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**The role of the C-terminus in defining the efficiency and
specificity of G-protein coupling**

A THESIS PRESENTED FOR

THE DEGREE OF

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BY

DALJIT SINGH BAHIA

DIVISION OF BIOCHEMISTRY AND MOLECULAR BIOLOGY

INSTITUTE OF BIOMEDICAL AND LIFE SCIENCES

UNIVERSITY OF GLASGOW

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Abstract

In combination with conferring resistance to ADP-ribosylation by Pertussis toxin, the substitution of a conserved cysteine residue (C351) four amino acids from the C-terminus of $G_{i\alpha 1}$ has been shown to modulate the efficiency of coupling to the α_2A adrenoceptor. Investigation of this phenomenon through systematic substitution of this cysteine residue for all other amino acids highlighted a relationship between the hydrophobicity of the substituted residue and the capacity of the α -subunit to functionally couple to the α_2A adrenoceptor. From the results of this investigation, it was noted that wild type $G_{i\alpha 1}$ did not display optimal coupling at this receptor. Relative to wild type $G_{i\alpha 1}$, coupling was enhanced by the substitution of a more hydrophobic residue at position C351, but diminished upon the substitution of a more hydrophilic residue. In contrast to this, substitution of proline or a charged residue at this position essentially attenuated functional coupling with the α_2A adrenoceptor. Similarly, pEC_{50} values of the mutants also showed a high degree of correlation with the hydrophobic nature of the substituted residue, with more hydrophobic residues reducing pEC_{50} values and more hydrophilic residues increasing pEC_{50} values respectively relative to wild type $G_{i\alpha 1}$. This change in coupling efficiency could not be attributed to a change in the affinity for nucleotides at the α -subunit or to a change in the rate of basal guanine nucleotide exchange. These data indicate that functional coupling of the $G_{i\alpha 1}$ subunit to the α_2A adrenoceptor is in part modulated by the physiochemical properties of residue 351 and that a relationship exists between the hydrophobicity of residue 351 and the capacity of the α -subunit to functionally couple to the α_2A adrenoceptor.

A series of fusion constructs composed of the α_2A adrenoceptor covalently linked to selected $G_{i\alpha 1}$ C351 mutants were used to assess the effects of substituting residue C351 in $G_{i\alpha 1}$ on agonist intrinsic activity at the α_2A adrenoceptor. The agonist UK14304 was shown to elicit a spectrum of responses at the fusion constructs, closely mirroring the order of coupling efficiency previously determined in the separately expressed components. While UK14304 essentially acted as a full agonist compared to adrenaline at the fusion construct composed of wild type $G_{i\alpha 1}$ (C351 $G_{i\alpha 1}$), it was shown to act as a partial agonist at an equivalent construct containing a glycine residue at position 351 in the $G_{i\alpha 1}$ moiety. In contrast to this, relative to the wild type $G_{i\alpha 1}$ fusion, UK14304 displayed greater relative intrinsic activity at the fusion construct containing

an isoleucine residue at position 351 in the $G_{i\alpha 1}$ moiety. Analysis of a more extensive range of partial agonists demonstrated that the order of agonist relative intrinsic activity at the respective fusion constructs was conserved regardless of the agonist assayed. This discrepancy of intrinsic activity at the fusion proteins could not be attributed to a change in the pharmacological profile of the receptor moiety or to a modification of its affinity for agonist. These data indicate that the intrinsic activity of partial agonists, relative to adrenaline, can be modulated by the physiochemical properties of residue 351 in the $G_{i\alpha 1}$ moiety.

The capacity of a series of chimeras containing substitutions of the last 6 C-terminal residues of $G_{i\alpha 1}$ for those of $G_{s\alpha}$, $G_{q\alpha}$ and $G_{16\alpha}$ were assessed for functional coupling with a range of non Gi-linked receptors. Functional coupling was demonstrated at the Gi/Gs chimera with the V_2 vasopressin receptor and β_2 adrenoceptor. Similarly, the Gi/Gq chimera was shown to functionally couple with the P_2Y_4 , and TRH, receptors. No functional coupling was detected for the Gi/G16 chimera. The inability of the Gi/G16 chimera to functionally couple to any of the receptors analysed was independent from its capacity to basally exchange guanine nucleotides, which was shown to be unchanged relative to the Gi/Gs, Gi/Gq chimeras and wild type $G_{i\alpha 1}$. The substitution of the 6 C-terminal residues of $G_{i\alpha 1}$ for those of $G_{s\alpha}$ also conferred resistance to ADP-ribosylation by both pertussis and cholera toxins. This was demonstrated by functional coupling of the Gi/Gs chimera to a FLAGTM-tagged version of the IP prostanoid receptor following treatment with both bacterial toxins. The $G_{i\alpha 1}G_{s\alpha}$ chimera was seen to couple more efficiently at the IP prostanoid receptor in the presence of these toxins than in non toxin treated samples, indicating that coupling efficiency of this chimera at the IP prostanoid receptor was not optimal. These findings indicate that the C-terminus of the G-protein α -subunit is an important determinant in defining G-protein/receptor coupling and that additional determinants, not present in the C-terminus of the α -subunit, are required for optimal coupling efficiency.

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Abbreviations

| | |
|------------------|--------------------------------------|
| AA | Amino acid |
| AC | Adenylyl cyclase |
| ADP | Adenosine 5'-diphosphate |
| App(NH)p | Adenylyl imido-diphosphate |
| ATP | Adenosine 5'-triphosphate |
| B. max | Maximal binding capacity |
| BSA | Bovine serum albumin |
| cAMP | Adenosine 3'-5'-cyclic monophosphate |
| CPM | Counts per minute |
| CTX | Cholera toxin |
| DAG | Sn-1,2-diacylglycerol |
| DMEM | Dulbeccos' modified Eagles' medium |
| DMSO | Dimethylsulphoxide |
| cDNA | Complementary DNA |
| EC ₅₀ | Median effective dose |
| <i>E.coli</i> | <i>Escherichia coli</i> |
| EDTA | Ethylenediaminetetraacetic acid |
| GDP | Guanosie diphosphate |
| G-protein | Guanine nucleotide binding protein |
| GTP γ S | Guanosine 5'-[3-o-thio]triphosphate |
| GTP | Guanosine 5'-triphosphate |

| | |
|------------------|--|
| HEPES | 4-[2-Hydroxyethyl]-1-piperazine-N'-2-ethane- Sulphonic acid |
| IC ₅₀ | Median inhibitory dose |
| IPTG | Isopropyl-1-thio-β-D-galactopyranoside |
| IP ₃ | D- <i>myo</i> -inositol-1,4,5-triphosphate |
| K _d | Equilibrium dissociation constant |
| kDa | Kilodaltons |
| mRNA | Messenger ribonucleic acid |
| NAD | Nicotinamide adenine dinucleotide |
| PKA | Protein kinase A |
| PKC | Protein kinase C |
| PLA | Phospholipase A |
| PLD | Phospholipase D |
| PTX | Pertussis toxin |
| S.D. | Standard deviation |
| S.E.M. | Standard error of the mean |
| SDS | Sodium dodecylsulphate |
| TCA | Trichloroacetic acid |
| TRH | Thyrotropin releasing hormone |
| Tris | Tris(hydroxymethyl)aminomethane |
| WT | Wild type |
| X-gal | 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside |

G-protein abbreviations

| | |
|------------------------------------|---|
| C351 $G_{i\alpha 1}$ | Wild type $G_{i\alpha 1}$ |
| C351X $G_{i\alpha 1}$ | Amino acid substitution of residue C351 in $G_{i\alpha 1}$ |
| $G_{i1\alpha}G_{s\alpha}$ | Chimera of $G_{i\alpha 1}$ and the 6 C-terminal amino acids of $G_{s\alpha}$ |
| $G_{i1\alpha}G_{q\alpha}$ | Chimera of $G_{i\alpha 1}$ and the 6 C-terminal amino acids of $G_{q\alpha}$ |
| $G_{i1\alpha}G_{16\alpha}$ | Chimera of $G_{i\alpha 1}$ and the 6 C-terminal amino acids of $G_{16\alpha}$ |
| α_2A -C351X $G_{i\alpha 1}$ | Fusion construct of the α_2A adrenoceptor and a $G_{i\alpha 1}$ cysteine 351 mutant of $G_{i\alpha 1}$ |

Symbols for amino acids

| | | |
|---|-----|---------------|
| A | Ala | Alanine |
| C | Cys | Cysteine |
| D | Asp | Aspartic acid |
| E | Glu | Glutamic acid |
| F | Phe | Phenylalanine |
| G | Gly | Glycine |
| H | His | Histidine |
| I | Ile | Isoleucine |
| K | Lys | Lysine |
| L | Leu | Leucine |
| M | Met | Methionine |
| N | Asp | Asparagine |
| P | Pro | Proline |
| Q | Gln | Glutamine |
| R | Arg | Arginine |
| S | Ser | Serine |
| T | Thr | Threonine |
| V | Val | Valine |
| W | Trp | Tryptophan |
| Y | Tyr | Tyrosine |

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Chapter 1

Introduction

1.1 Introduction

Cell surface receptors allow the transduction of extracellular signals across the lipid bilayer into the cell. These receptors can be broadly divided into two groups, those characterised by a single membrane spanning domain and those characterised by a seven transmembrane domain structure. Of these two groups, seven transmembrane domain receptors (or G-protein coupled receptors) constitute the largest family of cell surface receptors, with over 1000 known family members. This enormous number of family members reflects the diversity of ligands that are known to mediate responses through these receptors. Such ligands include – chromophores, nucleotides, cations, odours, small biogenic amines, large peptide hormones and chemokines. This list is by no means exhaustive. The constant cloning of new receptors has led to the discovery of receptors with no known ligands. These so-called “orphan” receptors serve to reflect the diverse nature of G-protein coupled receptors. This diversity is mirrored by the broad distribution of receptors throughout virtually every cell in the body. The combination of ligand diversity and tissue specific localisation of receptor subtypes allows G-protein coupled receptors to mediate functions as disparate as reproduction, metabolism, vision, smell, neurotransmission, cardiovascular regulation and immune response.

Considering the diversity of responses mediated by G-protein coupled receptors, it is not surprising to note that mutations in the components of the signal transduction cascade can give rise to a variety of diseases. While such mutations provide potential therapeutic targets for the pharmaceutical industry, they can also be used to elucidate some of the many mechanisms involved in the G-protein signal transduction pathways.

1.1.2 Historical overview

The path leading to the discovery of the G-protein mediated signal transduction pathway started in the late 1950's with Sutherland and Rall's observation that the enzyme adenylyl cyclase could be modulated in a concentration dependent manner by the addition of adrenaline or glucagon to preparations of canine liver (Sutherland *et al.*, 1958; Rall *et al.*, 1957). Early research continued to be focussed on the modulation of adenylyl cyclase activity, with Murad *et al.* (1962) not only showing that the stimulatory effects of adrenaline could be antagonised by “adrenergic blocking agents”, but also demonstrating the existence of partial agonists. Using a strategy of elimination, Sutherland *et al.* finally resolved the cellular location of adenylyl cyclase to the cell

membrane in 1962. Despite the wealth of information regarding the function of adenylyl cyclase, in the early 1960's adenylyl cyclase was still thought to be a membrane protein complex at which hormones exerted their effects through direct binding at regulatory subunits. It was not until 1969 that Birnbaumer and Rodbell concluded that hormones had discrete binding sites independent from the enzyme. Rodbell *et al.* (1971a) went on to demonstrate that the hormone glucagon has specific binding sites which were saturable and finite in number and that glucagon binding at these sites was proportional to adenylyl cyclase stimulation. It was not until 12 years later that Nathans *et al.* (1983) cloned the first G-protein coupled receptor, the bovine opsin receptor, using degenerate primers designed from the amino acid sequence of the bovine rhodopsin receptor. From analysis of the amino acid sequence, Nathans *et al.* proposed that the receptor was composed of seven hydrophobic membrane spanning domains that in turn were connected by alternating intracellular and extracellular loops more hydrophilic in character. In Nathans's theoretical model, the N and C-termini of the receptor were positioned on opposite faces of the lipid bilayer, with the N-terminus on the extracellular face and the C-terminus in contact with the cytoplasm. This early model was corroborated when the second G-protein coupled receptor, the β_2 adrenoceptor, was cloned 3 years later by Dixon *et al.* (1986). The true extent of receptor homology was not fully realised until 1993 when Baldwin analysed the sequences of over 100 cloned G-protein coupled receptors. From this study, Baldwin (1993) concluded that all G-protein coupled receptors shared a common seven transmembrane structure. Further evidence of a seven transmembrane structure was presented by Schertler *et al.* (1993) in the form of a high resolution (9 angstrom) electron density map of rhodopsin. This map not only illustrated the presence of seven membrane spanning regions of high electron density, but also provided the first insight into the relative orientation of the transmembrane domains. Due to the problems associated with purifying and crystallising receptors, no further G-protein coupled receptor crystal structures have been resolved to date.

It was not until the early 1970's that guanine nucleotides were recognised as important factors in cellular signalling. The first association between guanine nucleotides and receptor binding was made by Rodbell *et al.* (1971b) who demonstrated that GTP enhanced the dissociation of glucagon from its cognate receptor. Later, Pfeuffer and Helmreich (1975) linked the activation of adenylyl cyclase in turkey erythrocytes with the non-covalent binding of GTP at a separate regulatory protein. In

the following year, Cassel and Selinger (1976) demonstrated agonist stimulated GTP hydrolysis by this regulatory protein and linked this activity with the attenuation of adenylyl cyclase activation. Cassel and Selinger also demonstrated that abolition of this GTPase activity through cholera toxin treatment resulted in the constitutive activation of adenylyl cyclase. After further experimentation Cassel and Selinger concluded that the guanine nucleotide binding regulatory protein acted as a signalling intermediate between the hormone activated receptor and the enzyme adenylyl cyclase. In parallel with these observations, Ross and Gilman demonstrated that this guanine nucleotide binding protein could reconstitute hormone stimulated adenylyl cyclase activity in the mutant S49 murine lymphoma *cyc⁻* cell line (Ross and Gilman, 1977). This guanine nucleotide binding protein was finally purified and characterised by Northup, who demonstrated that this "regulatory component of adenylyl cyclase" was actually composed of more than one polypeptide chain (Northup *et al.*, 1980). Northup *et al.* (1980) also showed that the effects of cholera toxin, as observed by Cassel and Selinger (1977), were a result of ADP ribosylation of one of the guanine nucleotide binding protein subunits. The high resolution structure of the active conformation of the transducin α -subunit was finally resolved 13 years later by Noel *et al.* (1993). Analysis of this structure showed that the α -subunit was composed of two distinct domains separated by a deep cleft, which formed the guanine nucleotide binding pocket. One of these domains was determined as being predominantly α -helical in structure, while the other was shown to closely resemble the structure of the small nucleotide binding protein Ras. The conformational changes involved in G-protein activation were finally resolved a year later when Lambright *et al.* (1994) ascertained the crystal structure of the transducin α -subunit in the inactive GDP-bound conformation. Direct comparison of the two crystal structures of $G_{i\alpha}$ showed that conformational changes were localised to three conserved "switch" regions in the nucleotide binding portion of the α -subunit. In the following two years the crystal structures of free $\beta\gamma$ and $\beta\gamma$ as part of a G-protein heterotrimer were ascertained by Sondek *et al.* (1996) and Wall *et al.* (1995) respectively. Despite the extensive information provided by these X-ray diffraction studies, the structures of the extreme N-terminal helix and C-terminal tail still remain unresolved.

Characterisation of the basic components of the signal transduction pathway also led to the identification of further factors capable of regulating receptor and G-protein

activity. The first regulator of G-protein signalling (RGS), SST2, was identified in the yeast *Saccharomyces cerevisiae* by Chan and Othie (1982). SST2, like all RGS proteins, regulates G-protein activity by accelerating the rate of guanine nucleotide hydrolysis and so the length of time the G-protein remains in the active conformation. It was not until 1995 that the first human homologue GOS8 was isolated from a human cDNA library (Wu *et al.*, 1995), although this was initially incorrectly identified as a transcription factor. This discovery was rapidly followed by the isolation of another closely related human homologue, GAIP, by a yeast two hybrid screen with human $G_{i\alpha 3}$ as bait (DeVries *et al.*, 1995). Alignment of these RGS proteins has shown that members of the RGS family contain 3 highly conserved GOS8, or GH, homology domains (Dohlman *et al.*, 1996). At present 19 RGS proteins have been isolated from organisms as diverse as *S.cerevisiae* to *C.elegans*.

Knowledge in the field of G-protein coupled receptors has increased enormously since the first observations of Sutherland and Rall in the late 1950's. Many of the components of the signal transduction cascade have now been isolated, and numerous molecular mechanisms involved in signal propagation are now well characterised. However, despite our current level of understanding, the continuous discovery of novel receptors and regulatory components of the signal transduction cascade leaves no doubt that much still remains to be learned.

