAGAGTG TGAATGGAGA GTCGGGGAGC
AGGAACTGGA GGCTGGGCGT GGAGAGCAAG GCTGGGGGTG CTCTGTGCGC CAATGGCGCG
GTGAGGCAAG GTGACGATGG CGCCGCCCTG GAGGTGATCG AGGTGCACCG AGTGGGCAAC
TCCAAAGAGC ACTTGCCTCT GCCCAGCGAG GCTGGTCCTA CCCCTTGTGC CCCCGCCTCT
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AGGAAGACAG TGAAGACGCT GGGCATCATC ATGGGCACCT TCATCCTCTG CTGGCTGCCC
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CGCCAGGGAT CTATGGGATG TACTCTGAGC GCAGAGGAGA GAGCCGCCCT CGAGCGGAGC
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ACCATCCAGT CTCTGGCAGC CATTGTGCGG GCCATGGATA CTCTGGGCGT GGAGTATGGT
GACAAGGAGA GGAAGGCAGA CTCCAAGATG GTGTGTGACG TGGTGAGTCG CATGGAGGAC
ACTGAACCAT TCTCTGCAGA ACTGCTTTCT GCCATGATGC GACTCTGGGG CGACTCGGGG
ATCCAGGAGT GCTTCAACCG ATCTCGGGAG TATCAGCTCA ACGACTCTGC CAAATACTAC
CTGGACAGCT TGGATCGGAT TGGAGCCGCT GACTACCAGC CCACCGAGCA GGACATCCTC
CGAACCAGGG TCAAAACAAC TGGCATCGTA GAAACCCACT TCACCTTCAA GAACCTCCAC
TTCAGGCTGT TTGACGTTGG GGGCCAGCGA TCTGAACGTA AGAAGTGGAT CCACTGCTTC
GAGGATGTCA CGGCCATCAT CTTCTGTGTC GCACTCAGCG GCTATGACCA GGTGCTCCAC
GAGGACGAAA CCACGAACCG CATGCACGAG TCTCTCATGC TCTTCGACTC CATCTGTAAC
AACAAGTTTT TCATCGATAC CTCCATCATT CTCTTCCTCA ACAAGAAAGA CCTCTTTGGC
GAGAAGATTA AGAAGTCACC CTTGACCATC TGCTTTCCTG AATACCCAGG CTCCAACACC
TATGAAGACG CAGCTGCCTA CATCCAAACA CAGTTTGAAA GCAAAAACCG CTCACCCAAC
AAAGAAATTT ACTGTCACAT GACTTGTGCC ACAGACACGA ATAATATCCA GGTGGTATTC
GACGCCGTCA CCGACATCAT CATTGCCAAC AATCTCCGGG GCTGTGGCTT GTACTGA

5-HT_{1A}-receptor-$G_{o1}\alpha Cys^{3}Ser, Cys^{351}Ile$ ($C^{3}S$)

ATGGATGTGC TCAGCCCTGG TCAGGGCAAC AACACCACAT CACCACCGGC TCCCTTTGAG
ACCGGCGGCA ACACTACTGG TATCTCCGAC GTGACCGTCA GCTACCAAGT GATCACCTCT
CTGCTGCTGG GCACGCTCAT CTTCTGCGCG GTGCTGGGCA ATGCGTGCGT GGTGGCTGCC
ATCGCCTTGG AGCGCTCCCT GCAGAACGTG GCCAATTATC TTATTGGCTC TTTGGCGGTC
ACCGACCTCA TGGTGTCGGT GTTGGTGCTG CCCATGGCCG CGCTGTATCA GGTGCTCAAC
AAGTGGACAC TGGGCCAGGT AACCTGCGAC CTGTTCATCG CCCTCGACGT GCTGTGCTGC
ACCTCATCCA TCTTGCACCT GTGCGCCATC GCGCTGGACA GGTACTGGGC CATCACGGAC
CCCATCGACT ACGTGAACAA GAGGACGCCC CGGCGCGCCG CTGCGCTCAT CTCGCTCACT
TGGCTTATTG GCTTCCTCAT CTCTATCCCG CCCATGCTGG GCTGGCGCAC CCCGGAAGAC
CGCTCGGACC CCGACGCATG CACCATTAGC AAGGATCATG GCTACACTAT CTATTCCACC
TTTGGAGCTT TCTACATCCC GCTGCTGCTC ATGCTGGTTC TCTATGGGCG CATATTCCGA
GCTGCGCGCT TCCGCATCCG CAAGACGGTC AAAAAGGTGG AGAAGACCGG AGCGGACACC
CGCCATGGAG CATCTCCCGC CCCGCAGCCC AAGAAGAGTG TGAATGGAGA GTCGGGGAGC
AGGAACTGGA GGCTGGGCGT GGAGAGCAAG GCTGGGGGTG CTCTGTGCGC CAATGGCGCG
GTGAGGCAAG GTGACGATGG CGCCGCCCTG GAGGTGATCG AGGTGCACCG AGTGGGCAAC
TCCAAAGAGC ACTTGCCTCT GCCCAGCGAG GCTGGTCCTA CCCCTTGTGC CCCCGCCTCT
TTCGAGAGGA AAAATGAGCG CAACGCCGAG GCGAAGCGCA AGATGGCCCT GGCCCGAGAG
AGGAAGACAG TGAAGACGCT GGGCATCATC ATGGGCACCT TCATCCTCTG CTGGCTGCCC
TTCTTCATCG TGGCTCTTGT TCTGCCCTTC TGCGAGAGCA GCTGCCACAT GCCCACCCTG
TTGGGCGCCA TAATCAATTG GCTGGGCTAC TCCAACTCTC TGCTTAACCC CGTCATTTAC
GCATACTTCA ACAAGGACTT TCAAAACGCG TTTAAGAAGA TCATTAAGTG TAAGTTCTGC
CGCCAGGGAT CTATGGGAAG TACTCTGAGC GCAGAGGAGA GAGCCGCCCT CGAGCGGAGC
AAGGCGATTG AGAAAAATCT CAAAGAAGAT GGCATCAGCG CCGCCAAAGA CGTGAAATTA
CTCCTGCTGG GGGCTGGAGA ATCAGGAAAA AGCACCATTG TGAAGCAGAT GAAGATCATC
CATGAAGATG GCTTCTCTGG AGAAGACGTA AAGCAGTACA AGCCTGTCGT CTACAGCAAC
ACCATCCAGT CTCTGGCAGC CATTGTGCGG GCCATGGATA CTCTGGGCGT GGAGTATGGT
GACAAGGAGA GGAAGGCAGA CTCCAAGATG GTGTGTGACG TGGTGAGTCG CATGGAGGAC
ACTGAACCAT TCTCTGCAGA ACTGCTTTCT GCCATGATGC GACTCTGGGG CGACTCGGGG
ATCCAGGAGT GCTTCAACCG ATCTCGGGAG TATCAGCTCA ACGACTCTGC CAAATACTAC
CTGGACAGCT TGGATCGGAT TGGAGCCGCT GACTACCAGC CCACCGAGCA GGACATCCTC
CGAACCAGGG TCAAAACAAC TGGCATCGTA GAAACCCACT TCACCTTCAA GAACCTCCAC
TTCAGGCTGT TTGACGTTGG GGGCCAGCGA TCTGAACGTA AGAAGTGGAT CCACTGCTTC
GAGGATGTCA CGGCCATCAT CTTCTGTGTC GCACTCAGCG GCTATGACCA GGTGCTCCAC
GAGGACGAAA CCACGAACCG CATGCACGAG TCTCTCATGC TCTTCGACTC CATCTGTAAC
AACAAGTTTT TCATCGATAC CTCCATCATT CTCTTCCTCA ACAAGAAAGA CCTCTTTGGC
GAGAAGATTA AGAAGTCACC CTTGACCATC TGCTTTCCTG AATACCCAGG CTCCAACACC
TATGAAGACG CAGCTGCCTA CATCCAAACA CAGTTTGAAA GCAAAAACCG CTCACCCAAC
AAAGAAATTT ACTGTCACAT GACTTGTGCC ACAGACACGA ATAATATCCA GGTGGTATTC
GACGCCGTCA CCGACATCAT CATTGCCAAC AATCTCCGGG GCTGTGGCTT GTACTGA

5-HT_{1A}Cys^{417}Ser, Cys^{420}Ser-receptor-$G_{o1}\alpha$Cys^{3}Ser, Cys^{351}Ile ($C^{417}S$, $C^{420}S$, $C^{3}S$)