1.2 G-protein coupled receptors

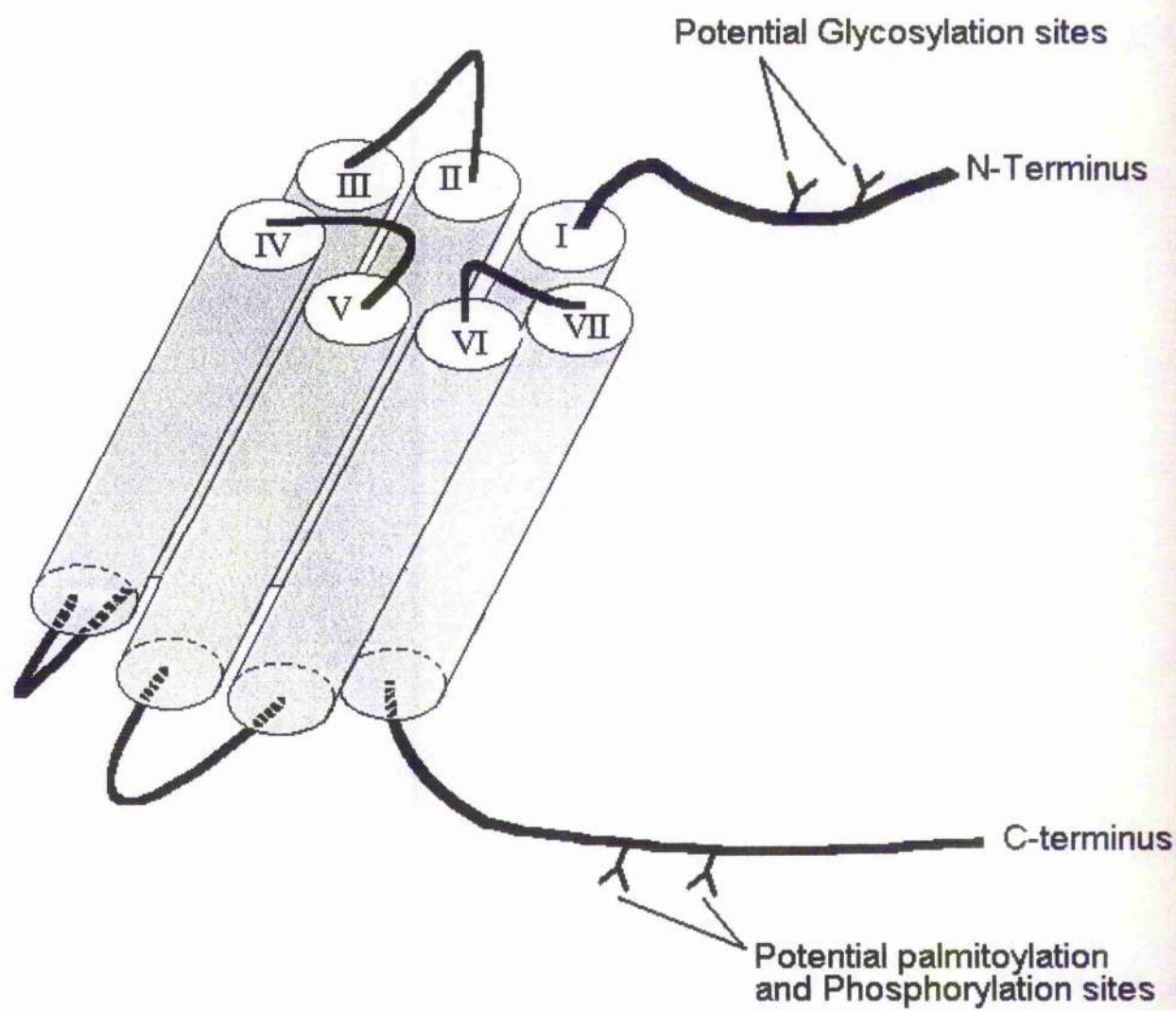
G-protein coupled receptors (GPCRs) play an important role sensing and transmitting extracellular signals across the cell membrane to intracellular effectors via GTP binding proteins (G-proteins). At present over 1000 G-protein coupled receptors have been identified. This enormous number of family members is reflected in their diversity of function and ligand specificity. Despite this diversity, all GPCRs share a common general structure of an N-terminal segment, seven putative transmembrane helices, which fold to form the transmembrane core, connected by three extracellular loops and at least three cytoplasmic loops (Fig. 1.1). The structure is completed by a C-terminal segment, which can be post-translationally modified to form a fourth cytoplasmic loop.

Amino acid analysis of the first cloned GPCR, the bovine opsin receptor, by Nathans *et al.* (1983) provided the first structural model of a G-protein coupled receptor. The amino acid sequence highlighted the presence of seven hydrophobic regions that were postulated to transverse the cell membrane as α -helical structures. These hydrophobic regions, while themselves relatively conserved in size, were thought to be linked by intracellular and extracellular loops more hydrophilic in nature and variable in length. From the subsequent cloning of the β -adrenergic receptor three years later (Dixon *et al.*, 1986) it became apparent that G-protein coupled receptors shared a great deal of structural homology. The extent of this homology was demonstrated by Baldwin (Baldwin, 1993) who analysed the sequences of over 100 cloned receptors and demonstrated that the seven hydrophobic, putative membrane spanning domains were common to all GPCRs. Up till 1993, all models of G-protein coupled receptors were based on the crystal structure of the functionally unrelated bacterial proton pump, bacterial rhodopsin. It was not until Schertler *et al.* (1993) produced the first high resolution (9 angstrom) crystal structure of rhodopsin that it became apparent that the arrangement of the transmembrane domains varied between the two proteins. From the X-ray structure resolved by Schertler *et al.* (1993) it could be observed that not all of the transmembrane domains were orientated perpendicular to the cell membrane as in bacterial rhodopsin. The transmembrane helices of rhodopsin appeared to cross the lipid bilayer at a variety of angles. Baldwin (1993) used this electron density map of rhodopsin in conjunction with sequence information of over 100 receptors to generate models of possible transmembrane domain arrangements. This model has since been

Figure 1.1 A diagrammatic representation of a typical G-protein coupled receptor

Figure 1.1 represents the structure of a typical G-protein coupled receptor. The N and C-termini of the receptor are positioned on opposite faces of the lipid bilayer separated by seven putative helical membrane spanning domains which are hydrophobic in nature. The N-terminus is located on the extracellular surface of the membrane and may contain potential glycosylation sites. In contrast to this, the C-terminus protrudes into the cytoplasm and is known to be post-translationally modified by both phosphorylation and palmitoylation. The intervening transmembrane domains, numbered I to VII, are arranged in a counter clockwise orientation as viewed from the extracellular surface of the cell and are themselves connected by alternating intracellular and extracellular loops more hydrophilic in character.

Figure 1.1



refined by labelling experiments, mutational analysis and various forms of spectroscopy to formulate a detailed understanding of receptor structure in relation to function.

1.2.1 GPCRs :Structure and Function

Despite their diversity, all G-protein coupled receptors share two common features. The first of these is that all G-protein coupled receptors are composed of the following structural elements. An N-terminal segment, seven transmembrane domains, three extracellular loops, three cytoplasmic loops and an intracellular C-terminal segment. Secondly, all G-protein coupled receptors have the same function, which is to transduce extracellular signals across the cell membrane to intracellular effectors. The complex relationship between these two features will now be discussed below for each of the structural components.

1.2.2 The N-terminus

The N-terminus is hydrophilic in nature and is located on the extracellular side of the cell membrane. This region of the G-protein coupled receptors can be quite variable in both length (7-595 aa) and primary amino acid sequence. In many G-protein coupled receptors the N-terminal region plays a significant role in determining the specificity of ligand binding. There are two basic mechanisms by which the N-terminus may be involved in ligand binding. The N-terminal region can either determine initial binding of the ligand, as in glycoprotein hormone receptors, or binding may involve synergistic contacts between both the N-terminus and the extracellular loops, such as for parathyroid hormone receptors (Ji *et al.* 1998). In the exceptional example of protease activated receptors, the N-terminus is cleaved to generate what is essentially a tethered ligand which subsequently binds to the receptor's extracellular loops. Ligand size has been shown to correlate weakly with the length of the GPCR N-terminus (Ji *et al.*, 1995). However, there are exceptions to this rule, the most striking being the calcium receptor, which has an N-terminal segment approximately 600 residues in length.

Glycosylation at the N-terminal segment can account for a significant percentage of the mass of the receptor. For example Rands *et al.* (1990) have demonstrated that endoglycosidase treatment of the β_2 adrenoceptor can decrease the molecular mass from 65 kDa to 49 kDa. Despite accounting for a significant percentage of the mass of the receptor, glycosylation does not appear to play any significant role in receptor function. Through the use of glycosylation defective mutants, Rands *et al.*

(1990) have demonstrated that glycosylation does not affect ligand binding, G-protein coupling or the capacity of the receptor to activate its cognate secondary effector. In contrast, glycosylation did seem to affect correct localisation of the β_2 adrenoceptor in the cell. Rands *et al.* (1990) observed that only half of the glycosylation defective mutants were correctly localised at the cell surface compared to the fully glycosylated wild type receptor.

1.2.3 The C-terminus

The C-terminal segment is located on the intracellular face of the cell membrane, where it has been shown to play a role in determining the specificity of G-protein coupling. In common with the N-terminal region, the C-terminal segment is variable in both length (12-359 residues) and primary sequence. The NMR structure of a peptide corresponding to the C-terminal segment of rhodopsin has revealed that the C-terminus is quite compact in nature (Yeagle *et al.*, 1996). This compact domain is thought to be orientated away from the membrane into the cytoplasm, where it can interact with both the G-protein α and $\beta\gamma$ subunits (Bourne, 1997). Other structural features of this compact domain include- an α -helix, presumed to be an extension of TM7, connected by a loop to cysteine residues, which are thought to be in close proximity with the cell membrane, followed by a hydrophilic β -sheet structure (Yeagle *et al.*, 1996)

Experimental evidence suggests that in certain receptors the C-terminal tail has the capacity to form a fourth cytoplasmic loop through palmitoylation at highly conserved cysteine residues (O'Dowd *et al.*, 1989). It is believed that the palmitate residue covalently linked to the conserved cysteine residue "anchors" itself to the cell membrane thereby forming an additional cytoplasmic loop. This theory would explain the close proximity between the cysteine residues and the cell membrane in the NMR structure of the C-terminal tail of rhodopsin (Yeagle *et al.*, 1996). The significance of this fourth cytoplasmic loop appears to vary from receptor to receptor. O'Dowd *et al.* (1989) have demonstrated that substitution of the conserved cysteine residue for glycine in the β_2 adrenoceptor results in a non-palmitoylated form of the receptor. This non-palmitoylated form exhibits a reduced capacity to stimulate adenylyl cyclase upon isoproterenol stimulation when compared to the wild type receptor. Conversely, the

equivalent mutation in rhodopsin does not appear to adversely affect receptor function (Karnik *et al.*, 1988).

In addition to G-protein coupling, the C-terminal tail also appears to play a crucial role in determining the specificity of receptor/G-protein interactions. Namba *et al.* (1993) have demonstrated that the primary sequence of the C-terminal tail can dictate G-protein coupling specificity in the EP3 prostanoid receptor. Four splice variants of the EP3 prostanoid receptor, differing only in the sequence of the C-terminal tail, were seen to couple to three different sets of G-proteins. Of the four splice variants, the EP3_A receptor coupled to G_{iα}, EP3_B coupled to G_{sα}, EP3_C coupled to both G_{sα} and G_{oα} whilst EP3_D coupled to G_{iα}, G_{sα} and G_{qα} (Namba *et al.*, 1993).

Evidence suggests that the C-terminal tail of the receptor may be an important phosphorylation site in the process of receptor desensitisation. Using reconstituted systems and site directed mutagenesis, Hausdorff *et al.* (1989) have shown that the C-terminal tail of the β_2 adrenoceptor can be phosphorylated by the two G-protein receptor kinases β ARK1 and β ARK2. Similarly, Premont *et al.* (1995) have identified two serine residues in the C-terminus of rhodopsin (Ser 338, Ser 343) which can be phosphorylated by either β ARK1/ β ARK2 or the G-protein receptor kinase GRK5.

1.2.4 Extracellular loops

The extracellular loops of a G-protein coupled receptor can vary in length from 2 to 230 residues. In many GPCRs the extracellular loops play an integral role in ligand binding, the nature of which varies from receptor to receptor. The extracellular loops can either play an auxiliary role in ligand binding in combination with other structures such as the TM core or the N-terminus, or they can be the sole sites for ligand binding.

Examples of receptor which use extracellular loops in ligand binding include the formyl peptide receptor, parathyroid hormone receptors and protease activated receptors. Each of these receptors utilises the extracellular loops in a distinct way in ligand binding.

For example, ligand binding at the formyl peptide receptor is distributed between the extracellular loops and the transmembrane core. Mutational studies have shown that the N-formyl moiety of the ligand binds in the transmembrane core, while the C-terminal region of the ligand binds to both the N-terminal region of the receptor

and the extracellular loops 1 and 2 (Fay *et al.*, 1993). In contrast, ligand binding at the parathyroid hormone receptor is determined more by the N-terminal segment while interactions with extracellular loop 2 and the transmembrane domain 3 are involved more in receptor activation (Bergwitz *et al.*, 1997). The mechanism of activation of protease activated receptors differs from the other two receptors in that the receptor itself becomes its own ligand. Vu *et al.* (1991) have shown that protease action results in the cleavage of a section of the N-terminal segment of the receptor. Following protease cleavage the remaining N-terminal region essentially acts as a tethered ligand that in turn activates the receptor by binding to the extracellular loops. The principal contact point required for activation of both the thrombin receptor, and the structurally related protease activated receptor 2, appears to be in extracellular loop 2 (Nanevics *et al.*, 1996; Lerner *et al.*, 1996).

In addition to ligand binding there is evidence to suggest that conformational changes in extracellular loop structure can influence G-protein selectivity. Gilchrist *et al.* (1996) have demonstrated that while a mutation in the extracellular loop of the luteinizing hormone receptor abolishes its capacity to activate G_{sx} , it has no discernible effect on the capacity of the receptor to bind ligand or activate G_{qx} .

1.2.5 Cytoplasmic loops

Intracellular, or cytoplasmic loops, play a role in defining G-protein receptor specificity as well as in the transmission of the activated state of the receptor to the cytoplasmic signalling molecule. In common with extracellular loops, cytoplasmic loops are variable in length and primary sequence. All G-protein coupled receptors possess at least 3 intracellular loops. In certain receptors, such as the β_2 adrenoceptor, a fourth cytoplasmic loop may be formed as a consequence of palmitoylation at conserved cysteine residues in the C-terminal tail (O'Dowd *et al.*, 1989). Intracellular loops from receptors known to activate similar subsets of G-proteins vary considerably in primary sequence, and as of yet it has not been possible to determine a common rule for predicting the G-protein coupling specificity of a receptor merely by analysing the primary amino acid sequence of the intracellular loops. Of the three loops, the first and second loops appear to be the most conserved, whilst the third intracellular loop linking transmembrane domains 5 and 6 appears to be the most variable in primary sequence and size. It is interesting to note that this variability in primary sequence can be related to the relative importance of the cytoplasmic loops in determining G-protein coupling

and specificity. The order of importance in ascending order appears to be loop1, loop2 and then loop3.

The importance of the cytoplasmic loops in determining G-protein specificity has been demonstrated through the use of chimeric receptors in which one receptor donates either a portion or a majority of the sequence for the third intracellular loop. Kobilka *et al.*, (1988) have demonstrated that a chimeric α_2 adrenoceptor containing regions of the β_2 adrenoceptor encoding most of transmembrane domain 5, intracellular loop3 and all of transmembrane domain 6 can stimulate adenylyl cyclase activity to a third of the level of that seen with the wild type β_2 adrenoceptor. In contrast, a similar chimera containing only a small portion of the third intracellular loop displayed no activity at all. This would indicate that other determinants are required to determine efficient G-protein coupling and specificity. This is clearly demonstrated by the experiments of Wong *et al.* (1994) in which the third intracellular loops of the muscarinic acetylcholine receptors 1 and 2, which normally activate $G_{q\alpha}$ and $G_{i\alpha}$ respectively, were replaced by the equivalent sequence of the β_1 adrenoceptor, a $G_{s\alpha}$ linked receptor. Interestingly, as opposed to activating the G-protein associated with the donor of the third intracellular loop, both chimeras activated all three classes of G-proteins with equal efficiency. Further introduction of both intracellular loops 1 and 2 of the β_1 adrenoceptor was required for the activation of $G_{s\alpha}$ exclusively. From these observations it appears that G-protein coupling determinants are scattered between the intracellular loops and that the three dimensional scaffold produced by all three intracellular loops may be more important than the contribution of any one loop alone (Bourne, 1997).

Many of the determinants involved in G-protein activation lie close to the junctions between the transmembrane domains and the intracellular loops. One well characterised example is the highly conserved glutamine/aspartate- arginine- tyrosine (ERY or DRY) sequence located at the junction of transmembrane domain 3 and intracellular loop 2. Substitution of the conserved arginine residue at the middle of this motif in rhodopsin has been shown to prevent activation of bound $G_{t\alpha}$ (Ernst *et al.*, 1995). The arginine residue of this DRY motif is thought to be constrained in a hydrophobic pocket formed by the interactions between polar residues from transmembrane domains 1, 2 and 7 (Scheer *et al.*, 1996). It has been suggested that activation of the α_1B adrenoceptor causes this arginine residue to leave the hydrophilic

pocket and in doing so expose previously inaccessible sequences in the second and third intracellular loops (Scheer *et al.*, 1996). Similar residues in the carboxyl end of the third intracellular loop of the β_2 adrenoceptor have also been shown to play an important role in G-protein activation. Deletion of residues at this position have been shown to significantly reduce the capacity of the receptor to activate adenylyl cyclase (Hausdorff *et al.*, 1990)

While many receptors contain only three intracellular loops, certain receptors, such as the β_2 adrenoceptor and rhodopsin, can form a fourth intracellular loop through palmitoylation of conserved cysteine residues in the C-terminal segment (O'Dowd *et al.*, 1989). Where present, the relevance of the fourth intracellular loop appears to differ between receptors. O'Dowd *et al.* (1989) have demonstrated that substitution of conserved cysteine residues for glycine in the β_2 adrenoceptor results in a non-palmitoylated form of the receptor which displays a reduced capacity to activate adenylyl cyclase in response to isoproterenol stimulation. In contrast, the equivalent substitution in rhodopsin has been shown to have no discernible effect on receptor activity (Karnik *et al.*, 1988).

1.2.6.1 Transmembrane domains

All G-protein coupled receptors possess 7 putative transmembrane domains (TM's) which are thought to span the cell membrane as α -helical structures. These transmembrane domains bundle together to form the transmembrane core. Not all of the α -helical transmembrane domains intersect the cell membrane at right angles. Analysis of the two dimensional crystal structure of rhodopsin by Schertler *et al.* (1993) provides evidence that some of the α -helices cross the lipid bilayer at angles of up to 30° . The α -helices vary from 20 to 27 residues in length and may extend into both the cytoplasm and the extracellular environment. Evidence suggests that the α -helical structure may be further projected out of the cell membrane through a continuation of the helical structure by the connecting loops. Nuclear magnetic resonance (NMR) analysis of the parathyroid hormone receptor by Mirke *et al.* (1996) has shown an α -helical structure at the amino terminus of the third intracellular loop. Similarly, NMR analysis of the C-terminal segment of rhodopsin by Yeagle *et al.* (1996) has shown that the C-terminal segment of the receptor partially extends the α -helix of transmembrane domain 7. Not all α -helices are of equal hydrophobicity, TMs 1, 4, and 7 are generally more

hydrophobic than TMs 2, 3, 5 and 6. Differences in the hydrophobic potential of the helices may help determine the relative orientation of the transmembrane domains in the lipid bilayer.