ATGGATGTGC TCAGCCCTGG TCAGGGCAAC AACACCACAT CACCACCGGC TCCCTTTGAG
ACCGGCGGCA ACACTACTGG TATCTCCGAC GTGACCGTCA GCTACCAAGT GATCACCTCT
CTGCTGCTGG GCACGCTCAT CTTCTGCGCG GTGCTGGGCA ATGCGTGCGT GGTGGCTGCC
ATCGCCTTGG AGCGCTCCCT GCAGAACGTG GCCAATTATC TTATTGGCTC TTTGGCGGTC
ACCGACCTCA TGGTGTCGGT GTTGGTGCTG CCCATGGCCG CGCTGTATCA GGTGCTCAAC
AAGTGGACAC TGGGCCAGGT AACCTGCGAC CTGTTCATCG CCCTCGACGT GCTGTGCTGC
ACCTCATCCA TCTTGCACCT GTGCGCCATC GCGCTGGACA GGTACTGGGC CATCACGGAC
CCCATCGACT ACGTGAACAA GAGGACGCCC CGGCGCGCCG CTGCGCTCAT CTCGCTCACT
TGGCTTATTG GCTTCCTCAT CTCTATCCCG CCCATGCTGG GCTGGCGCAC CCCGGAAGAC
CGCTCGGACC CCGACGCATG CACCATTAGC AAGGATCATG GCTACACTAT CTATTCCACC
TTTGGAGCTT TCTACATCCC GCTGCTGCTC ATGCTGGTTC TCTATGGGCG CATATTCCGA
GCTGCGCGCT TCCGCATCCG CAAGACGGTC AAAAAGGTGG AGAAGACCGG AGCGGACACC
CGCCATGGAG CATCTCCCGC CCCGCAGCCC AAGAAGAGTG TGAATGGAGA GTCGGGGAGC
AGGAACTGGA GGCTGGGCGT GGAGAGCAAG GCTGGGGGTG CTCTGTGCGC CAATGGCGCG
GTGAGGCAAG GTGACGATGG CGCCGCCCTG GAGGTGATCG AGGTGCACCG AGTGGGCAAC
TCCAAAGAGC ACTTGCCTCT GCCCAGCGAG GCTGGTCCTA CCCCTTGTGC CCCCGCCTCT
TTCGAGAGGA AAAATGAGCG CAACGCCGAG GCGAAGCGCA AGATGGCCCT GGCCCGAGAG
AGGAAGACAG TGAAGACGCT GGGCATCATC ATGGGCACCT TCATCCTCTG CTGGCTGCCC
TTCTTCATCG TGGCTCTTGT TCTGCCCTTC TGCGAGAGCA GCTGCCACAT GCCCACCCTG
TTGGGCGCCA TAATCAATTG GCTGGGCTAC TCCAACTCTC TGCTTAACCC CGTCATTTAC
GCATACTTCA ACAAGGACTT TCAAAACGCG TTTAAGAAGA TCATTAAGTC TAAGTTCTCC
CGCCAGGGAT CTATGGGAAG TACTCTGAGC GCAGAGGAGA GAGCCGCCCT CGAGCGGAGC
AAGGCGATTG AGAAAAATCT CAAAGAAGAT GGCATCAGCG CCGCCAAAGA CGTGAAATTA
CTCCTGCTGG GGGCTGGAGA ATCAGGAAAA AGCACCATTG TGAAGCAGAT GAAGATCATC
CATGAAGATG GCTTCTCTGG AGAAGACGTA AAGCAGTACA AGCCTGTCGT CTACAGCAAC
ACCATCCAGT CTCTGGCAGC CATTGTGCGG GCCATGGATA CTCTGGGCGT GGAGTATGGT
GACAAGGAGA GGAAGGCAGA CTCCAAGATG GTGTGTGACG TGGTGAGTCG CATGGAGGAC
ACTGAACCAT TCTCTGCAGA ACTGCTTTCT GCCATGATGC GACTCTGGGG CGACTCGGGG
ATCCAGGAGT GCTTCAACCG ATCTCGGGAG TATCAGCTCA ACGACTCTGC CAAATACTAC
CTGGACAGCT TGGATCGGAT TGGAGCCGCT GACTACCAGC CCACCGAGCA GGACATCCTC
CGAACCAGGG TCAAAACAAC TGGCATCGTA GAAACCCACT TCACCTTCAA GAACCTCCAC
TTCAGGCTGT TTGACGTTGG GGGCCAGCGA TCTGAACGTA AGAAGTGGAT CCACTGCTTC
GAGGATGTCA CGGCCATCAT CTTCTGTGTC GCACTCAGCG GCTATGACCA GGTGCTCCAC
GAGGACGAAA CCACGAACCG CATGCACGAG TCTCTCATGC TCTTCGACTC CATCTGTAAC
AACAAGTTTT TCATCGATAC CTCCATCATT CTCTTCCTCA ACAAGAAAGA CCTCTTTGGC
GAGAAGATTA AGAAGTCACC CTTGACCATC TGCTTTCCTG AATACCCAGG CTCCAACACC
TATGAAGACG CAGCTGCCTA CATCCAAACA CAGTTTGAAA GCAAAAACCG CTCACCCAAC
AAAGAAATTT ACTGTCACAT GACTTGTGCC ACAGACACGA ATAATATCCA GGTGGTATTC
GACGCCGTCA CCGACATCAT CATTGCCAAC AATCTCCGGG GCTGTGGCTT GTACTGA

8.3 Amino acid sequences of α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ constructs

The amino acid sequence of each α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ fusion construct is given below. The GPCR portion is shown in red font. The G protein portion is shown in blue font. Potential palmitoylation sites are shown in underlined black font.

α_{2A}-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ **(WT)**

MGSLQPEAGNASWNGTEAPGGGARATPYSLQVTLTLVCLAGLLMLFTVFGNVLVIIAVF
TSRALKAPQNLFLVSLASADILVATLVIPFSLANEVMGYWYFGKAWCEIYLALDVLFCT
SSIVHLCAISLDRYWSITQAIEYNLKRTPRRIKAIIVTVWVISAVISFPPLISIEKKAG
GGGQQPAEPRCEINDQKWYVISSCIGSFFAPCLIMILVYVRIYQIAKRRTRVPPSRRGP
DAAAALPGGAERRPNGLGPERGVGRVGAEAEPLPVQLNGAPGEPAPAGPRDADGLDLEE
SSSSEHAERPPGPRRSERGPRAKSKARASQVKPGDSLPRRGPGAPGPGAPATGAGEERG
GVAKASRWRGRQNREKRFTFVLAVVIGVFVVCWFPFFFTYTLTAVGCSVPPTLFKFFFW
FGYCNSSLNPVIYTIFNHDFRRAFKKILCRGDRKRIVMGCTLSAEERAALERSKAIEKN
LKEDGISAAKDVKLLLLGAGESGKSTIVKQMKIIHEDGFSGEDVKQYKPVVYSNTIQSL
AAIVRAMDTLGVEYGDKERKADSKMVCDVVSRMEDTEPFSAELLSAMMRLWGDSGIQEC
FNRSREYQLNDSAKYYLDSLDRIGAADYQPTEQDILRTRVKTTGIVETHFTFKNLHFRL
FDVGGQRSERKKWIHCFEDVTAIIFCVALSGYDQVLHEDETTNRMHESLMLFDSICNNK
FFIDTSIILFLNKKDLFGEKIKKSPLTICFPEYPGSNTYEDAAAYIQTQFESKNRSPNK
EIYCHMTCATDTNNIQVVFDAVTDIIIANNLRGCGLY