Many GPCR models are based upon the structure of the functionally unrelated bacterial transporter, bacterial rhodopsin. A comparison of the high resolution crystal structure of bacterial rhodopsin with the structure of adrenergic receptors shows that in both proteins the transmembrane domains are arranged in a counter clockwise closed loop formation when viewed from the extracellular surface (Mizobe *et al.*, 1996). Interactions between the transmembrane domains form the transmembrane core, which is thought to be stabilised by extensive intermolecular bonds including hydrogen bonding and salt bridges (Pebay-Peyroula *et al.*, 1997). These strong molecular interactions are thought to constrain GPCRs in their normally inactive conformation. Evidence of this comes from Robinson *et al.* (1992) who have shown that the disruption of a presumed salt bridge between TMs 3 and 7 in rhodopsin results in constitutive activation of the receptor in the absence of chromophore. It is presumed that this same salt bridge is broken upon normal receptor activation. Conversely, restriction of movement between TMs 3 and 6 in rhodopsin through zinc binding at engineered metal binding sites has been shown to prevent receptor activation (Sheikh *et al.*, 1996).

1.2.6.2 Functions of the transmembrane domains

Transmembrane domains are unique amongst all of the components of the G-protein coupled receptor in so far as that they can be involved in up to three different functions in the receptor. These include ligand binding, receptor activation and G-protein coupling. This multifunctional capability is due to the fact that the transmembrane domains are not only in contact with the lipid bilayer but also the extracellular environment and the cytoplasm. These three proprietary functions of the transmembrane domains are examined in detail below.

1.2.6.3 Ligand binding

Ligand binding can either occur exclusively at the TM core, or in conjunction with ligand binding determinants present on the extracellular loops. Examples of receptors in which the ligand binds exclusively to the TM core include- biogenic amine receptors, nucleoside and nucleotide receptors, eicosanoid receptors and rhodopsin. Of these receptors rhodopsin and biogenic amine receptors are probably the best

characterised. In rhodopsin the ligand 11-cis retinal is covalently bound as an inverse agonist through the formation of a Schiff's base with a lysine residue in the middle of TM 7 (Strader *et al.*, 1994). This protonated Schiff's base is in turn paired with a conserved glutamine residue present at the junction of TM3 and exoloop1 (Strader *et al.*, 1994). Other interactions known to stabilise 11-cis-retinal in the TM core include associations between the β - ionone moiety and residues in TMs 3, 5 and 6 (Han *et al.*, 1997).

Biogenic amine receptors have also been widely used to model ligand binding in the TM core. Of this subset of receptors, the β_2 adrenoceptor is the one that is probably the best characterised. Experiments using photoaffinity labelling (Wang *et al.*, 1988) and fluorescent antagonists (Tota and Strader, 1990) have shown that catecholamine binding occurs 9 angstroms into the transmembrane core. The principal sites of ligand interaction being an aspartate residue in TM3 (asp 113) and the amine group of the catecholamine ligand. Additional H-bonding between the meta and para hydroxyl groups of the catechol ring and serine residues in TM5 (ser 204, ser 207) are thought to constrain TM5 relative to TM3 and have been shown to be involved in receptor activation (Strader *et al.*, 1994)

1.2.6.4 Receptor activation

In the absence of ligand the receptor is thought to be constrained in it's inactive state by extensive intermolecular bonding in the transmembrane core. Evidence for this model comes from mutational studies in which intermolecular bonds in the TM core are either disrupted or strengthened. The introduction of additional bonds constraining the relative movement of the TM domains has been shown to prevent receptor activation even in the presence of extracellular stimuli. Elling *et al.* (1995) have demonstrated that zinc binding at engineered metal binding sites between transmembrane domains 5 and 6 in the NK-1 receptor can prevent receptor activation. Similarly, Sheikh *et al.* (1996) have shown that zinc binding at equivalent sites between TMs 3 and 6 in rhodopsin has an equally restrictive effect. Activation of rhodopsin can also be prevented by the introduction of additional disulphide bonds into the TM core under oxidative conditions (Yu *et al.*, 1995).

Ligand binding is thought to induce a conformational change in the receptor, which results in the disruption of constraining bonds in the TM core and so receptor

activation. Once again evidence supporting this idea originates from mutational studies. Robinson *et al.* (1992) have shown that the disruption of a putative salt bridge between a lysine residue in TM7 and a conserved glutamate residue in TM3 of rhodopsin results in constitutive activity in the absence of chromophore. It is of interest to note that the same lysine residue in TM7 is thought to be linked to the ligand 11-*cis*-retinal through a protonated Schiff's base, and it is this bond which is thought to be broken upon isomerisation of 11-*cis*-retinal to the trans isomer.

The disruption of bonds in the TM core is thought to be followed by a movement of the TM domains into the plane of the cell membrane resulting in receptor activation. Data supporting the movement of TM helices originates from site directed spin labelling experiments and spectroscopic methods. Using dual site directed spin labelling Farrens *et al.* (1996) have demonstrated that activation of rhodopsin results in the movement of TMs 3 and 6 away from each other, and the rest of the helical bundle, into the plane of the cell membrane. They also suggest that the activation of rhodopsin causes TM6 to rotate 30° in a counter clockwise direction (when viewed from the extracellular surface) upon it's own axis. This observation corroborates those of Lin and Sakmar (1996) who used tryptophan UV absorbance spectroscopy to arrive at the same conclusion.

1.2.6.5 G-protein coupling

Although there is no definitive proof of G-protein coupling at the TM domains, increasing evidence indicates that this may be a possibility. Through site directed spin labelling experiments Farrens *et al.* (1996) have demonstrated that activation of rhodopsin results in the separation of TM6 from TM3 at the cytoplasmic face of the receptor. Given the lack of movement seen in the rest of the transmembrane bundle, the movement of these two helices could open up a crevice to the TM core. The finding of Javitch *et al.* (1997) supports the existence of such a crevice. Javitch *et al.* (1997) have shown that a cysteine residue in TM6 of a constitutively active β_2 adrenoceptor is susceptible to modification by charged sulfhydryl specific reagents while the equivalent residue in the inactive wild type receptor is protected from such modifications. Photochemical cross linking experiments can position both G-protein α - and β subunits in close proximity to the third intracellular loop of the α - adrenoceptor (Taylor *et al.*, 1994). Considering the close proximity of the G-protein with TM domains that have

been shown to move upon receptor activation, it is not inconceivable that certain portions of the G-protein may protrude into the transmembrane core.

1.3 G-proteins

Heterotrimeric GTP binding proteins, G-proteins, mediate the transduction of a signal from activated cell surface receptors, characterised by seven membrane spanning domains, to effector molecules. G-proteins are composed of three subunits designated α , β and γ that vary in mass from 39-52, 35-36 and 7-8 kDa respectively. The α -subunit is folded to form a guanine nucleotide binding pocket, which can accommodate either GDP or GTP. G-proteins can exist in either active or inactive conformations. In the inactive conformation the α -subunit contains GDP in the nucleotide binding pocket and is in tight association with the $\beta\gamma$ subunits. G-protein activation occurs through receptor catalysed guanine nucleotide exchange in the α -subunit, resulting in the exchange of GDP for GTP. This guanine nucleotide exchange event causes a conformational change in the β -subunit binding site on the α -subunit. This conformational change in the β -subunit binding site results in a lower affinity for the $\beta\gamma$ dimer and consequently $\beta\gamma$ dissociation. The GTP bound α -subunit and the $\beta\gamma$ subunits are then free to interact with downstream effector molecules. G-protein signalling is attenuated by the hydrolysis of the terminal phosphate of GTP to yield GDP in the guanine nucleotide binding pocket. The hydrolysis of GTP to GDP results in a conformational change in the α -subunit that allows $\beta\gamma$ subunit re-association and so reversion back to the inactive heterotrimeric state.

1.3.1 G-protein α -subunits

At present 20 members of the G-protein α -subunit superfamily have been identified encoded by at least 16 different genes. Family members vary in mass from 39 to 52 kDa and constitute the largest single subunit in the G-protein heterotrimer. The α -subunit superfamily can be subdivided into four distinct classes based upon sequence homology and effector specificity. These four groups are designated $G_{s\alpha}$, $G_{i\alpha}$, $G_{q\alpha}$ and $G_{12/13\alpha}$. Of these four groups G-proteins from the G_i and G_s class have been shown to have opposing functions, with G_s and G_i stimulating and inhibiting the activity of the secondary effector adenylyl cyclase respectively. Other classes of α -subunits couple to alternate effectors, for instance members of the $G_{q\alpha}$ subclass are known to stimulate the secondary effector phospholipase C- β . In contrast to the other classes of α -subunit, the functional roles of $G_{12\alpha}$ and $G_{13\alpha}$ are poorly defined, although it has been proposed that

these G-proteins may mediate activation of voltage dependent Ca^{2+} channels (Wilk-Blaszczak *et al.*, 1997) and regulate Na^+/H^+ exchange (Hooley *et al.*, 1996).

1.3.2. Gs family

The term $G_{s\alpha}$ refers to the capacity of subunits in this class of G-protein to activate the secondary effector adenylyl cyclase. Adenylyl cyclase is an integral membrane protein which catalyses the formation of the secondary messenger cyclic AMP (cAMP). Cyclic AMP acts as a signalling molecule, relaying the initial signal to additional components of the signal transduction cascade. Signalling by cyclic AMP is attenuated by cAMP specific phosphodiesterases which hydrolyse cAMP to 5'-AMP.

The capacity of Gs α -subunits to activate adenylyl cyclase was first observed by Pfeuffer and Helmreich (1975) who saw that adenylyl cyclase activity in pigeon erythrocyte membranes could be stimulated by GTP binding at a specific GTP binding protein. This protein was later purified and characterised by Northup *et al.* (1980) who demonstrated that this "regulatory component of adenylyl cyclase" was composed of more than one polypeptide chain. It is now known that alternate splicing of a single $G_{s\alpha}$ gene results in the production of four splice variants, termed $G_{s\alpha1}$, $G_{s\alpha2}$, $G_{s\alpha3}$ and $G_{s\alpha4}$, which collectively show ubiquitous tissue distribution (Bray *et al.*, 1986). This gene is approximately 20 Kb long and is subdivided into 13 exons and 12 introns (Kozasa *et al.*, 1988). Each of the splice variants differs in amino acid length, ranging from 380-381 residues for $G_{s\alpha3}$ and $G_{s\alpha4}$ to 394-395 residues for $G_{s\alpha1}$ and $G_{s\alpha2}$ respectively (Bray *et al.*, 1986). Analysis of the primary sequence of these splice variants has highlighted many similarities between the splice variants. For example, with the exception of a 15 amino acid sequence, $G_{s\alpha3}$ and $G_{s\alpha4}$ are identical. The loss of this sequence is a consequence of alternate splicing of exon 3 of the $G_{s\alpha}$ gene. A similar relationship exists between $G_{s\alpha4}$ and $G_{s\alpha2}$. In addition to this variation, $G_{s\alpha1}/G_{s\alpha2}$ and $G_{s\alpha3}/G_{s\alpha4}$ are identical bar an extra amino acid which can be accounted for by 3 nucleotides (CAG) which reside 5' to the end of exon 4.

The four splice variants are commonly known as $G_{s\alpha}$ long, $G_{s\alpha(L)}$ ($G_{s\alpha1}/G_{s\alpha2}$) and $G_{s\alpha}$ short $G_{s\alpha(S)}$ ($G_{s\alpha3}/G_{s\alpha4}$) depending on the length of their sequence. Despite the diversity of these splice variants, no functional significance can be attributed to the insertion/deletion of amino acid sequences, and all four splice variants retain the

capacity to activate the 9 known mammalian adenylyl cyclases. The splice variants are also equally good substrates for ADP ribosylation by the bacterial toxin CTX.

The second subunit in this class of G-protein, $G_{olf\alpha}$, is quite distinct from the splice variants of $G_{s\alpha}$ in that it is largely localised to the olfactory neuroepithelium, where it is thought to mediate signalling by the large family of olfactory receptors. Despite this centralised tissue distribution, $G_{olf\alpha}$ shares 88% amino acid identity with $G_{s\alpha}$ and also activates adenylyl cyclase (Hepler *et al.*, 1992).

1.3.3.1, Gi family

Although the term Gi stands for inhibitor of adenylyl cyclase activity, not all of the G-proteins in this class of G-protein can regulate this function. In fact α -subunits in this class of G-protein probably modulate the most diverse set of effectors seen in any class of G-protein. Inclusion into this family is based more upon sequence homology than similarity in effector modulation. A noticeable similarity between all of the members of this group, with the exception of $G_{z\alpha}$, is their susceptibility to pertussis toxin catalysed ADP-ribosylation. Pertussis toxin, a toxin from the bacterium *Bordetella pertussis*, catalyses the transfer of the nucleoside ADP ribose from NAD to a conserved cysteine residue located four residues from the C-terminus in almost all of the α -subunits in this class of G-protein. This covalent modification results in the uncoupling of the G-protein from its cognate receptor. No such modification is seen in $G_{z\alpha}$, which contains an isoleucine residue at this position.

At present the Gi class of G-proteins contains three $G_{i\alpha}$ family members, termed $G_{i\alpha1}$, $G_{i\alpha2}$ and $G_{i\alpha3}$ (Jones and Reed, 1987). Although all three members have been shown to directly inhibit the activity of the secondary effector adenylyl cyclase *in vitro* (Taussig *et al.*, 1993), their physiological roles are thought to be more diverse.

1.3.3.2 $G_{i\alpha1}$

At 40.3 kDa, $G_{i\alpha1}$ is the smallest of the three $G_{i\alpha}$ subunits. $G_{i\alpha1}$ shows no distinct localisation, although it tends to be highly expressed in neural and endocrine tissues. With the exception of $G_{z\alpha}$, Gi family members have some of the highest catalytic activities of any of the α -subunits. The catalytic activity of $G_{i\alpha1}$ has been estimated to be $\text{kcat}(\text{min}^{-1}) = 2.4$ at 20 °C. This feature makes them particularly well

suited for analysis by guanine nucleotide exchange based assays such as binding of the non-hydrolysable GTP analogue, [35 S]GTP γ S ([35 S]GTP γ S binding) or hydrolysis of 32 P labelled GTP (GTPase). In common with most of the α -subunits of this class of G-protein, $G_{i\alpha 1}$ contains a conserved cysteine residue 4 amino acids from the C-terminal tail. This residue allows the potential use of pertussis toxin (PTX) as a tool to functionally uncouple this G-protein from its cognate receptors.

The physiological role of $G_{i\alpha 1}$ is thought to be quite distinct from its capacity to inhibit adenylyl cyclase activity. The high neuronal tissue concentration of $G_{i\alpha 1}$ has been linked to its capacity to indirectly regulate ATP sensitive and inwardly rectifying potassium channels through $\beta\gamma$ subunit dissociation.

1.3.3.3 $G_{i\alpha 2}$

$G_{i\alpha 2}$ has a slightly greater mass than $G_{i\alpha 1}$ at 40.5kDa and a catalytic activity of $k_{cat}(\text{min}^{-1})$ 2.7 at 20°C. Of the three $G_{i\alpha x}$ subunits, $G_{i\alpha 2}$ shares the least homology with $G_{i\alpha 1}$ with 88% amino acid identity. However, in common with other α -subunits of this class $G_{i\alpha 2}$ contains the conserved PTX ADP-ribosylation site at its C-terminal tail. As with the other $G_{i\alpha x}$ family members, $G_{i\alpha 2}$ can be found in almost every tissue type.

Although all three $G_{i\alpha x}$ subunits can directly inhibit adenylyl cyclase activity *in vitro* (Taussig *et al.*, 1993) evidence suggests that $G_{i\alpha 2}$ is the main regulator of this function *in vivo*. For example McKenzie and Milligan (1990) have shown that the inhibition of forskolin stimulated adenylyl cyclase activity can be attenuated by a $G_{i\alpha 2}$ specific antiserum in a NG-108-15 hybrid cell line. The inhibition of adenylyl cyclase activity may not be the only physiological role of this subunit. Yatani *et al.* (1988) have demonstrated that the addition of activated $G_{i\alpha 2}$ to membrane patches from cardiac myocytes stimulates potassium channel opening in a PTX sensitive manner.

1.3.3.4 $G_{i\alpha 3}$

Although $G_{i\alpha 3}$ has the same molecular mass as $G_{i\alpha 2}$ (40.5kDa) it shares significantly more amino acid identity with $G_{i\alpha 1}$ (94%) than $G_{i\alpha 2}$. As with the other two $G_{i\alpha x}$ subunits, this protein is nearly ubiquitous in all tissue types and is a substrate for pertussis toxin catalysed ADP-ribosylation. At $k_{cat}(\text{min}^{-1})$ of 1.8 at 20 °C, $G_{i\alpha 3}$ has the lowest catalytic activity of the three $G_{i\alpha x}$ subunits. While $G_{i\alpha 3}$ can inhibit the activity of

adenylyl cyclase *in vitro*, its physiological role is thought to involve the activation of ion channels. For example, Schwiebert *et al* (1990) have observed $G_{i\alpha 3}$ mediated stimulation of a large conductance renal apical Cl^- channel in rabbit kidney CCD cells. Yatani *et al* (1988) have also shown that $G_{i\alpha 3}$ can activate potassium channels in cardiac myocytes. Evidence also suggests that $G_{i\alpha 3}$ may modulate membrane trafficking in certain cell lines. For example, Stow *et al.* (1991) have shown that over expression and localisation of $G_{i\alpha 3}$ in the Golgi apparatus of the LLC-PK1 kidney cell line reduces the rate of protein secretion and trafficking in a PTX sensitive manner.