$\alpha_{2A}Cys^{442}Ala$-adrenoceptor-$G_{o1}\alpha Cys^{351}Ile$ **($C^{442}A$)**

MGSLQPEAGNASWNGTEAPGGGARATPYSLQVTLTLVCLAGLLMLFTVFGNVLVIIAVF
TSRALKAPQNLFLVSLASADILVATLVIPFSLANEVMGYWYFGKAWCEIYLALDVLFCT
SSIVHLCAISLDRYWSITQAIEYNLKRTPRRIKAIIVTVWVISAVISFPPLISIEKKAG
GGGQQPAEPRCEINDQKWYVISSCIGSFFAPCLIMILVYVRIYQIAKRRTRVPPSRRGP
DAAAALPGGAERRPNGLGPERGVGRVGAEAEPLPVQLNGAPGEPAPAGPRDADGLDLEE
SSSSEHAERPPGPRRSERGPRAKSKARASQVKPGDSLPRRGPGAPGPGAPATGAGEERG
GVAKASRWRGRQNREKRFTFVLAVVIGVFVVCWFPFFFTYTLTAVGCSVPPTLFKFFFW
FGYCNSSLNPVIYTIFNHDFRRAFKKILARGDRKRIVMGCTLSAEERAALERSKAIEKN
LKEDGISAAKDVKLLLLGAGESGKSTIVKQMKIIHEDGFSGEDVKQYKPVVYSNTIQSL
AAIVRAMDTLGVEYGDKERKADSKMVCDVVSRMEDTEPFSAELLSAMMRLWGDSGIQEC
FNRSREYQLNDSAKYYLDSLDRIGAADYQPTEQDILRTRVKTTGIVETHFTFKNLHFRL
FDVGGQRSERKKWIHCFEDVTAIIFCVALSGYDQVLHEDETTNRMHESLMLFDSICNNK
FFIDTSIILFLNKKDLFGEKIKKSPLTICFPEYPGSNTYEDAAAYIQTQFESKNRSPNK
EIYCHMTCATDTNNIQVVFDAVTDIIIANNLRGCGLY

α_{2A}-adrenoceptor-$G_{o1}\alpha$Cys^{3}Ser,Cys^{351}Ile (**C^{3}S**)

MGSLQPEAGNASWNGTEAPGGGARATPYSLQVTLTLVCLAGLLMLFTVFGNVLVIIAVF
TSRALKAPQNLFLVSLASADILVATLVIPFSLANEVMGYWYFGKAWCEIYLALDVLFCT
SSIVHLCAISLDRYWSITQAIEYNLKRTPRRIKAIIVTVWVISAVISFPPLISIEKKAG
GGGQQPAEPRCEINDQKWYVISSCIGSFFAPCLIMILVYVRIYQIAKRRTRVPPSRRGP
DAAAALPGGAERRPNGLGPERGVGRVGAEAEPLPVQLNGAPGEPAPAGPRDADGLDLEE
SSSSEHAERPPGPRRSERGPRAKSKARASQVKPGDSLPRRGPGAPGPGAPATGAGEERG
GVAKASRWRGRQNREKRFTFVLAVVIGVFVVCWFPFFFTYTLTAVGCSVPPTLFKFFFW
FGYCNSSLNPVIYTIFNHDFRRAFKKILCRGDRKRIVMGSTLSAEERAALERSKAIEKN
LKEDGISAAKDVKLLLLGAGESGKSTIVKQMKIIHEDGFSGEDVKQYKPVVYSNTIQSL
AAIVRAMDTLGVEYGDKERKADSKMVCDVVSRMEDTEPFSAELLSAMMRLWGDSGIQEC
FNRSREYQLNDSAKYYLDSLDRIGAADYQPTEQDILRTRVKTTGIVETHFTFKNLHFRL
FDVGGQRSERKKWIHCFEDVTAIIFCVALSGYDQVLHEDETTNRMHESLMLFDSICNNK
FFIDTSIILFLNKKDLFGEKIKKSPLTICFPEYPGSNTYEDAAAYIQTQFESKNRSPNK
EIYCHMTCATDTNNIQVVFDAVTDIIIANNLRGCGLY

α_{2A}Cys^{442}Ala-adrenoceptor-$G_{o1}\alpha$Cys^{3}Ser, Cys^{351}Ile (**C^{442}A, C^{3}S**)