1.3.3.5 $G_{o\alpha}$

$G_{o\alpha}$ or "G-other" proteins were first purified from bovine brain where they account for a remarkable 1-2% of membrane protein. At present two splice variants are known, $G_{o\alpha 1}$ and $G_{o\alpha 2}$, both of which share 73% amino acid identity with $G_{i\alpha 1}$. Both splice variants originate from the same gene and arise from alternate splicing of exons 7 and 8 (Kaziro *et al.*, 1991). The resultant α -subunits differ in both C-terminal sequence and molecular mass ($G_{o\alpha 1}$ =40kDa, $G_{o\alpha 2}$ =40.1kDa). More significantly, unlike the splice variants of $G_{s\alpha}$, the two alternately spliced $G_{o\alpha}$ subunits appear to discriminate between different receptors. Evidence of this comes from Kleuss *et al.* (1991) who observed that calcium channel activity in rat GH3 pituitary cells can be attenuated by $G_{o\alpha 1}$ coupling to the muscarinic receptor or $G_{o\alpha 2}$ coupling to the somatostatin receptor respectively. In common with $G_{i\alpha 3}$, $G_{o\alpha}$ has an estimated catalytic activity of $k_{cat}(\text{min}^{-1})$ 1.8 at 20°C

1.3.3.6 $G_{t\alpha}$

At present two isoforms of $G_{t\alpha}$, or G-transducin, have been identified both of which share a 68% amino acid identity with $G_{i\alpha 1}$. The two isoforms, termed $G_{t\alpha 1}$ and $G_{t\alpha 2}$, are highly localised to two specific tissue types associated with light and colour perception. $G_{t\alpha 1}$ is found exclusively in retinal rod cells where it couples with the visual receptor rhodopsin, while $G_{t\alpha 2}$ is located in retinal cone cells where it couples to cone opsin. Despite their discrete locations, both subunits are thought to activate a common secondary effector, a cyclic GMP (cGMP) specific phosphodiesterase (Chambre and Deterre, 1989). The activated phosphodiesterase hydrolyses the secondary messenger cGMP, which in the absence of agonist has been shown to maintain various ion

channels in an open state (Fresenko *et al.*, 1985). Depletion in the levels of cGMP results in the closure of potassium ion channels and so membrane polarisation.

$G_{i\alpha 1}$ displays the highest catalytic activity of any of the G-protein α -subunits ($k_{cat}(\text{min}^{-1})=3.6$ at 20°C). It is also one of the two members of the G_i family which can be post translationally modified by both pertussis toxin and cholera toxin.

1.3.3.7 $G_{\text{gust}\alpha}$

G-gustducin, or $G_{\text{gust}\alpha}$, was first identified by McLaughlin *et al* (1992) in taste receptor cells where its expression appears to be limited to 40% of the cellular population. Evidence suggests that $G_{\text{gust}\alpha}$ is involved in sweet/bitter taste perception (McLaughlin *et al.*, 1992). Gustducin is thought to be the taste receptor homologue of transducin, this is reflected by its tissue specific location and the fact that it shares greater homology with $G_{i\alpha}$ (80%) than with $G_{i\alpha 1}$ (68%). Additional evidence from Hoon *et al.* (1995) has shown that $G_{\text{gust}\alpha}$ and $G_{i\alpha}$ share many functional similarities. Using a baculovirus expression system in insect Sf9 cells, Hoon *et al.* (1995) have demonstrated that $G_{i\alpha}$ and $G_{\text{gust}\alpha}$ are functionally identical with regard to interactions with bovine rhodopsin, $\beta\gamma$ heterodimers, cGMP specific phosphodiesterase activation and nucleotide exchange rate. In common with $G_{i\alpha}$, $G_{\text{gust}\alpha}$ is thought to be modified by both PTX and CTX.

1.3.3.8 $G_{z\alpha}$

$G_{z\alpha}$ displays highly specific tissue distribution in platelets, chromaffin and neuronal cells with long axonal processes (Fong *et al.*, 1988; Casey *et al.*, 1990, Matsuoka *et al.*, 1988). Of all of the G_i family members, $G_{z\alpha}$ shares the least amino acid identity with $G_{i\alpha 1}$ (60%), this is reflected in its unique biochemical properties.

In addition to having a slow guanine nucleotide exchange rate ($k_{off} = 0.02/\text{min}^{-1}$ at 30°C), $G_{z\alpha}$ possesses one of the lowest catalytic activities seen in any of the α -subunits ($k_{cat}(\text{min}^{-1})=0.05$ at 30°C). This low catalytic activity, which is approximately 200 times lower than that of $G_{i\alpha 1}$, and guanine nucleotide exchange rate may be due to its unusually high Mg^{2+} requirements for guanine nucleotide binding. A similar Mg^{2+} dependence for GDP binding in small nucleotide binding proteins such as Ras has been shown to reduce GDP dissociation by up to four orders of magnitude (John *et al.*, 1993).

G_{zα} is also the only α-subunit in the Gi family that cannot be modified by PTX treatment. An isoleucine residue as opposed to a cysteine residue four amino acids from the C-terminal tail confers insensitivity to this bacterial toxin.

Evidence suggests that G_{zα} may play a role in regulating adenylyl cyclase activity. Wong *et al.* (1992) have shown that co-transfection of G_{zα} with the D₂ dopamine, A₁ adenosine, lysophosphatidic acid or α₂ adrenergic receptors into HEK293 cells allows PTX resistant inhibition of adenylyl cyclase activity upon activation of these receptors. Another study using intracerebroventricular injection of antisense G_{zα} oligonucleotides into rats has highlighted a possible nociceptive role for this α-subunit (Sanchez-Blazquez *et al.*, 1995).

1.3.4 G_{qα} family

The G_{qα} family contains 5 subunits designated G_{qα}, G_{11α}, G_{14α}, G_{15α} and G_{16α}. All family members lack the conserved cysteine residue seen in the C-terminal tail of Gi family members and are so resistant to PTX modification. Additionally, unlike the Gi family members, all members of the G_q family share a common effector, phospholipase C-β (PLC-β). Phospholipase C-β catalyses the hydrolysis of phosphatidylinositol 4,5 bisphosphate to generate two secondary messengers, inositol 1,4,5 trisphosphate (IP₃) and diacylglycerol (DAG). IP₃ regulates the flow of Ca²⁺ from the endoplasmic reticulum to the cytosol, where in combination with the secondary messenger DAG it can activate the secondary messenger kinase, protein kinase C (PKC). Evidence suggests that members of this G-protein family activate at least two of the three isoforms of PLC-β, PLC-β₁ and PLC-β₂ (Lee *et al.*, 1992). Out of the five G_{qα} family members only G_{qα} and G_{11α} show ubiquitous tissue distribution. Expression of G_{14α} appears to be more localised to stromal cells, while G_{15α} and G_{16α} expression is restricted to hematopoietic B and T cells respectively (Wilkie *et al.*, 1991; Amatruda *et al.* 1991).

Differences in the N-terminal regions of G_{qα} and G_{11α} account for most of the disparity in sequence homology between these two proteins. The 12% difference in amino acid identity between G_{qα} and G_{11α} lies in a region known to be concerned with nucleotide exchange and hydrolysis as well as βγ subunit binding. It has therefore been postulated that these differences may play a role in determining PLC-β isoform

specificity as well as the catalytic activity of the G-proteins (Simon *et al.*, 1991). $G_{q\alpha}$ and $G_{11\alpha}$ also contain a unique 6 amino acid extension at the N-terminus of the protein. This extension may help define receptor selectivity, as a deletion or mutation at this region has been shown to allow coupling of these α -subunits to non $G_{q\alpha}$ coupled receptors (Kostenis *et al.*, 1997; Kostenis *et al.*, 1998). Other members of this subfamily are more distantly related to $G_{q\alpha}$, with $G_{14\alpha}$, $G_{15\alpha}$ and $G_{16\alpha}$ displaying 79%, 57% and 58% amino acid identity respectively.

Despite their restrictive expression patterns in hematopoietic B and T cells, $G_{16\alpha}$ and the murine homologue $G_{15\alpha}$ appear to couple to a wide array of receptors. Offermans *et al.* (1995) have demonstrated functional coupling of $G_{16\alpha}$ and $G_{15\alpha}$ to the M_2 muscarinic, β_2 adrenergic, V_2 vasopressin, dopamine D_2 and adenosine A_2 receptors. In all cases stimulation of the receptor in question led to the activation of the $G_{15/16\alpha}$ secondary effector PLC- β . This promiscuity in receptor coupling has led to the suggestion that $G_{16\alpha}$ may function as a universal G-protein adapter (Milligan *et al.*, 1996). The potential use of such an adapter protein has been realised in the development of a novel assay to detect the activation of G-protein coupled receptors via an aequorin reporter system (Green *et al.*, 1997). In addition to its unusual receptor promiscuity, $G_{16\alpha}$ appears to discriminate between different PLC- β isoforms. Lcc *et al.* (1992) have demonstrated that while $G_{q\alpha}$ and $G_{11\alpha}$ preferentially activate PLC- β_1 , $G_{16\alpha}$ shows greater stimulation of PLC- β_2 . The activation of PLC- β may also serve to autoregulate the activity of $G_{16\alpha}$. As described earlier, activation of PLC- β generates the secondary messengers IP_3 , which increases cytoplasmic Ca^{2+} levels, and DAG that in combination with Ca^{2+} activates the secondary messenger kinase PKC. Aragay *et al.* (1999) have shown that direct stimulation of PKC activity can attenuate $G_{16\alpha}$ mediated TRH signalling in COS-7 cells co-transfected with $G_{16\alpha}$ and the TRH receptor. This effect can also be mimicked by repeated stimulation of the TRH receptor and is correlated with an increase in PLC- β activity. Furthermore, this attenuation of TRH response is not seen in cells co-transfected with a mutant $G_{16\alpha}$ isoform lacking four PKC phosphorylation sites.

1.3.5 G_{12/13α} family

G_{12α} and G_{13α} are both 44kDa proteins and share a 67% common amino acid identity. In common with G_{2α}, G_{12α} and G_{13α} display low rates of guanine nucleotide exchange (Fields *et al.*, 1997). Despite their extensive tissue distribution, very little is known about the physiological roles of G_{12α} and G_{13α}. Recent studies have revealed several possible roles for these subunits, although none of these have been proved conclusively. Since both proteins are ubiquitously expressed it has been suggested that they regulate a diverse array of effectors. Increasing evidence suggests that both G_{12α} and G_{13α} can regulate Na⁺/H⁺ exchange. Dhanasekaran *et al.* (1994) have demonstrated that transfection of mutationally activated G_{12α} or G_{13α} subunits can increase Na⁺/H⁺ activity 2.5 fold in COS-7 cells. A similar effect has been observed upon activation of a G_{13α}/G_{2α} chimera by Gi coupled receptors (Voyno-Yasenetskaya *et al.*, 1994a). G_{13α} stimulation of Na⁺/H⁺ exchange is thought to be mediated through Cdc42/MEKK and Rho dependent pathways (Hooley *et al.*, 1996).

Experimental evidence also suggests that G_{12α} and G_{13α} are also involved in growth regulation. Aragay *et al.* (1995) have demonstrated increased AP-1 mediated transcriptional activation in 1321 astrocytoma cells upon expression of a constitutively active G_{12α} mutant. Constitutively active mutants of G_{12α} and G_{13α} have also been shown to transform NIH 3T3 and RAT1 fibroblasts (Chan *et al.*, 1993; Jiang *et al.*, 1993; Xu *et al.*, 1993; Voyno-Yasentskaya *et al.*, 1994b).

Conversely, many effectors have been excluded from direct regulation by either G_{12α} or G_{13α}. Using purified α-subunits, Kozaza *et al.* (1995) and Singer *et al.* (1994) have demonstrated that neither G_{12α} or G_{13α} can regulate the effectors PLC(β₁, β₂, β₃ and δ₁), phospholipase D, adenylyl cyclase (types I, II and IV) or phosphoinositidase 3-kinase activity in *in vitro* based assays.

1.3.6.1 Post translational modification of α-subunits

Following translation, the α-subunit may be modified by a variety of factors both endogenous (e.g. PKC) or of foreign origin (e.g. PTX). Endogenous factors have been shown to be involved in regulation of α-subunit activity and are required for correct G-protein function. In contrast, external factors which regulate G-protein activity are invariantly of pathogenic origin and therefore detrimental to G-protein

signalling. There are four main post-translational events that affect G-protein α -subunits; these include phosphorylation, ADP-ribosylation, palmitoylation and myristoylation. Each of these events and their associated factors will now be discussed in detail below.

1.3.6.2 Phosphorylation

Regulation of G-protein mediated signalling by phosphorylation at the level of the receptor is a highly characterised, well-established phenomenon. Increasing evidence now suggests that phosphorylation of the G-protein α -subunit may play a similar role in the attenuation of signalling by this receptor superfamily. At present, phosphorylation has only been detected in four of the 20 known α -subunits; these include $G_{7\alpha}$, $G_{12\alpha}$, $G_{16\alpha}$ and the murine homologue $G_{15\alpha}$. The limited number of α -subunits which are substrates for phosphorylation suggests that phosphorylation of the α -subunit may not be a universal system of G-protein regulation. In instances where no G-protein phosphorylation is detected, signalling may be attenuated by alternate means such as receptor phosphorylation.

Numerous studies suggest that the secondary messenger kinase PKC is the principal component in α -subunit phosphorylation. Evidence of this was first presented by Katada *et al.* (1985) who demonstrated phosphorylation of a 44 kDa G_i α -subunit in platelet membranes upon the addition of purified PKC. This phosphorylated α -subunit was later shown to be $G_{7\alpha}$ through systematic elimination of all other α -subunits in this G-protein family (Loansbury *et al.*, 1991). The mechanism by which phosphorylation attenuated $G_{7\alpha}$ signalling was not resolved until 1995 when Fields and Casey (1995a) demonstrated that phosphorylation at the α -subunit inhibited $\beta\gamma$ subunit binding and so the formation of the G-protein heterotrimer. This was followed by a similar observation for $G_{12\alpha}$ (Fields and Casey, 1995b), suggesting that phosphorylation may attenuate signalling through a single common mechanism.

The secondary messenger kinase, PKC, is activated by a combination of Ca^{2+} and diacylglycerol (DAG), the intracellular levels of which are elevated upon activation of the secondary effector PLC- β . Since PLC- β is itself activated by the $G_{q\alpha}$ family members $G_{16\alpha}$ and $G_{15\alpha}$, phosphorylation may serve as an autoregulatory feature at these G-proteins. Evidence of this autoregulatory feature has been demonstrated in two

G_{qα} family members G_{16α} and G_{15α}, by Aragay *et al.* (1999). Aragay *et al.* (1999) have demonstrated that repeated stimulation of the G_{qα} linked TRH receptor results in eventual signal attenuation. This effect has been correlated to increased phosphorylation at G_{16α} and the murine homologue G_{15α}. The observation that this effect can be mimicked by PKC activators implicates the involvement of PKC in this regulatory pathway. The involvement of PKC was corroborated by the fact that attenuation of signalling was lost when a mutant G_{16α} subunit lacking PKC phosphorylation sites was used in the above experiments. On a final note, this autoregulatory feature appears to be peculiar to G_{16α} and G_{15α} as none of the other G-proteins in this family were seen to be phosphorylated (Aragay *et al.*, 1999).

1.3.6.3 ADP-ribosylation

Various members of the G-protein superfamily have been shown to be substrates for ADP-ribosylation by bacterial toxins. Since ADP-ribosylation has the effect of disrupting signalling through susceptible G-proteins, bacterial toxins have proved to be useful tools in elucidating G-protein signalling pathways. Two bacterial toxins which have been used extensively in such studies are pertussis toxin (PTX) from the bacterial *Bordetella pertussis* and cholera toxin (CTX) from the pathogen *Vibrio cholerae*. Both of these toxins catalyse ADP-ribosylation at different residues on the α-subunit and therefore have vastly different effects. They also demonstrate a high degree of substrate selectivity, with very little overlap between the two toxins.

Pertussis toxin, from *Bordetella pertussis*, was first discovered by Kadota and Ui in 1977. This toxin was previously designated “islet activating protein” in accordance with its capacity to abolish α₂-adrenergic signalling in the pancreas. Pertussis toxin has since been shown to exert its effect through the Gi family of G-proteins. All members of this G-protein family, with the exception of G_{zα} that contains an isoleucine residue, possess a cysteine residue four amino acids from the C-terminal tail. Pertussis toxin catalyses the transfer of an ADP-ribose group from a NAD⁺ molecule to this cysteine residue (West *et al.*, 1985). This catalytic event involves the formation of a PTX/NAD⁺ transition state complex before final docking to the G-protein acceptor (Scheuring *et al.*, 1998). It appears that βγ subunits are required for efficient PTX catalysed ADP-ribosylation, although it should be noted that they have little effect upon the catalytic rate of the toxin (Scheuring *et al.*, 1998). This has led to the suggestion that βγ subunits

may aid docking of the PTX/NAD⁺ complex to the G-protein. Alternatively, $\beta\gamma$ subunits may preserve the C-terminal tail of the α -subunit in a disordered conformation so allowing toxin access to the conserved cysteine residue. The acceptor site for PTX catalysed ADP-ribosylation lies in the C-terminal tail of the α -subunit. Numerous studies have shown this region to be a critical determinant in receptor coupling and specificity. It is therefore not surprising to learn that ADP-ribosylation at this site blocks G-protein receptor coupling through presumably unfavourable steric interactions. Unable to couple to an activated receptor, the ADP-ribosylated heterotrimer is locked in the inactive GDP bound state.

The second toxin, cholera toxin (CTX), from *Vibrio cholerae* is quite distinct from PTX in both its site of ADP-ribosylation and its modulatory effect. Cholera toxin catalyses the transfer of ADP-ribose from NAD⁺ to a conserved arginine (201) located in the middle of the $G_{s\alpha}$ / $G_{i\alpha}$ subunit. This arginine residue is proposed to stabilise a negative charge on the terminal phosphate of GTP, so aiding GTP hydrolysis (Sprang, 1997). It is therefore not surprising to note that ADP-ribosylation at this arginine residue abolishes the capacity of the subunit to hydrolyse GTP. Since this modification does not alter the capacity of the subunit to bind GTP, once activated the α -subunit is locked in its activated state. In common with PTX, CTX requires $\beta\gamma$ subunits for efficient ADP-ribosylation. Although the requirements for $\beta\gamma$ subunits are unclear, it has been proposed that these subunits aid docking of the toxin or stabilise the α -subunit in a conformation amenable to the toxin. Despite their substrate specificities, both toxins can ADP-ribosylate transducin, which contains both acceptor sites (Van Dop *et al.*, 1983; West *et al.*, 1985).