MGSLQPEAGNASWNGTEAPGGGARATPYSLQVTLTLVCLAGLLMLFTVFGNVLVIIAVF
TSRALKAPQNLFLVSLASADILVATLVIPFSLANEVMGYWYFGKAWCEIYLALDVLFCT
SSIVHLCAISLDRYWSITQAIEYNLKRTPRRIKAIIVTVWVISAVISFPPLISIEKKAG
GGGQQPAEPRCEINDQKWYVISSCIGSFFAPCLIMILVYVRIYQIAKRRTRVPPSRRGP
DAAAALPGGAERRPNGLGPERGVGRVGAEAEPLPVQLNGAPGEPAPAGPRDADGLDLEE
SSSSEHAERPPGPRRSERGPRAKSKARASQVKPGDSLPRRGPGAPGPGAPATGAGEERG
GVAKASRWRGRQNREKRFTFVLAVVIGVFVVCWFPFFFTYTLTAVGCSVPPTLFKFFFW
FGYCNSSLNPVIYTIFNHDFRRAFKKILARGDRKRIVMGSTLSAEERAALERSKAIEKN
LKEDGISAAKDVKLLLLGAGESGKSTIVKQMKIIHEDGFSGEDVKQYKPVVYSNTIQSL
AAIVRAMDTLGVEYGDKERKADSKMVCDVVSRMEDTEPFSAELLSAMMRLWGDSGIQEC
FNRSREYQLNDSAKYYLDSLDRIGAADYQPTEQDILRTRVKTTGIVETHFTFKNLHFRL
FDVGGQRSERKKWIHCFEDVTAIIFCVALSGYDQVLHEDETTNRMHESLMLFDSICNNK
FFIDTSIILFLNKKDLFGEKIKKSPLTICFPEYPGSNTYEDAAAYIQTQFESKNRSPNK
EIYCHMTCATDTNNIQVVFDAVTDIIIANNLRGCGLY

8.4 Amino acid sequences of 5-HT$_{1A}$-receptor-$G_{o1}\alpha$Cys^{351}Ile constructs

The amino acid sequence of each 5-HT$_{1A}$-receptor-$G_{o1}\alpha$Cys^{351}Ile fusion construct is given below. The GPCR portion is shown in red font. The G protein portion is shown in blue font. Linker residues are shown in green font. Potential palmitoylation sites are shown in underlined black font.

5-HT_{1A}-receptor-$G_{o1}\alpha Cys^{351}$Ile (**WT**)

MDVLSPGQGNNTTSPPAPFETGGNTTGISDVTVSYQVITSLLLGTLIFCAVLGNACVVA
AIALERSLQNVANYLIGSLAVTDLMVSVLVLPMAALYQVLNKWTLGQVTCDLFIALDVL
CCTSSILHLCAIALDRYWAITDPIDYVNKRTPRPRALISLTWLIGFLISIPPILGWRTP
EDRSDPDACTISKDHGYTIYSTFGAFYIPLLLMLVLYGRIFRAARFRIRKTVKKVEKTG
ADTRHGASPAPQPKKSVNGESGSRNWRLGVESKAGGALCANGAVRQGDDGAALEVIEVH
RVGNSKEHLPLPSEAGPTPCAPASFERKNERNAEAKRKMALARERKTVKTLGIIMGTFI
LCWLPFFIVALVLPFCESSCHMPTLLGAIINWLGYSNSLLNPVIYAYFNKDFQNAFKKI
IKCNFCRQGSMGCTLSAEERAALERSKAIEKNLKEDGISAAKDVKLLLLGAGESGKSTI
VKQMKIIHEDGFSGEDVKQYKPVVYSNTIQSLAAIVRAMDTLGVEYGDKERKADSKMVC
DVVSRMEDTEPFSAELLSAMMRLWGDSGIQECFNRSREYQLNDSAKYYLDSLDRIGAAD
YQPTEQDILRTRVKTTGIVETHFTFKNLHFRLFDVGGQRSERKKWIHCFEDVTAIIFCV
ALSGYDQVLHEDETTNRMHESLMLFDSICNNKFFIDTSIILFLNKKDLFGEKIKKSPLT
ICFPEYPGSNTYEDAAAYIQTQFESKNRSPNKEIYCHMTCATDTNNIQVVFDAVTDIII
ANNLRGCGLY

5-$HT_{1A}Cys^{417}$Ser-receptor-$G_{o1}\alpha Cys^{351}$Ile (**$C^{417}S$**)

MDVLSPGQGNNTTSPPAPFETGGNTTGISDVTVSYQVITSLLLGTLIFCAVLGNACVVA
AIALERSLQNVANYLIGSLAVTDLMVSVLVLPMAALYQVLNKWTLGQVTCDLFIALDVL
CCTSSILHLCAIALDRYWAITDPIDYVNKRTPRPRALISLTWLIGFLISIPPILGWRTP
EDRSDPDACTISKDHGYTIYSTFGAFYIPLLLMLVLYGRIFRAARFRIRKTVKKVEKTG
ADTRHGASPAPQPKKSVNGESGSRNWRLGVESKAGGALCANGAVRQGDDGAALEVIEVH
RVGNSKEHLPLPSEAGPTPCAPASFERKNERNAEAKRKMALARERKTVKTLGIIMGTFI
LCWLPFFIVALVLPFCESSCHMPTLLGAIINWLGYSNSLLNPVIYAYFNKDFQNAFKKI
IKSNFCRQGSMGCTLSAEERAALERSKAIEKNLKEDGISAAKDVKLLLLGAGESGKSTI
VKQMKIIHEDGFSGEDVKQYKPVVYSNTIQSLAAIVRAMDTLGVEYGDKERKADSKMVC
DVVSRMEDTEPFSAELLSAMMRLWGDSGIQECFNRSREYQLNDSAKYYLDSLDRIGAAD
YQPTEQDILRTRVKTTGIVETHFTFKNLHFRLFDVGGQRSERKKWIHCFEDVTAIIFCV
ALSGYDQVLHEDETTNRMHESLMLFDSICNNKFFIDTSIILFLNKKDLFGEKIKKSPLT
ICFPEYPGSNTYEDAAAYIQTQFESKNRSPNKEIYCHMTCATDTNNIQVVFDAVTDIII
ANNLRGCGLY