1.3.6.4 Palmitoylation and myristoylation

Although palmitoylation and myristoylation occur independently, both modifications contribute to the membrane attachment of α -subunits. Palmitoylation and myristoylation appear to play different roles in this process, in so far as palmitoylation appears to be a more temporary modification than myristoylation.

The post-translational process of myristoylation involved the addition of a 14-carbon fatty acid, myristate, to the N-terminus of the G-protein α -subunit. This event occurs at a well-defined consensus sequence characterised by a glycine residue followed

by a hydroxylamine residue four amino acids further downstream. G-protein α -subunits known to be myristoylated include $G_{i\alpha1}$, $G_{i\alpha2}$, $G_{i\alpha3}$, $G_{t\alpha}$ and $G_{z\alpha}$. Notable exceptions to this list are $G_{s\alpha}$ and $G_{q\alpha}$, both of which have been shown to be alternatively modified by palmitoylation. In contrast to palmitoylation, myristoylation is a permanent event and is therefore not expected to play a regulatory role in G-protein signalling.

Palmitoylation and myristoylation are not mutually exclusive events, and many of the α -subunits known to be myristoylated are also palmitoylated. Subunits known to be palmitoylated include $G_{o\alpha}$, $G_{i\alpha1}$, $G_{i\alpha2}$, $G_{i\alpha3}$, $G_{z\alpha}$, $G_{gust\alpha}$, $G_{q\alpha}$ and $G_{s\alpha}$. Palmitoylation involves the addition of palmitate, a 16 carbon fatty acid, to a cysteine residue located at the N-terminus of the G-protein α -subunit. Cysteine residues known to be palmitoylated include cysteine 3 in $G_{s\alpha}$ and $G_{o\alpha}$ (Degtyarev *et al.*, 1993; Parenti *et al.*, 1993) and cysteine 9 and 10 in $G_{q\alpha}$ (Wedegaertner *et al.*, 1993). Palmitoylation is a reversible process, whose half-life has been estimated to be 50-90 minutes at the inactive α -subunit or 2 minutes upon G-protein activation (Wedegaertner and Bourne, 1994). This has led to the suggestion that palmitoylation may be used to regulate G-protein activity by varying the affinity of the α -subunit for the cell membrane. This could presumably attenuate signalling by removing the inactive G-protein from the immediate environment of the activated receptor (Wedegaertner *et al.*, 1995).

1.3.7 $\beta\gamma$ subunits

Although the $\beta\gamma$ heterodimer is composed of two independent polypeptides, it functionally acts as a monomer that cannot be divided into its individual components except in strongly denaturing conditions. At present six β and 12 γ subunits have been identified, ranging in molecular mass from 35 to 36 kDa for the β subunit and 6-9 kDa for the γ subunit respectively. β subunits show less sequence diversity (50-90% amino acid identity) than the significantly smaller γ subunits that share between 30 and 80% amino acid identity. Not all combinations of β and γ are possible. For example, the $G_{t\alpha}$ associated γ_1 subunit can form a dimer with β_1 but not β_2 despite the 93% amino acid homology between these two β -subunits (Iniguez-Lluhi *et al.*, 1992; Pronin and Gautam, 1992; Schmidt *et al.*, 1992). Chimeric studies have highlighted a Phe residue at position 40 in the γ_1 subunit that mediates the specificity of $\beta\gamma$ heterodimer combinations (Lee *et al.*, 1995). It is assumed that the side chain of the Phe 40 residue

slots into a complimentary cavity which is defined by a Leu residue at position 300 on the β_1 -subunit. The strength of this association is thought to be governed by the size of the side chain of residue 40 and the depth of the cavity on the β_1 subunit. (Sondek *et al.*, 1996).

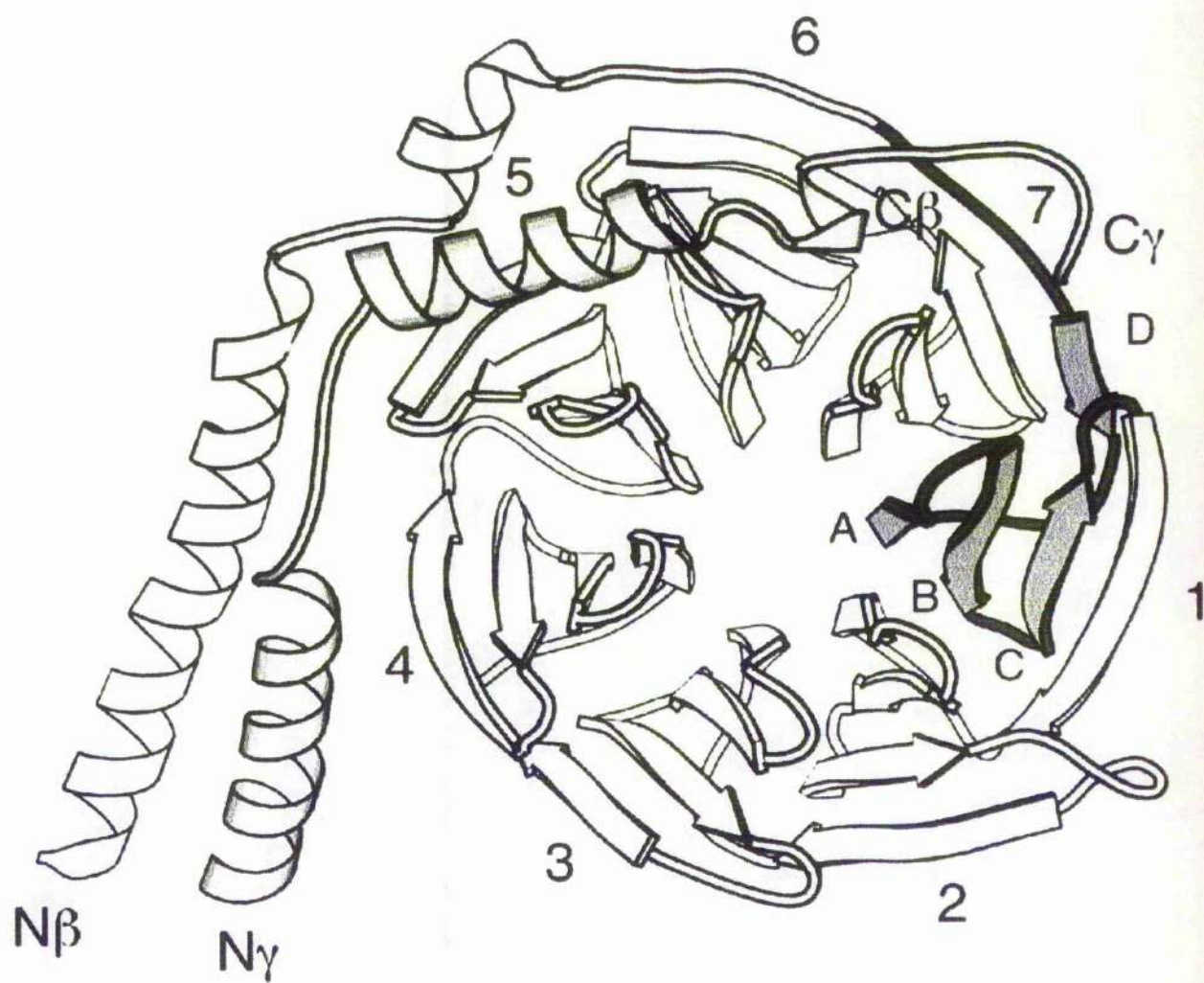
Resolution of the crystal structures of both free $\beta_1\gamma_1$ and $\beta_1\gamma_2$ as part of a $G_{i\alpha 1}$ heterotrimer suggest that different isoforms of $\beta\gamma$ share many common structural determinants (Sondek *et al.*, 1996; Wall *et al.*, 1995). Structurally, the β subunit appears to be composed of two distinct domains, an N-terminal helical domain and a significantly larger “ β -propeller” domain (Fig. 1.2). The N-terminal domain is composed of a relatively simple helical structure approximately 20 residues in length. In contrast to this, the β -propeller domain is approximately 300 residues long and is composed of seven repeating WD motifs that fold to form a seven bladed β -propeller structure. Each of the WD repeats is approximately 43 residues long and is characterised by a conserved core of amino acids which are flanked by Gly-His (GH) and Trp-Asp (WD) motifs. The WD repeats are separated by a region more variable in length (6-94 residues). Each blade of the β -propeller structure is composed of four anti-parallel strands, which associate to form a four-stranded twisted β -sheet. No single WD repeat corresponds to a β -propeller blade or any stable independently expressible domain. Instead, each WD repeat comprises the inner three strands of a single blade and the C-terminal strand of the adjacent propeller blade. The outer strand of the seventh blade is provided by the N-terminal strand of the first WD repeat. This serves to close and presumably stabilise the seven bladed circular structure. Additional stabilisation of the structure may be provided by hydrogen bonding between highly conserved Asp residues in the middle of the WD repeat and the histidine residue in the GH flanking sequence.

In contrast to the β subunit, the γ subunit has little or no tertiary structure and is composed of two helical segments joined together by a loop. The γ subunit is subject to post-translational modification and may be isoprenylated at a CAAX (C-aliphatic-aliphatic-any amino acid) motif at the C-terminus. Isoprenylation may take the form of farnesylation or geranylgeranylation depending upon the identity of the terminal amino acid in the motif (Casey *et al.*, 1994). Isoprenylation is considered to be important for membrane localisation of the $\beta\gamma$ dimer and so correct function of this subunit. The

Figure 1.2 A 3-Dimensional representation of the $\beta_1\gamma_2$ heterodimer

Figure 1.2 represents the three dimensional structure of the $\beta_1\gamma_2$ heterodimer as resolved by Wall *et al.*(1995). The γ subunit is represented as a darkened region extended across the circumference of the β -subunit. The first WD repeat in the β -subunit is also shown as a darkened region, with shading heavier than at the γ subunit. The propeller blades of the β -subunit are numbered 1-7 from the N-terminus of the protein.

Figure 1.2



association between the β and γ subunits is stabilised at two distinct points. The first of these is a coiled coil interaction between the N-terminal helices of both subunits. Secondly the γ subunit extends across the circumference of the β -propeller where it makes extensive hydrophobic interactions with the outer strands of blades 1, 5, 6 and 7 (Fig. 1.2). The lack of intrachain interactions in the γ subunit may explain the strong association between the $\beta\gamma$ subunits.

The G-protein α -subunit is thought to associate with the $\beta\gamma$ dimer through two principal contact sites, the N-terminal helix and the switch II region. The N-terminal helix of the G_{α} subunit is thought to dock alongside the residues in the C-D strands of first propeller blade of the β subunit, parallel to the central tunnel of the β -propeller. This interaction is presumed to be stabilised by hydrophobic modifications at the C-terminus of the γ subunit and the N-terminus of the α -subunit. These modifications are thought to localise the two subunits in the same general vicinity of the lipid bilayer (Lambright *et al.*, 1996; Wall *et al.*, 1995). Removal of the N-terminus has been shown to disrupt heterotrimer formation in $G_{i\alpha}$, $G_{o\alpha}$, $G_{i\alpha 1}$ and $G_{s\alpha}$ (Navon *et al.*, 1987; Graf *et al.*, 1992; Journot *et al.*, 1991). The switch II region of G_{α} is orientated directly above the central tunnel formed by the blades of the β -propeller. In the β_1 subunit, hydrophobic interactions are thought to be centred around the conserved Trp 99 and Trp 332 residues. This is supported by evidence that a mutation of a residue equivalent to that of Trp 99 (Trp 136) in the β -subunit of *S.cerevisiae* to glycine results in constitutive activation of the mating pathway by presumably disrupting heterotrimer formation (Whiteway *et al.*, 1994). The switch II region of the α -subunit is known to undergo a conformational change upon binding GTP. It is therefore presumed that nucleotide exchange in the α -subunit results in the switch II region being drawn into the α -subunit, thereby disrupting α - $\beta\gamma$ subunit interactions and so allowing heterotrimer dissociation. Dissociation of the G-protein heterotrimer is thought to uncover important effector contact sites on both the α - and the $\beta\gamma$ subunits.

A functional role for $\beta\gamma$ was not discovered until 1987 when Logothetis *et al.* Demonstrated activation of cardiac K^+ channels by $\beta\gamma$ subunits purified from bovine brain. Since then the list of effectors known to be regulated by $\beta\gamma$ has grown to the point where $\beta\gamma$ subunits are recognised as important signalling molecules in their own right. $\beta\gamma$ subunits can function both independently of the G_{α} subunit or they can act in

combination with the $G\alpha$ signalling molecule in either a synergistic or antagonistic manner. Examples of effectors known to be modulated by $\beta\gamma$ subunits include phospholipase A_2 , yeast mating response, Ca^{2+} channels, adenylyl cyclases, phospholipase C and receptor kinases (Clapham and Neer, 1993). Of these examples, adenylyl cyclase is probably the best characterised effector known to be modulated by a combination of both α - and $\beta\gamma$ subunits. The effects of $\beta\gamma$ have been shown to be both antagonistic (Type I) and synergistic (Types II, IV) for the various adenylyl cyclase isoforms (Tang *et al.*, 1991). However, the effects of $\beta\gamma$ on these isoforms is conditional upon prior activation by $G_{s\alpha}$. In contrast, the synergistic or antagonistic activity of $\beta\gamma$ subunits on the secondary effector PLC- β are not conditional on priming by either subunit. These observations suggest that convergence of signalling between $\beta\gamma$ and α -subunits may provide a "fine tuning" mechanism to modulate the intracellular effects of an agonist.

At present, the effector contact sites on the $\beta\gamma$ subunit are not well defined. However, the similarity in the structure of the free $\beta\gamma$ subunit with its counterpart in the G-protein heterotrimer suggests that the principal effector contact sites are sequestered by α -subunit binding. Evidence supporting this comes from Chen *et al.* (1995) who demonstrated that a peptide from adenylyl cyclase type II can block $\beta\gamma$ subunit activation of both PLC- β and an inward rectifier K^+ channel. This peptide was later cross linked to a region on the β -subunit known to be involved in α -subunit binding (Chen *et al.*, 1997). Other regions thought to be involved in effector regulation include the N-terminal coiled coil and β -propeller blades 1 and 7 (Hamm, 1998).

1.3.8 G-protein α -subunit structure

The three dimensional crystal structures have been resolved for a variety of GTP binding proteins complexed with both guanine nucleotides and guanine nucleotide analogues. Analysis of the crystal structures of Ras, $G_{i\alpha 1}$ and $G_{t\alpha}$ have provided insights into the relationship between G-protein structure and function (Pai *et al.*, 1990; Coleman *et al.*, 1994; Noel *et al.*, 1993). The G-protein α -subunit appears to be composed of two distinct domains designated the GTPase, or G domain and an α -helical domain (Fig. 1.3(a)). The GTPase domain is a highly conserved domain that is structurally similar to the nucleotide binding domain of the small nucleotide binding protein p21^{Ha-ras}. As the name implies, the GTPase domain is primarily involved in

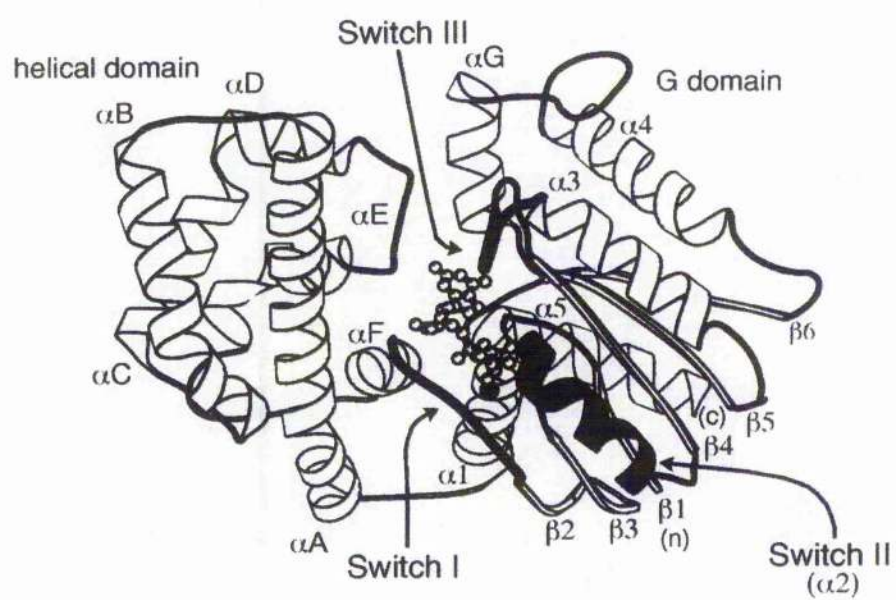
Figure 1.3 A 3-Dimensional representation of $G_{1\alpha}$ in both the active and inactive conformations

Figure 1.3 represents the two crystal structures of $G_{1\alpha}$ in both the GTP γ S bound "active" (**Fig. 1.3 (a)**) and the GDP bound "inactive" (**Fig. 1.3(b)**) conformations as resolved by Noel *et al.*(1993) and Lambright *et al.*(1994) respectively. The three switch regions associated with G-protein activation are represented as darkened regions on the active representation of $G_{1\alpha}$ (**Fig. 1.3 (a)**). Other regions annotated on the active representation include the helices of the α -helical domain (designated A to F from the N-terminus) and the β -sheets of the G-domain (labelled 1-6 from the N-terminus of the subunit).

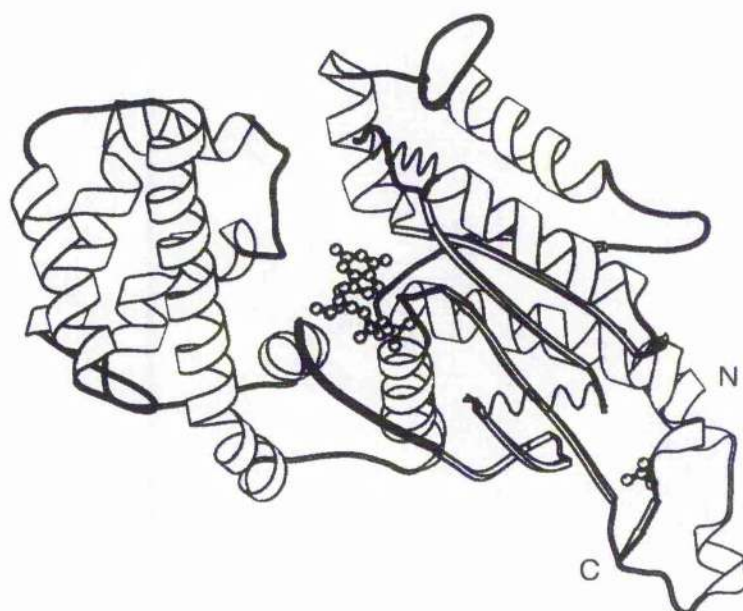
Figure reproduced from Sprang (1997)

Fig. 1.3

(a)



(b)



guanine nucleotide binding and hydrolysis, although it also contains sites necessary for receptor, effector and $\beta\gamma$ subunit binding. The GTPase domain is composed of 5- α -helices surrounding a six stranded β -sheet, each of which are designated α -1 to 5 and β 1 to 6 respectively. In common with other GTPase proteins, the guanine nucleotide binding site is formed from 5 polypeptide loops (P- or G-loops) designated G1 to G5. These loops are the most highly conserved structural elements across all members of the G-protein superfamily. These G-loops contain the consensus sequences known to be involved in GTP binding (GXGXXGKS in G1), Mg^{2+} binding (DXXG in G3) and binding of the guanine ring (NKXD in G4).

The second domain, the α -helical domain, as its name suggests, is composed entirely of α -helices. Six α -helices, designated A to E, bundle together to form an independently folded domain which can be stably expressed in the absence of the G-domain (Markby *et al.*, 1993). The helical domain is connected to the GTPase domain by two polypeptide linker segments 4 and 6 residues in length. The second domain appears to be unique to heterotrimeric GTP-binding proteins, and as of yet no structural equivalent has been detected in the smaller GTP binding proteins. The function of the helical domain is poorly defined, although it is expected to act as a "lid" shielding and stabilising the GTP/ Mg^{2+} complex in the guanine nucleotide binding pocket (Noel *et al.*, 1993). It has also been postulated that the α -helical domain may function as a GAP and facilitate nucleotide hydrolysis and exchange (Sprang, 1997).

1.3.9 G-protein structure vs function

In the inactive state, the G-protein is present as a heterotrimer of α , β and γ subunits and contains GDP in the nucleotide binding pocket (**Fig. 1.3(b)**). In order to switch from the inactive to the active state, the inactive G-protein must associate with an activated cell surface receptor. Association of the G-protein with an activated receptor is aided by lipid modifications at the α - and γ subunits, which not only attach the G-protein to the cell membrane but also define its orientation relative to the activated receptor. Although the conformational change is transmitted to the α -subunit, increasing evidence suggests that all three subunits may be involved in receptor coupling.

Experimental evidence suggests that the extreme C-terminus of the α -subunit defines the specificity of G-protein receptor coupling. The substitution of as little as 3

amino acids in the C-terminal tail of $G_{q\alpha}$ for the equivalent sequence of $G_{i\alpha 2}$ has been shown to be sufficient to switch the selectivity of the chimera to Gi coupled receptors (Conklin *et al.*, 1993). Similarly, a 5 amino acid substitution of the C-terminal tail of $G_{13\alpha}$ for $G_{2\alpha}$ creates a chimera that can activate Na^+/H^+ exchange upon stimulation of the dopamine D_2 receptor (Voyno-Yasentskaya *et al.*, 1994). This principal has been conclusively demonstrated by Komatsuzaki *et al.* (1997) who have shown that $G_{s\alpha}/G_{10\alpha(1/2)}$, $G_{s\alpha}/G_{14\alpha}$, and $G_{s\alpha}/G_{16\alpha}$ chimeras can channel stimulation of the somatostatin SSTR3 receptor to the $G_{s\alpha}$ effector adenylyl cyclase. In all of these examples, substitution of the C-terminal sequence resulted in a switch of receptor coupling specificity but did not prevent the chimera from interacting with its natural effector. The efficiency of receptor/chimera interactions appears to be related to the length of the C-terminal substitution. Conklin *et al.* (1993) have demonstrated that increasing the length of the amino acid substitutions in the C-terminal tail of $G_{q\alpha}$ to those of $G_{i\alpha 2}$ first increased receptor coupling efficiency, but then resulted in a gradual loss of receptor/G-protein coupling. It is interesting to note that the optimal number of substitutions was around 8 amino acids, which is exactly the same number of residues which are observed as being disordered in the G-protein crystal structure (Noel *et al.*, 1993). It has been proposed that these C-terminal residues adopt an ordered conformation in the free α -subunit which prevents their association with the activated receptor (Kisselev *et al.*, 1998). Finally, antibodies directed against the C-terminus of $G\alpha$ and PTX catalysed ADP-ribosylation of a cysteine residue four amino acids from the C-terminus in most Gi family members has been shown to disrupt G-protein receptor coupling (Simonds *et al.*, 1989; Gilman *et al.*, 1987).

Potential points of α -subunit/receptor interaction have been mapped through chimeric experiments and photochemical cross linking. Liu *et al.* (1995) have demonstrated that a C-terminal 5 amino acid substitution of $G_{q\alpha}$ for those of a Gi family member is sufficient to allow coupling of this chimera to Gi coupled M_2 muscarinic receptor. However, this same $G_{q\alpha}$ /Gi chimera cannot couple to the wild type form of the $G_{q\alpha}$ specific M_3 muscarinic receptor. By substituting varying length of intracellular loop 3 (IC3) of the Gi coupled M_2 muscarinic receptor, Liu *et al.* were able to generate a mutant M_3 muscarinic receptor capably of coupling to the $G_{q\alpha}$ /Gi chimera. Subsequent M_2 / M_3 muscarinic receptor mutants were used to map the points of interaction between the C-terminus of the chimera to four non-contiguous residues (Val 385, Thr 386, Ile

389 and Leu 390) located at the C-terminus in IC3. This four amino acid motif is conserved between both the M₂ and the M₄ Gi coupled muscarinic receptors. IC3 has also been implicated in other receptor/G-protein contact sites. Evidence suggests that IC3 does not interact exclusively with the C-terminus of the α -subunit. Taylor *et al.* (1994) have demonstrated that the N-terminal 17 residues of G_{o α} can be chemically cross linked to a peptide corresponding to the C-terminal region of α -adrenoceptor IC3.

Alanine scan mutagenesis has also highlighted other residues in the C-terminal half of G_{1 α} and G_{1 α} that may be involved in stabilising receptor coupling. For example Mazzoni and Hamm (1996) have demonstrated that the Arg 310 residue located in the $\alpha_2\beta_4$ loop of G_{1 α} is protected from tryptic cleavage in the presence of activated rhodopsin. Similarly, the $\alpha_2\beta_6$ loop has been implicated in Gi coupling to the 5HT_{1B} serotonin receptor (Bae *et al.*, 1997).

$\beta\gamma$ subunits may also contribute significantly to heterotrimeric G-protein/receptor interactions. Taylor *et al.* (1996) have demonstrated that a peptide corresponding to the C-terminal region of the α_2 adrenergic receptor can be chemically cross linked to the C-terminal 60 residues of G β . Although there is no direct evidence of γ subunit/receptor coupling, experiments have shown that the γ subunit is able to influence receptor specificity through the prenyl group at its C-terminus (Yasuda *et al.*, 1996). In addition to direct coupling to the receptor, the $\beta\gamma$ subunits also contribute to the efficiency and specificity of receptor coupling by localising the heterotrimer at the membrane in an orientation that can easily interact with the receptor and by maintaining the C-terminus of the α -subunit in a disordered conformation.

Upon coupling with an activated receptor, the G-protein undergoes a conformational change that allows exchange of the GDP molecule in the nucleotide binding pocket for GTP. This nucleotide exchange event is thought to involve movement of the α -helical "lid" domain and the C-terminal tail. This exposes the nucleotide binding pocket and allows GDP to dissociate and Mg²⁺/GTP to enter. Analysis of the crystal structures of active (GTP γ S bound) and inactive (GDP bound) forms of G_{1 α} have shown that the γ phosphate of the GTP/Mg²⁺ complex triggers a substantial re-arrangement of three discrete regions in the G-domain. These three regions, designated switch I, II and III, are the only points of re-arrangement in the virtually identical crystal structures (Lambright *et al.*, 1994). Interactions of the γ

phosphate of GTP with $G_{1\alpha}$ induces the switch II region to turn and connect with switch III. Switch III in turn draws switch II towards the γ phosphate group. The conformational changes at switch II and III are stabilised by ionic interactions between basic residues in the two switch regions. During this conformational change, switch I is pulled upwards into the nucleotide binding pocket.

Movement of the switch regions has two effects on α -subunit activity. First, the re-arrangement of the switch regions disrupts bonding between the α -subunit switch region II and the central tunnel of the β -propeller in the β -subunit, causing the α -subunit to dissociate from its $\beta\gamma$ partner. The GTP-bound α -subunit and free $\beta\gamma$ subunits are then able to engage with and activate secondary effectors. Secondly, the re-arrangement of switch regions exposes previously hidden determinants involved in effector coupling. Chimeric studies by Berlot and Bourne (1992) have identified three sites on the α -subunit that are involved in effector coupling. The first of these, the C-terminus of α -helix 2, corresponds to a portion of the G-domain encompassing the switch II region. The second segment is a loop connecting α -helix 3 to β -strand 5. The third region corresponds to the $\alpha 4\beta 6$ loop. An independent observation has shown that a peptide corresponding to this loop can activate the effector cGMP-PDE *in vitro* (Rarick *et al.*, 1992).

The period of G-protein/effector coupling is regulated by the intrinsic GTPase activity of the α -subunit. Upon GTP hydrolysis, the three switch regions revert back to their conformations in the inactive state. The loss of the γ phosphate, by the hydrolysis of GTP to GDP, destabilises bonds between switch regions II and III, which collapse back to their disordered state and so disengage effector coupling. Switch region II twists back to a conformation in which it can interact with free $\beta\gamma$ subunits. $\beta\gamma$ subunit re-association, in combination with the loss of effector determinants, serves to attenuate further G-protein/effector coupling. The heterotrimeric GDP-bound G-protein is then once again ready to transduce a signal from an activated cell surface receptor.

1.4 α -adrenergic receptors

Adrenergic receptors mediate the effects of the catecholamines such as adrenaline and noradrenaline by coupling to several of the major signalling pathways modulated by G-proteins. The distinction between α and β adrenoceptor subtypes was made as long ago as 1948 by Ahlquist on the basis of their pharmacological characteristics. Despite this early progress, for many years the distinction between different members in a receptor subtype remained blurred due to the lack of suitable subtype-selective ligands. This situation was not resolved until molecular cloning techniques were used to isolate the first GPCR, the β_2 adrenoceptor, by Dixon *et al.* in 1986. Since then, the isolation of additional family members has allowed the various adrenoceptor family members to be classified in the basis of sequence information as well as compound selectivity. The current classification system indicated the presence of two α -adrenoceptor subtypes, designated α_1 and α_2 . Each of these subtypes and their associated family members will now be discussed below, with special emphasis being placed on the α_2A adrenoceptor.

1.4.1 α_1 adrenoceptors

α_1 adrenoceptors can be distinguished from α_2 adrenoceptors on the basis of the potency of the two antagonists yohimbine and prazosin. Prazosin is more potent than yohimbine at the α_1 adrenoceptor, while the reverse is true at the α_2 adrenoceptors. Evidence suggests the presence of four subtypes of α_1 adrenoceptor, designated α_{1A} , α_{1B} , α_{1C} and α_{1D} . As with other adrenoceptor subtypes, studies into the distribution of these receptors has been hampered by the lack of selective ligands. Therefore, distribution studies have relied on the detection of receptor mRNA. Based upon Northern analysis on rat and bovine tissue, the α_{1B} and α_{1D} subtypes appear to be widely distributed with high levels of expression in peripheral tissues such as lung, kidney (α_{1B}), heart and spleen (α_{1D}). Radioligand binding has also shown a similar peripheral tissue distribution for the α_{1A} adrenoceptor. All three adrenoceptors appear to show high expression in CNS tissues such as the hippocampus, cerebral cortex and brainstem. In contrast to the above three receptors, Northern analysis has failed to accurately map α_{1C} distribution in either rat or bovine tissue. This has led to the suggestion that α_{1C} receptors either display highly specialised tissue distribution, or are only expressed during development. Finally, α_1 adrenoceptors are thought to be coupled

to the phosphoinositide signalling pathway through the pertussis toxin insensitive $G_{q\alpha}/G_{11\alpha}$ class of G-proteins.

Pharmacological characterisation of the α_1 adrenoceptor subtypes has been hampered by the lack of suitable selective ligands. However, by a process of elimination, all four members of the α_1 family can be resolved through the use of three selective antagonists. The α_{1A} receptor can be distinguished from the other three subtypes on the grounds that it cannot be irreversibly inactivated by the antagonist chloroethylclonidine. Similarly, the α_{1B} receptor can be identified by using the antagonist WB4104, which is 100 times more potent at the α_{1A} , α_{1C} and α_{1D} subtypes. Finally, the agonist 5-methylurapidil, which displays a 100 times greater potency at α_{1C} and α_{1D} than at α_{1A} and α_{1B} , can be used to distinguish the α_1 adrenoceptor subtypes α_{1C} and α_{1D} .

1.4.2.1 α_2 adrenoceptors

The α_{2A} adrenoceptor is one of three α_2 adrenoceptor subtypes designated α_{2A} , α_{2B} and α_{2C} . The human α_{2A} adrenoceptor was initially cloned by Kobilka *et al.* in 1987, a year after the cloning of the β_2 adrenoceptor (Dixon *et al.*, 1986). Kobilka identified this receptor as the principal receptor in human platelets and termed it α_{2C10} in accordance with its location at chromosome 10 in the human genome. Further cloning of the two other family members α_{2B} (α_{2C2}) and α_{2C} (α_{2C4}) showed that all three genes were encoded by intronless genes located at different chromosomes in the human genome (chromosome 2 for α_{2B} and chromosome 4 for α_{2C}) (Regan *et al.*, 1988; Lomasney *et al.*, 1990).

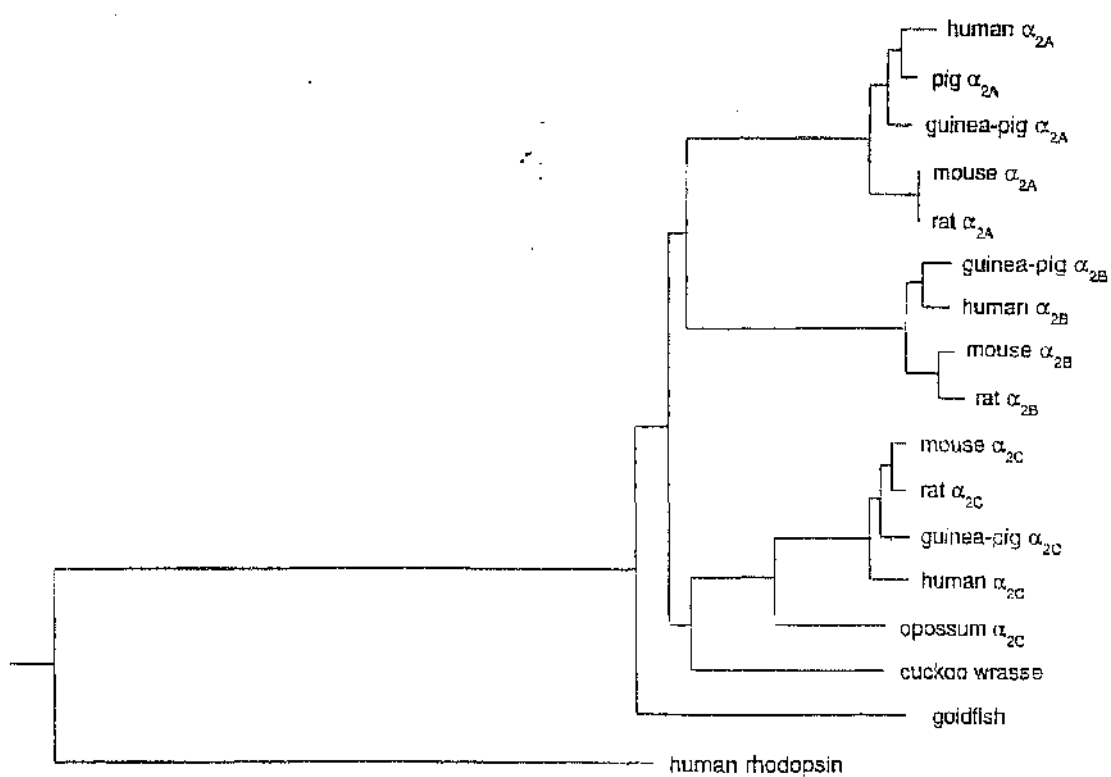
Cloning rapidly progressed from the human subtypes to other homologues in a wide range of species including- guinea pig, opossum, rat, cow chicken and pig (Svensson *et al.*, 1996; Blaxall *et al.*, 1994; Chalberg *et al.*, 1990; Blaxall *et al.*, 1993 and Guyer *et al.*, 1990). Subsequent cloning of these receptors highlighted a limitation of the original nomenclature as devised by Kobilka. Therefore, the α_2 nomenclature was revised to the current system based upon the phylogenetic analysis of the receptor sequences (Figure 1.4). While this system appears to be adequate for mammalian α_2 receptor subtypes, the α_2 adrenoceptors of the fish, cuckoo wrasse and goldfish, which show little sequence similarity with the mammalian subtypes, are difficult to position in

Figure 1.4 A phylogenetic representation of the α_2 adrenoceptor subtypes

Figure 1.4 represents the hypothetical phylogenetic tree of different α_2 adrenoceptor subtypes from a wide variety of species. The tree was generated from the alignment of multiple adrenoceptor primary sequences and was rooted using the amino acid sequence of human rhodopsin. The large, divergent third intracellular loop was excluded from this study. Sequence divergence is represented through the length of the horizontal lines.