5-$HT_{1A}Cys^{417}$Ser-receptor-$G_{o1}\alpha Cys^{3}$Ser, Cys^{351}Ile (**$C^{417}S$, $C^{3}S$**)

MDVLSPGQGNNTTSPPAPFETGGNTTGISDVTVSYQVITSLLLGTLIFCAVLGNACVVA
AIALERSLQNVANYLIGSLAVTDLMVSVLVLPMAALYQVLNKWTLGQVTCDLFIALDVL
CCTSSILHLCAIALDRYWAITDPIDYVNKRTPRPRALISLTWLIGFLISIPPILGWRTP
EDRSDPDACTISKDHGYTIYSTFGAFYIPLLLMLVLYGRIFRAARFRIRKTVKKVEKTG
ADTRHGASPAPQPKKSVNGESGSRNWRLGVESKAGGALCANGAVRQGDDGAALEVIEVH
RVGNSKEHLPLPSEAGPTPCAPASFERKNERNAEAKRKMALARERKTVKTLGIIMGTFI
LCWLPFFIVALVLPFCESSCHMPTLLGAIINWLGYSNSLLNPVIYAYFNKDFQNAFKKI
IKSNFCRQGSMGSTLSAEERAALERSKAIEKNLKEDGISAAKDVKLLLLGAGESGKSTI
VKQMKIIHEDGFSGEDVKQYKPVVYSNTIQSLAAIVRAMDTLGVEYGDKERKADSKMVC
DVVSRMEDTEPFSAELLSAMMRLWGDSGIQECFNRSREYQLNDSAKYYLDSLDRIGAAD
YQPTEQDILRTRVKTTGIVETHFTFKNLHFRLFDVGGQRSERKKWIHCFEDVTAIIFCV
ALSGYDQVLHEDETTNRMHESLMLFDSICNNKFFIDTSIILFLNKKDLFGEKIKKSPLT
ICFPEYPGSNTYEDAAAYIQTQFESKNRSPNKEIYCHMTCATDTNNIQVVFDAVTDIII
ANNLRGCGLY

5-HT_{1A}Cys^{420}Ser-receptor-$G_{o1}\alpha$Cys^{351}Ile (**C^{420}S**)

MDVLSPGQGNNTTSPPAPFETGGNTTGISDVTVSYQVITSLLLGTLIFCAVLGNACVVA
AIALERSLQNVANYLIGSLAVTDLMVSVLVLPMAALYQVLNKWTLGQVTCDLFIALDVL
CCTSSILHLCAIALDRYWAITDPIDYVNKRTPRPRALISLTWLIGFLISIPPILGWRTP
EDRSDPDACTISKDHGYTIYSTFGAFYIPLLLMLVLYGRIFRAARFRIRKTVKKVEKTG
ADTRHGASPAPQPKKSVNGESGSRNWRLGVESKAGGALCANGAVRQGDDGAALEVIEVH
RVGNSKEHLPLPSEAGPTPCAPASFERKNERNAEAKRKMALARERKTVKTLGIIMGTFI
LCWLPFFIVALVLPFCESSCHMPTLLGAIINWLGYSNSLLNPVIYAYFNKDFQNAFKKI
IKCNFSRQGSMGCTLSAEERAALERSKAIEKNLKEDGISAAKDVKLLLLGAGESGKSTI
VKQMKIIHEDGFSGEDVKQYKPVVYSNTIQSLAAIVRAMDTLGVEYGDKERKADSKMVC
DVVSRMEDTEPFSAELLSAMMRLWGDSGIQECFNRSREYQLNDSAKYYLDSLDRIGAAD
YQPTEQDILRTRVKTTGIVETHFTFKNLHFRLFDVGGQRSERKKWIHCFEDVTAIIFCV
ALSGYDQVLHEDETTNRMHESLMLFDSICNNKFFIDTSIILFLNKKDLFGEKIKKSPLT
ICFPEYPGSNTYEDAAAYIQTQFESKNRSPNKEIYCHMTCATDTNNIQVVFDAVTDIII
ANNLRGCGLY

5-HT_{1A}Cys^{420}Ser-receptor-$G_{o1}\alpha$Cys^{3}Ser, Cys^{351}Ile (**C^{420}S, C^{3}S**)