Adapted from MacDonald *et al.*(1997)

Figure 1.4



the α_2 adrenoceptor phylogenetic tree. This has led to the proposal of an alternate fish subtype termed α_2F (Svensson *et al.*, 1993). Despite their structural differences, the fish subtypes appear to be most closely related to the mammalian α_2C subtypes (Figure 1.4).

1.4.2.2 The α_2A adrenoceptor

The gene for the human α_2A adrenoceptor encodes a protein 450 residues in length with a predicted molecular mass of 49 kDa. The receptor is subject to a variety of post translational modifications including glycosylation at two Asn residues near the N-terminus (Asn 10 and 14) and palmitoylation at a conserved cysteine residue in the C-terminal tail (Cys 442). In addition to this, the receptor is thought to form a disulphide bond between two cysteine residues in exoloops 1 and 2 (Cys 106 and Cys 188). In common with other members of the α_2 adrenoceptor family, the α_2A adrenoceptor has an unusually large third intracellular loop (157 residues) and a short C-terminal tail (20 residues). Phylogenetic analysis shows the human α_2A adrenoceptor to be closely related to other mammalian homologues. Analysis of the sequence of the human α_2A adrenoceptor with its porcine analogue has shown a 93% amino acid identity (Guyer *et al.*, 1990).

The α_2A adrenoceptor can be pharmacologically distinguished from the other two α_2 adrenoceptor subtypes by the selectivity of the weak partial agonist oxymetazoline which shows 50-100 times greater affinity at this receptor than at the α_2B or α_2C subtypes. The antagonists prazosin and ARC 239 also exhibit a 10-100 fold lower selectivity for the α_2A adrenoceptor relative to the α_2B or α_2C subtypes. The rat and mouse homologues of the human α_2A adrenoceptor can be characterised by a 20 fold lower affinity for the agonist yohimbine. This reduction in affinity for yohimbine has been attributed to a serine residue at position 210 instead of a cysteine residue which is present at the equivalent position in both the human and porcine receptors (Link *et al.*, 1992). Other determinants affecting ligand binding have been mapped to regions of transmembrane domain 7. For example, Suryanarayana *et al.* (1991) have shown that a Phe to Asn substitution at residue 341 in the seventh transmembrane domain of the α_2A adrenoceptor increases the affinity of the receptor for a variety of β receptor agonists and antagonists by as much as 3000 fold.

In common with other members of the α_2 adrenoceptor family, the α_2A adrenoceptor has been shown to mediate a variety of responses including- the inhibition of adenylyl cyclase, the inhibition of voltage gated Ca^{2+} channels and the opening of K^+ channels. In addition to this, the α_2A adrenoceptor has also been implicated in the activation of phospholipase isoforms C and D, the activation of adenylyl cyclase as well as the PTX insensitive inhibition of adenylyl cyclase (Limbird *et al.*, 1988; Wong *et al.*, 1992). This wide range of effectors has prompted a great deal of research into divergent signalling through this receptor in terms of both agonist channelling and G-protein coupling.

Divergent signalling through the α_2A adrenoceptor appears to be modulated by two primary factors, agonist concentration and agonist structure. For example, Eason *et al.*, (1992) have shown that the agonist UK14304 exhibits a biphasic response at the α_2A adrenoceptor in transfected CHO cells. At low concentrations of UK14304, this agonist mediated the inhibition of adenylyl cyclase by the α_2A adrenoceptor, whilst at high concentrations the agonist mediated the activation of adenylyl cyclase activity. In a similar experiment in HEK 293 cells, Chabre *et al.*, (1994) have demonstrated that the agonist UK14304 can force sequential coupling of the α_2A adrenoceptor to the G-proteins Gi, Gq and Gs respectively in a concentration dependent manner. The capacity of the agonist to promote divergent coupling also appears to be a function of its potency. Eason *et al.* (1994) analysed the capacity of a wide range of agonists to stimulate adenylyl cyclase activity through the α_2A adrenoceptor. From their findings they concluded that agonists vary in their capacity to elicit $\alpha_2A/G_{s\alpha}$ coupling, with full agonists such as UK14304 displaying a greater capacity to stimulate divergent coupling than weak partial agonists such as ozymetazoline. The structure of the ligand also appears to play an important role in determining the preference of G-protein coupling at the α_2A adrenoceptor. In contrast to the biphasic response exhibited by UK14304 (Eason *et al.*, 1992) Airriess *et al.* (1997) have demonstrated that phenolamines such as octopamine and synephrine, are only able to couple the α_2A adrenoceptor to a concentration dependent inhibition of adenylyl cyclase activity.

G-protein coupling at the α_2A adrenoceptor has been demonstrated through the use of various techniques including immuno-co-precipitation, photochemical cross linking and chimeric studies. The first direct evidence that a G-protein other than Gi

could couple to the α_2A adrenoceptor was presented by Eason *et al.* (1992) who co-precipitated an agonist- α_2A -G_s complex following UK14304 stimulation of the α_2A adrenoceptor. The receptor coupling determinants involved in this interaction were later resolved through a series of chimeric receptor studies based upon the initial observation that a deletion of residues 218-235 in the α_2A adrenoceptor N-terminal region of the third intracellular loop abolishes Gs coupling (Eason *et al.*, 1994). Eason *et al.* (1994) generated a series of α_2A receptor chimeras in which residues 218-235 were substituted with the equivalent sequence of either the Gi coupled 5HT_{1A} receptor or the predominantly Gs coupled β_2 adrenoceptor. Upon functional analysis of these chimeras Eason discovered that while Gs coupling was lost in both of the substitutions, Gi coupling was generally unaffected. In order to precisely map which residues were involved in Gs coupling, Eason *et al.* (1994) created two more chimeras, this time containing 5HT_{1A} receptor sequence in either positions 218-228 or 229-235. When these chimeras were analysed for functional coupling, only the chimera containing the limited substitution at residues 229-235 showed functional coupling to both Gi and Gs. By a process of elimination, Eason *et al.* (1994) concluded that while residues 218-228 of the α_2A adrenoceptor are necessary for Gs coupling, they are not essential for Gi coupling.

In a continuation of this work, Eason and Liggett (1996) modified the α_2A chimeras to include substitutions of the whole of intracellular loop 2 (residues 133-149) and the C-terminal region of the intracellular loop 3 (residues 335-371) with the equivalent sequence from the Gi coupled 5HT_{1A} receptor and the Gs coupled β_2 adrenoceptor. Eason and Liggett then assayed each of the chimeras with regard to functional Gi and Gs coupling. From these experiments, they discovered that by replacing the 2nd intracellular loop of the α_2A adrenoceptor with the equivalent sequence of the Gi coupled 5HT_{1A} receptor they could abolish Gs coupling without affecting Gi coupling. Similarly, substitution of the same region with the equivalent sequence of the Gs coupled β_2 adrenoceptor depressed Gi coupling while leaving Gs coupling fully intact. From this Eason and Liggett concluded that the 2nd intracellular loop of the α_2A adrenoceptor contains determinants necessary for functional coupling to both Gi and Gs α -subunits. In another experiment, Eason and Liggett (1996) substituted either the carboxy or amino terminal segments of the 3rd intracellular loop of the α_2A adrenoceptor with the equivalent sequence of the 5HT_{1A} receptor. Analysis of these chimeras showed that while Gi coupling was unaffected, a substitution at either of these

regions ablated agonist stimulation of adenylyl cyclase activity. In contrast to this, a substitution of β_2 adrenoceptor sequence at either of these positions had no appreciable effect on Gi coupling. However, substitution of β_2 adrenoceptor sequence at both of these regions significantly impaired the capacity of the chimera to inhibit adenylyl cyclase activity. From these observations, Eason and Liggett(1996) concluded that both the N and C-terminal regions of the 3rd intracellular loop of the α_2A adrenoceptor are capable of independently supporting Gi coupling.

To summarise, Liggett and co-workers have identified three determinants involved in functional coupling of the α_2A adrenoceptor to the G-proteins Gi and Gs. Determinants necessary for Gs functional coupling appear to be distributed between the 2nd intracellular loop and the C-terminal (residues 355-371) and N-terminal (residues 218-228) regions of intracellular loop 3. In contrast to this, functional coupling of the α_2A receptor to Gi appears to be far more flexible. Functional coupling to Gi requires determinants in the 2nd intracellular loop in combination with determinants in either the N- or C-terminal regions of intracellular loop 3.

The observations of Eason and Liggett (1996) have in part been corroborated by photochemical cross linking experiments by Taylor *et al.* (1996). Taylor *et al.*(1996) have demonstrated that a 14 residue peptide corresponding to the C-terminal region of the 3rd intracellular loop of the α_2A adrenoceptor can be photochemically cross linked to a region corresponding to the N-terminal 17 residues of the Gi family member $G_{\alpha i}$. In combination with this, Taylor *et al.* (1994; 1996) have also shown photochemical cross linking of the same region of the α_2A adrenoceptor with the last 60 C-terminal residues of $G\beta$.

1.4.2.3 The α_2B and α_2C adrenoceptor subtypes

The α_2B and α_2C adrenoceptors share many of the features of the α_2A subtype. The genes for all three subtypes encode proteins of similar length (α_2A 450 residues, α_2B 450 residues, α_2C 461 residues) and molecular mass (49kDa). Analysis of the amino acid sequence has shown that all three receptors contain an unusually large 3rd intracellular loop (α_2A 157 residues, α_2B 179 residues, α_2C 151 residues) and a short C-terminal tail (α_2A 20 residues, α_2B 21 residues, α_2C 21 residues). The three receptors have also been shown to regulate a common set of effectors through the Gi/o family of

G-proteins. Responses known to be mediated by α_2B and α_2C subtypes include – inhibition of adenylyl cyclase activity, inhibition of voltage gated Ca^{2+} channels and the opening of K^+ channels. The three subtypes do however differ in post translational processing. The α_2A appears to be the only subtype which is covalently modified by both palmitoylation and glycosylation, the other receptors being either exclusively palmitoylated (α_2B) or glycosylated (α_2C). The α_2B and C subtypes also appear to differ in tissue distribution, with the α_2B being localised to peripheral tissues such as the liver and kidney and the α_2C being exclusively expressed in central nervous tissues such as the hippocampus and cerebral cortex. In contrast to this , the α_2A subtype has been detected in both peripheral tissues as well as the central nervous system.

The α_2B and α_2C adrenoceptor subtypes can be pharmacologically distinguished from the α_2A adrenoceptor through the use of the two antagonists prazosin and ARC 239 which exhibit a 10-100 fold greater affinity for these receptor subtypes than for the α_2A adrenoceptor. The extensive pharmacological overlap between the α_2B and α_2C subtypes means that at present, these receptors cannot be separated by pharmacological means. Alternately they can be identified by either sequence analysis or through their differential post translational processing.

1.5 Aims of Research

The C-terminus of the G-protein α -subunit has been shown to be an important determinant in defining the efficiency and specificity of G-protein/receptor coupling. Through alanine scan mutagenesis, Osawa and Weiss (1995) have identified several residues in the C-terminus of $G_{i\alpha}$, the mutation of which negatively affect coupling of the G-protein with its cognate receptor. One of the residues identified by Osawa and Weiss (1995) is a cysteine residue, located four amino acids from the extreme C-terminus of $G_{i\alpha}$. This cysteine residue is known to be conserved across the G_i family of G-proteins, with the exception of $G_{z\alpha}$ which contains an isoleucine residue at this position. Substitution of this residue for tyrosine (C347Y) was seen to result in a loss of G-protein/receptor coupling. Similarly, ADP-ribosylation of this residue by the bacterial toxin, pertussis toxin, has been shown to have an equally detrimental effect on receptor coupling. It could be postulated that since this residue is conserved across the range of G_i family members it plays a vital role in G-protein function. However, mutations of the equivalent residue (C351) in other G_i family members have shown this not to be the case. Substitution of this cysteine residue to glycine in $G_{i\alpha1}$ (Senogles, 1994) or serine in $G_{i\alpha3}$ (Hunt *et al.*, 1994) has been shown to result in a less marked decrease in G-protein/ receptor coupling, the extent of which has yet to be determined.

In parallel with the above observations, Osawa and Weiss (1995) demonstrated that the activity of the C347Y $G_{i\alpha}$ mutant could be restored by substituting the residues adjacent to the point mutation with those of $G_{q\alpha}$ (D346E, C347Y, G348N, F350V). From this study, it would appear that the C-terminus of the α -subunit is more tolerant to extensive substitutions than to selective point mutations. Evidence supporting this comes from Conklin *et al.* (1993) who have shown that while a substitution of the last 3 C-terminal amino acids of $G_{q\alpha}$ for those of $G_{i\alpha2}$ switches the specificity of receptor coupling towards G_i linked receptors, it does not affect the capacity of the G-protein to be activated or to interact with its cognate effector. The equivalent effect has also been demonstrated in more extensive C-terminal substitutions in $G_{13\alpha}$ and $G_{s\alpha}$ (Voynoyasentskya *et al.*, 1994a; Komatsuzaki *et al.*, 1997), but not as of yet in any of the G_i family members.

Based upon the above findings, the aim of this research project was to quantitatively determine how mutations and substitutions in the extreme C-terminus of

$G_{i\alpha 1}$ affect the efficiency and specificity of G-protein/receptor coupling. In the first results chapter, by generating a series of C351 $G_{i\alpha 1}$ mutants similar to those of Senogles (1994), I aim to quantify the effects of substituting cysteine residue 351 in $G_{i\alpha 1}$ on the efficiency of coupling to the α_2A adrenoceptor. In the second results chapter, using a series of Gi chimeras similar to those generated by Komatsuzaki *et al.* (1997), I aim to investigate the role the α -subunit C-terminus plays in defining the specificity of G-protein/receptor coupling. Finally, in the third results chapter, I aim to quantify how the C351 $G_{i\alpha 1}$ mutants affect the intrinsic activity of agonists at the α_2A adrenoceptor. This property will be investigated using fusion proteins between the α_2A adrenoceptor and the C351 $G_{i\alpha 1}$ mutants based on the design of Bertin *et al.* (1994).

Chapter 2

Materials and Methods

2.1 Materials

2.1.1 General Reagents

Alexis Corporation Ltd., Bingham, Nottingham, U. K.

DTT

Amersham International plc., Buckinghamshire, U. K.

Enhanced chemiluminescence reagent, ECL, detection film

BDH

Ammonium persulphate, glucose, glycine, Na_2HPO_4

Boehringer Mannheim U. K. Ltd., Lewes, East Sussex, U. K.

App(NH)p, aprotinin, creatine phosphokinase, GDP, $\text{GTP}\gamma\text{S}$ and restriction enzymes

Calbiochem-Novabiochem Ltd., Beeston, Nottingham, U. K.

Geneticin (G-418)

FMC BioProducts, Rockland, USA

Agarose

Genosys, Cambridge, U. K.

Oligonucleotides

Gibco BRL Life Technologies Inc., Paisley, U. K.

Lipofectamine™, TRIS, 1 kb DNA ladder, oligonucleotides, optimem 1

Invitrogen, San Diego, CA, U. S. A.

pcDNA3

Merck Ltd., Poole, Dorset, U. K.

Agar, NaOH

Oxoid Ltd., Hampshire, U. K.

Tryptone, yeast extract

Packard Instruments, BV, Netherlands

Ultima Gold XR liquid scintillation cocktail

Premier Beverages, Stafford, U. K.

Marvel

Promega Ltd., Southampton, U. K.

Restriction enzymes, Wizard™ Miniprep kit, Wizard™ DNA clean-up kit, X-Gal, IPTG

Qiagen Ltd., West Sussex, U. K.

Quiagen maxiprep kit

Sigma Chemical Company, Poole, Dorset, U. K.

Alumina, ampicillin, cholera toxin, DOWEX AG50 W-X4 (200-400 mesh), forskolin, imidazole, mineral oil, pertussis toxin, TEMED, thimerosal, TRICINE
Acetic acid, DMSO, EDTA, HEPES, hydrochloric acid, KCl, KH₂PO₄, K₂HPO₄, MgCl₂, NaCl, Na₂CO₃, NaHCO₃, NaH₂PO₄, sucrose, SDS, trichloroacetic acid

Stratagene Ltd., Cambridge, U. K.

Pfu DNA Polymerase, pCR-Script cloning system

Whatman International Ltd., Maidstone, U. K.

Brandell GF/C Glassfibre filters

2.1.2 Radiochemicals

Amersham International plc., Buckinghamshire, U. K.

[³H]RS-79948-197 (specific activity: 79 Ci/mmol)

Du Pont NEN Ltd., Stevenage, Hertfordshire, U. K.

[γ -³²P]GTP (specific activity: 30 Ci/mmol)

[³⁵S]GTP γ S (specific activity: 1250 Ci/mmol)

2.1.3 Plasmids

The porcine α_2A adrenoceptor which was obtained from Dr. L.E. Limbird (Vanderbilt University, TN). All other receptors, with the exception of the FLAG™ tagged IP prostanoid receptor which was produced in house by Mr. Chee-Wai Fong, were obtained from Glaxo Wellcome (Stevenage, Herts, UK).

2.2 Cell growth

Cells were cultivated in a continuous monolayer in 75cm² sterile tissue culture flasks (Costar, Cambridge, USA). The Growth medium used was Dulbecco's Eagle's minimal essential medium (DMEM) supplemented with 2mM L-glutamine and 10%(v/v) new born calf serum (NBCS). Buffering of media was achieved by growing the cells in a 95% air 5% CO₂ atmosphere. Cells were cultured in 10ml of the above medium in a Nuair model Nu-2700 incubator (Jencons, Minnesota, USA) at 37°C and allowed to reach confluency. Medium was changed every 3 days or when expended, as indicated by a change in the phenol red in the medium, whichever was the sooner.

2.3 Maintenance of cells

COS 7, HEK 293 and HEK293 Large T cells (Glaxo-Wellcome UK) were grown in DMEM containing 10%(v/v) new born calf serum.