MDVLSPGQGNNTTSPPAPFETGGNTTGISDVTVSYQVITSLLLGTLIFCAVLGNACVVA
AIALERSLQNVANYLIGSLAVTDLMVSVLVLPMAALYQVLNKWTLGQVTCDLFIALDVL
CCTSSILHLCAIALDRYWAITDPIDYVNKRTPRPRALISLTWLIGFLISIPPILGWRTP
EDRSDPDACTISKDHGYTIYSTFGAFYIPLLLMLVLYGRIFRAARFRIRKTVKKVEKTG
ADTRHGASPAPQPKKSVNGESGSRNWRLGVESKAGGALCANGAVRQGDDGAALEVIEVH
RVGNSKEHLPLPSEAGPTPCAPASFERKNERNAEAKRKMALARERKTVKTLGIIMGTFI
LCWLPFFIVALVLPFCESSCHMPTLLGAIINWLGYSNSLLNPVIYAYFNKDFQNAFKKI
IKCNFSRQGSMGSTLSAEERAALERSKAIEKNLKEDGISAAKDVKLLLLGAGESGKSTI
VKQMKIIHEDGFSGEDVKQYKPVVYSNTIQSLAAIVRAMDTLGVEYGDKERKADSKMVC
DVVSRMEDTEPFSAELLSAMMRLWGDSGIQECFNRSREYQLNDSAKYYLDSLDRIGAAD
YQPTEQDILRTRVKTTGIVETHFTFKNLHFRLFDVGGQRSERKKWIHCFEDVTAIIFCV
ALSGYDQVLHEDETTNRMHESLMLFDSICNNKFFIDTSIILFLNKKDLFGEKIKKSPLT
ICFPEYPGSNTYEDAAAYIQTQFESKNRSPNKEIYCHMTCATDTNNIQVVFDAVTDIII
ANNLRGCGLY

5-HT_{1A}Cys^{417}Ser, Cys^{420}Ser-receptor-$G_{o1}\alpha$Cys^{351}Ile (**C^{417}S, C^{420}S**)

MDVLSPGQGNNTTSPPAPFETGGNTTGISDVTVSYQVITSLLLGTLIFCAVLGNACVVA
AIALERSLQNVANYLIGSLAVTDLMVSVLVLPMAALYQVLNKWTLGQVTCDLFIALDVL
CCTSSILHLCAIALDRYWAITDPIDYVNKRTPRPRALISLTWLIGFLISIPPILGWRTP
EDRSDPDACTISKDHGYTIYSTFGAFYIPLLLMLVLYGRIFRAARFRIRKTVKKVEKTG
ADTRHGASPAPQPKKSVNGESGSRNWRLGVESKAGGALCANGAVRQGDDGAALEVIEVH
RVGNSKEHLPLPSEAGPTPCAPASFERKNERNAEAKRKMALARERKTVKTLGIIMGTFI
LCWLPFFIVALVLPFCESSCHMPTLLGAIINWLGYSNSLLNPVIYAYFNKDFQNAFKKI
IKSNFSRQGSMGCTLSAEERAALERSKAIEKNLKEDGISAAKDVKLLLLGAGESGKSTI
VKQMKIIHEDGFSGEDVKQYKPVVYSNTIQSLAAIVRAMDTLGVEYGDKERKADSKMVC
DVVSRMEDTEPFSAELLSAMMRLWGDSGIQECFNRSREYQLNDSAKYYLDSLDRIGAAD
YQPTEQDILRTRVKTTGIVETHFTFKNLHFRLFDVGGQRSERKKWIHCFEDVTAIIFCV
ALSGYDQVLHEDETTNRMHESLMLFDSICNNKFFIDTSIILFLNKKDLFGEKIKKSPLT
ICFPEYPGSNTYEDAAAYIQTQFESKNRSPNKEIYCHMTCATDTNNIQVVFDAVTDIII
ANNLRGCGLY

5-HT_{1A}-receptor-$G_{o1}\alpha$$Cys^{3}$Ser, Cys^{351}Ile (**$C^{3}S$**)

MDVLSPGQGNNTTSPPAPFETGGNTTGISDVTVSYQVITSLLLGTLIFCAVLGNACVVA
AIALERSLQNVANYLIGSLAVTDLMVSVLVLPMAALYQVLNKWTLGQVTCDLFIALDVL
CCTSSILHLCAIALDRYWAITDPIDYVNKRTPRPRALISLTWLIGFLISIPPILGWRTP
EDRSDPDACTISKDHGYTIYSTFGAFYIPLLLMLVLYGRIFRAARFRIRKTVKKVEKTG
ADTRHGASPAPQPKKSVNGESGSRNWRLGVESKAGGALCANGAVRQGDDGAALEVIEVH
RVGNSKEHLPLPSEAGPTPCAPASFERKNERNAEAKRKMALARERKTVKTLGIIMGTFI
LCWLPFFIVALVLPFCESSCHMPTLLGAIINWLGYSNSLLNPVIYAYFNKDFQNAFKKI
IKCNFCRQGSMGSTLSAEERAALERSKAIEKNLKEDGISAAKDVKLLLLGAGESGKSTI
VKQMKIIHEDGFSGEDVKQYKPVVYSNTIQSLAAIVRAMDTLGVEYGDKERKADSKMVC
DVVSRMEDTEPFSAELLSAMMRLWGDSGIQECFNRSREYQLNDSAKYYLDSLDRIGAAD
YQPTEQDILRTRVKTTGIVETHFTFKNLHFRLFDVGGQRSERKKWIHCFEDVTAIIFCV
ALSGYDQVLHEDETTNRMHESLMLFDSICNNKFFIDTSIILFLNKKDLFGEKIKKSPLT
ICFPEYPGSNTYEDAAAYIQTQFESKNRSPNKEIYCHMTCATDTNNIQVVFDAVTDIII
ANNLRGCGLY

5-$HT_{1A}$$Cys^{417}$Ser, Cys^{420}Ser-receptor-$G_{o1}\alpha$$Cys^{3}$Ser, Cys^{351}Ile (**$C^{417}S$, $C^{420}S$, $C^{3}S$**)

MDVLSPGQGNNTTSPPAPFETGGNTTGISDVTVSYQVITSLLLGTLIFCAVLGNACVVA
AIALERSLQNVANYLIGSLAVTDLMVSVLVLPMAALYQVLNKWTLGQVTCDLFIALDVL
CCTSSILHLCAIALDRYWAITDPIDYVNKRTPRPRALISLTWLIGFLISIPPILGWRTP
EDRSDPDACTISKDHGYTIYSTFGAFYIPLLLMLVLYGRIFRAARFRIRKTVKKVEKTG
ADTRHGASPAPQPKKSVNGESGSRNWRLGVESKAGGALCANGAVRQGDDGAALEVIEVH
RVGNSKEHLPLPSEAGPTPCAPASFERKNERNAEAKRKMALARERKTVKTLGIIMGTFI
LCWLPFFIVALVLPFCESSCHMPTLLGAIINWLGYSNSLLNPVIYAYFNKDFQNAFKKI
IKSNFSRQGSMGSTLSAEERAALERSKAIEKNLKEDGISAAKDVKLLLLGAGESGKSTI
VKQMKIIHEDGFSGEDVKQYKPVVYSNTIQSLAAIVRAMDTLGVEYGDKERKADSKMVC
DVVSRMEDTEPFSAELLSAMMRLWGDSGIQECFNRSREYQLNDSAKYYLDSLDRIGAAD
YQPTEQDILRTRVKTTGIVETHFTFKNLHFRLFDVGGQRSERKKWIHCFEDVTAIIFCV
ALSGYDQVLHEDETTNRMHESLMLFDSICNNKFFIDTSIILFLNKKDLFGEKIKKSPLT
ICFPEYPGSNTYEDAAAYIQTQFESKNRSPNKEIYCHMTCATDTNNIQVVFDAVTDIII
ANNLRGCGLY

8.5 B_{max} calculation

The first step is the calculation of the concentration of the radioligand added to each reaction from a vial that received only radioligand:

DPM/DPM per fmol = fmol added per reaction

fmol added/assay volume = concentration of radioligand added (nM)

The second step is to calculate expression level using the specific counts per reaction and correcting for that amount of protein added:

(specific binding counts (DPM)) / (DPM/fmol) x 1000/μg protein added
= expression in fmol/mg of protein

8.6 K_i calculation

Calculation of K_i uses a derivation of the Cheng-Prusoff equation (Cheng and Prusoff 1973):

$$K_i = IC_{50}/(1+L/K_d)$$

Where L is the ligand concentration, K_d is the equilibrium dissociation constant, and the IC_{50} is the concentration of inhibitor required to inhibit half the specific binding.

8.7 GTPase Eadie Hofstee calculation

This analysis of high affinity GTPase activity data allows the calculation of both the V_{max} and the K_m for the GTPase reaction. It requires the assay of GTPase activity over a range of GTP concentrations (25nM – 3000nM approximately).

For each assay the concentration of GTP in each triplicate reaction is first worked out. The first triplicate reaction receives only GTP from the [^{32}P]-GTP in the reaction mixture. This varies and is defined by the half-life of ^{32}P of 14 days and is calculated for each experiment. The second triplicate reaction receives a saturating concentration of GTP that defines non-specific GTP hydrolysis. Further treatments range from the addition of 25nM to 3000nM "cold" GTP.

The CPM values from each reaction are meaned and the standard deviation calculated. The non-specific GTPase activity defined by the "High GTP" treatment are subtracted from the mean of each triplicate sample identifying the Specific CPM and the standard deviation for these mean results.

The next step corrects for the ratio between the [^{32}P]-GTP and the "cold" GTP:

Specific CPM x (total [GTP](nM)/[^{32}P-GTP](nM)) = corrected CPM

Followed by calculating the concentration of GTP hydrolysed:

Corrected CPM/CPM per pmol = pmols

The V (rate/velocity) of the reaction was calculated as follows:

(1000/1.5μg protein) x (1/40minutes) x (1000/300μl) = V

Where 1.5μg of protein was added to each reaction that was incubated for 40 minutes, from which a final volume of 300μl was counted on the Topcount.

In order to calculate the K_m using an Eadie Hofstee plot, V/S was calculated. This is simply V (pmol/mg/min) divided by the total GTP concentration (nM) in the reaction.

Chapter 9

References

9.0 References

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