2.4 Passaging of confluent cell cultures

Upon reaching confluency a flask of cells was subcultured into 4 to 10 flasks depending on the particular cell line's growth characteristics. Medium was removed and the cell monolayer was first washed and then incubated with 2ml of a sterile trypsinisation solution containing 0.1%(w/v) trypsin, 10mM glucose and 0.67mM EDTA, pH7.4. The trypsinisation process was terminated upon cell detachment from the flask by the addition of 5ml of the appropriate growth medium. The cell suspension

was transferred from the flask to a sterile 13ml polypropylene tube and centrifuged at 1000rpm for 5 minutes in a Super Minor centrifuge (MSE). The supernatant was discarded and the cell pellet was resuspended in 1ml of the appropriate growth medium by pipetting. The cell resuspension was further diluted with additional growth medium to give a final volume of 1ml per flask, 1ml aliquots of resuspended cells were dispensed into an appropriate number of sterile 75cm² tissue culture flasks each containing 9ml of appropriate growth medium. The flasks were then placed into an incubator and grown as above.

2.5 Storage and recovery of cells

Cell lines not in constant use were cryogenically preserved in liquid nitrogen. The cells were first trypsinised and centrifuged as detailed in **Section 2.4** before being resuspended in 1ml per flask of freezing medium consisting of 90% (v/v) NBCS and 10% (v/v) DMSO. The resuspended cells were dispensed in 1ml aliquots into sterile 1.8ml cryogenic storage vials (Nunc, Denmark). A two stage freezing process was used to prevent the formation of ice crystals in the cells. In the first stage the vials were placed in an insulated polystyrene box and stored at -80°C for 18 hours, the second stage involved transferring the vials to a liquid nitrogen vat for long term storage.

Cells were recovered from their cryogenic storage by removing the vials from the liquid nitrogen vats and thawing the contents at room temperature. The contents of the vial were then transferred to a sterile polypropylene tube and diluted with 9ml of DMEM growth medium containing 10%(v/v) NBCS before being centrifuged at 1000 rpm for 5 minutes. After centrifugation the supernatant was discarded and the cell pellet was re-suspended into 1ml of DMEM growth medium before being dispensed into a sterile 75cm² tissue culture flask containing 9ml of DMEM growth medium supplemented with 10%(v/v) NBCS. Cells were maintained as described in **Section 2.2**.

2.6 Bacterial toxin treatment of cells

2.6.1 Pertussis toxin treatment of cells

Cells were treated with pertussis toxin *in vivo* 18 hours prior to harvesting. Medium was removed by aspiration and replaced with fresh medium containing pertussis toxin at a final concentration of 50 ng/ml. The toxin was prepared as a 10x stock in DMEM and was added to the growth medium prior to use. Following toxin

treatment, cells were essentially maintained as described in **Section 2.2** prior to harvesting.

2.6.2 Dual toxin treatment with pertussis and cholera toxin

Cells were treated with a combination of pertussis and cholera toxin essentially as described in **Section 2.6.1**. Pertussis toxin was used at a final concentration of 50ng/ml while cholera toxin was used at a final concentration of 200ng/ml.

2.7 Harvesting of cells

Upon reaching confluency or after toxin treatment cells were harvested as follows. Medium was removed by aspiration and cells were washed with 2ml of ice cold phosphate buffered saline (PBS) (137mM NaCl, 4mM Na₂HPO₄, 0.27mM KCl, 0.15mM KH₂PO₄ (pH 7.4)). The cell monolayer was scraped into 2ml ice cold PBS using a disposable cell scraper (Costar,USA). The scraping procedure was repeated with a further 2ml of ice cold PBS and the cell suspensions were pooled into a 13ml polypropylene tube chilled on ice. The harvested cells were centrifuged at 2000rpm for 5 minutes at 4°C in a TJ-6 bench top cooled centrifuge (Beckman Instruments, California, USA). The supernatant was discarded and the pellet was re-suspended in 1ml ice-cold PBS and transferred to a 1.5ml microcentrifuge tube. The re-suspended cells were centrifuged at 21000g for 5 minutes at 4°C in an IEC Micromase RF centrifuge (International Equipment Company, MA, USA). The supernatant was discarded and the pellet was stored at -80°C until required.

2.8 Transient transfection of mammalian cell lines

COS 7, HEK 293 or HEK 293 large T cells were used for the transient expression of receptor, G-protein or chimeric receptor/G-protein cDNA's. Cells to be transfected were seeded at 60% confluency in a 10cm diameter sterile tissue culture dishes (Costar) 24 hours prior to transfection. On the day of transfection cDNA to be transfected was diluted to 0.1µg/ml in sterile nuclease free H₂O. An amount of cDNA between 4-10µg was added to a sterile 13ml polypropylene tube and diluted with optimem 1 (Gibco BRL) to a final volume of 600µl. A solution consisting of 20µl lipofectamine 580µl optimem 1 was added to the tube and mixed by flicking the tube. The cDNA mix was incubated at room temperature for 30 minutes to allow DNA liposome complexes to form. During this incubation period medium was aspirated off

the cells to be transfected and the monolayer was first washed with 5ml optimum 1 before finally being incubated with another 5ml of fresh optimum 1. At the end of the DNA/lipofectamine incubation period 4.8ml of optimum 1 was added to the polypropylene tube. The optimum 1 was aspirated off the cells and replaced by the DNA/lipofectamine mix. Cells were incubated for 5 hours to allow DNA to enter the cells after which 6ml of DMEM supplemented with 20%(v/v) NBCS was added to the cells. The medium was replaced with 10%(v/v) NBCS DMEM 24 hours after transfection and cells were incubated for a further 72 hours before being harvested as described in **Section 2.7**.

2.9 Preparation of plasma membrane fractions

Frozen cell pastes were thawed on ice and re-suspended in 1ml of ice cold TE (10mM Tris (pH7.4), 0.1M EDTA) before being transferred to a pre-chilled 13ml polypropylene tube. Cells were homogenised for 10 seconds using an Ultra Turrax homogeniser (IKA) fitted with a S8n-8g dispersing element. The resulting homogenate was transferred to a 1.5ml microcentrifuge tube and re-suspended 10 times with a 1ml syringe fitted with a 25 gauge needle.

The homogenate was centrifuged at 100g for 10 minutes at 4°C in an IEC micromase RF centrifuge. The pellet was transferred to a fresh microcentrifuge tube and centrifuged at 21000g for 30 minutes at 4°C. The supernatant was discarded and the pellet was re-suspended in 500µl of ice cold TE. Samples were then stored in 100µl aliquots at -80°C until required.

2.10 Determination of protein concentration

Protein concentration was determined by the method of Smith et al.(1985) in a 96 well plate format. BSA was used as a protein standard. The optical density of the samples at 492nm were read using a Spectra plate reader (SLT-Lab instruments, Salzburg, Austria).

2.11 SDS polyacrylamide gel electrophoresis

2.11.1 Lower resolving gel

SDS-PAGE was performed using vertical slab gels of dimensions 11cm x 14cm x 1.5mm containing 10%(w/v) polyacrylamide using the discontinuous buffer system of

Laemmli (1970). Resolving gels were prepared from a stock solution of 30%(w/v) acrylamide, 0.8%(w/v) N,N'-methylene bisacrylamide and contained 0.375M Tris-HCl (pH8.8) and 0.1% SDS. Polymerisation was initiated by the addition of 0.0375(v/v) TEMED and 0.05(w/v) freshly prepared ammonium persulphate. A solution of 0.1%(w/v) SDS was overlayed on the gels to aid polymerisation. Polymerisation was deemed to be complete when a clear interface could be seen between the polyacrylamide gel and the 0.1%(w/v) SDS solution. Upper stacking gels were prepared as in **Section 2.11.2** and electrophoresis was performed as described in **Section 2.11.3**.

2.11.2 Upper stacking gels

The stacking gel was prepared from a stock solution of 30%(w/v) acrylamide, 0.8%(w/v) N,N'-methylene bisacrylamide and contained 0.125M Tris-HCl (pH6.7) and 0.1%(w/v) SDS giving a final concentration of 4%(w/v) acrylamide. Polymerisation was performed as detailed in **Section 2.11.1**.

2.11.3 Electrophoresis running conditions

Electrophoresis was carried out in a Tris-glycine buffer containing 25mM Tris-HCl (pH8.9), 0.192M glycine and 0.1%(w/v) SDS. Electrophoresis was conducted towards the anode at 45mA per gel slab until the bromophenol blue dye front was 1cm from the end of the gel.

2.12 Western Blotting

2.12.1 Transfer of proteins to nitrocellulose membranes

SDS-PAGE separated proteins were electroblotted from the polyacrylamide resolving gel membranes according to the method described by Towbin *et al.* (1979). Transfer was performed towards the anode in a LKB 2005 Transphor electrophoresis unit (Wallac, Finland) at 2A for 1 hour. The transfer buffer consisted of 25mM Tris-HCl (pH8.9) and 0.192M glycine. The gel/nitrocellulose arrangement was constructed according to manufacturers instructions, care was taken not to introduce any air pockets between the polyacrylamide gel and the nitrocellulose membrane. Following electroblotting proteins were visualised using ponceau red stain by incubating the nitrocellulose filter in a 0.1%(w/v) ponceau S, 3%(w/v) trichloroacetic acid solution for 5 minutes. The nitrocellulose membrane was first de-stained with two 50ml washes of de-ionised water after which the membrane was trimmed to remove any unused areas.

The re-shaped membrane was then fully destained by washing with used electroblotting transfer buffer until no more ponceau stain could be seen. Any remaining transfer buffer was washed away with two subsequent washes of 50ml of dH₂O.

2.12.2 Incubation of nitrocellulose membranes with antiserum

Unless otherwise stated all antibody incubations were carried out with gentle agitation at room temperature. Non-specific binding sites on the membrane were blocked by incubation in 5%(w/v) Marvell in TBST (0.02M Tris-HCl (pH7.5), 0.15M NaCl, 0.1%(v/v) Tween-20) at 4°C for 18 hours with gentle agitation. The nitrocellulose membrane was then incubated sequentially for one hour with a 1:1000 dilution of primary antibody (as indicated in **Table 2.1**) followed by a 1:1000 dilution of donkey anti-rabbit IgG horse radish peroxidase conjugated secondary antibody. All antibodies were diluted in 'TBST' containing 5%(w/v) Marvell. Nitrocellulose membranes were first rinsed twice with at least 50ml TBST and then washed sequentially for 1x 15 minutes and 2x 5 minutes at room temperature with 50ml TBST between non-specific blocking and antibody incubations.

2.13 Imaging of immunoblots

Specific binding of antiserum to the nitrocellulose bound proteins was determined using the ECL Western Blotting analysis system (Amersham Life Science Ltd.) according to the manufacturers instructions. Equal volumes of detection solution 1 and 2 were mixed just prior to detection to give a final volume of 0.125ml/cm² membrane. All excess TBST wash was drained from the membrane by holding it vertically from one corner with a pair of forceps and then touching the opposite corner on a piece of absorbent tissue before being placed protein side up in a clean 20cm diameter petri dish. The nitrocellulose membrane was then incubated with the detection reagents for exactly one minute with gentle agitation. After incubation excess detection reagent was drained off as above and the nitrocellulose membrane was sandwiched between two transparent pieces of polythene and the nitro-cellulose membrane by gently smoothing across the top of the polythene with a piece of tissue, this also helped to remove excess detection reagent. Under darkroom safe light conditions a sheet of Hyperfilm ECL autoradiography film was placed on top of the nitro-cellulose membrane and exposed for 1 minute before being processed in an X-Omat machine

Table 2.1 Specificity of antisera

The antisera used in this study are listed in **Table 2.1** in combination with the corresponding peptide sequence to which the antisera were raised, the location of the peptide sequence in the α -subunit, the α -subunits which the antisera identify and the references regarding their production. All antisera, with the exception of SM1 which was a gift from Smithkline Beecham, were produced in house.

Table 2.1

| Antiserum | Peptide | Amino acid sequence | Identification | Reference |
|-----------|------------|--|--|-------------------------------|
| SG1 | KENLKDCGLF | G ₁₀₁ 341-350 | G ₁₀₁ , G ₁₀₂ G ₁₀₁ , G ₁₀₂ | McKenzie & Milligan (1990) |
| IIC | LDRIAQPNYI | G ₁₀₁ 160-169 | G ₁₀₁ | Green <i>et al.</i> (1990) |
| SM1 | ARYLDQINLL | G ₁₆₀ 363-373 last 10 aa | G ₁₅₀ /G ₁₆₀ | None Available |
| CS3 | RMHLRQYELL | G ₅₀ 385-394 | G ₅₀ | Milligan & Unson (1989) |
| CQ5 | QLNLKEYNIV | G ₁₀₀ 351-360 | G ₁₀₀ , G ₁₁₀ | Mitchell <i>et al.</i> (1991) |

(Kodak). The exposure time was varied by referring to the level of background exposure on the processed film in order to get a better signal to noise ratio.

2.14 Densitometric analysis of G-protein expression

Autoradiography images of ECL processed immunoblots were densitometrically scanned on a Bio-Rad GS-360 imaging densitometer and analysed on an Apple Macintosh Quadra 800 microcomputer pre-loaded with Bio-Rad GS-360 software.

2.15 Radioligand binding assays

2.15.1 Determination of α_2 A adrenergic receptor expression

α_2 A adrenoceptor expression was determined as according to Wise and Milligan (1997). The assay was routinely performed with approximately 1.4nM [3 H] RS-79948-197 at 30°C for 30 minutes in 10mM Tris-HCl (pH7.4), 50mM sucrose, 20mM MgCl₂ (binding buffer A) in the absence and presence of 100 μ M idazoxan to define maximal and non-specific binding respectively. Binding experiments were terminated by rapid filtration through Brandell FP-200 GF/C filters using a Brandell cell harvester. All FP-200 filters were pre soaked for at least 10 minutes in binding buffer A prior to use. The initial rapid filtration was followed by three washes of the filter with 3x 5ml volumes of ice cold binding buffer A. Filters were left to dry at room temperature for 2 hours before being soaked overnight in 5ml of Ultima Gold XR liquid scintillation cocktail prior to being counted on a scintillation counter (Beckman Instruments Inc., CA, USA).

2.15.2 Agonist stimulated binding of [35 S] GTP γ S

Agonist stimulated [35 S]GTP γ S binding assays were performed according to Wieland and Jakobs (1994). Membrane suspensions (5-10 μ g) were incubated with 50-100 nCi of [35 S] GTP γ S in a reaction mix consisting of 20mM HEPES (pH7.4) 5mM MgCl₂, 100mM NaCl and 5 μ M GDP in a total reaction volume of 100 μ l. To prevent decomposition of the nucleotides the GDP was added to the reaction mix just prior to performing the binding and was stored as a 100mM stock in Tricine (pH 7.4) at -20°C. The same was true for the labelled and unlabelled [35 S] GTP γ S which was stored in tricine (pH 7.6), 10mM DTT at -80°C. Unless otherwise stated the reaction was incubated at 30°C for 30 minutes in the presence and absence of agonist. Non-specific binding was assayed by replacing agonist with 10 μ M unlabelled GTP γ S.

Reactions were terminated by vacuum filtration through Brandell FP-200 GF/C filters pre-soaked in filter wash buffer (20mM HEPES (pH 7.4), 5mM MgCl₂). Unbound ligand was removed from the filters by three subsequent 5ml washes with ice cold filter wash buffer. The filters were then prepared as in **Section 2.15.1** before being counted. Assays were performed in triplicate.

2.16 High affinity GTPase assay

The high affinity GTPase assay was performed essentially as described by Koski and Klee (1981). Aliquots of reaction mix (100 μ l) (2mM App(NH)p, 2mM ATP, 2mM ouabian, 20mM creatine phosphate, 0.1U/ml creatine phosphokinase, 200 mM NaCl, 10 mM MgCl₂, 4mM DTT, 0.2mM EDTA, 80mM Tris-HCl (pH 7.5), 0.5 μ M [γ -³²P]GTP) were added to tubes on ice containing 5-10 μ g of membrane protein and either agonist, water or GTP (1mM). The reaction was initiated by removing the tubes from the ice slurry, vortexing them and then transferring them to a 37°C water bath. The assay tubes were incubated at 37°C for 20 min, after which the reaction was terminated by the addition of 900 μ l of ice cold charcoal mix (5% activated charcoal in 10mM H₃PO₄). After centrifugation at 15000g for 5 min, radioactivity was measured in a 500 ml sample by Cherenkov counting on a 1219 Rackbeta liquid scintillation counter (Wallac, Finland). The rate of high affinity GTP hydrolysis (pmol/mg/min) was calculated by subtracting the reading from the GTP control tube and by taking into account the specific activity of the labelled [γ -³²P]GTP, the concentration of unlabelled GTP, the membrane concentration and the incubation time.

2.17 Trypsinisation protection assay

The trypsinisation protection assay was performed essentially as described by Denker *et al.* (1995). Samples (100 μ l) were incubated in parallel in the presence or absence of 100 μ M GTP γ S for 15 minutes at 37°C in buffer A (20mM Tris-HCl (pH8.0), 25mM MgCl₂, 2mM DTT and 0.1mM EDTA). At the end of the incubation period, the samples were once again incubated in parallel in the presence or absence of TPCK inactivated trypsin for a further 15 minutes at 37, after which the trypsinisation process was terminated by the addition of soya bean trypsin inhibitor to a final concentration of 0.5mg/ml. Following the termination of the reaction, the samples were precipitated by the addition of ice cold buffer B (6% TCA, 0.5% deoxycholate), incubated on ice for 15 minutes and then centrifuged for a further 10 minutes at 15000g. Following the removal

of the supernatant was then removed, the samples were re-suspended in equivalent volumes of 2M Tris-HCl and Laemmli sample buffer, before being boiled at 100°C for 5 minutes. The samples were finally resolved by SDS-PAGE gel electrophoresis (Section 2.11) and transferred to nitrocellulose (Section 2.12.1) before being probed with appropriate antiserum as detailed in Table 2.1.

2.18 Growth of *Escherichia coli* strains

Escherichia coli (*E. coli*) strains were grown at 37°C either as liquid cultures or on agar plates. *E. coli* strains grown on agar plates were incubated in a temperature controlled room while liquid cultures were propagated in a Stuart Scientific Si50 orbital incubator with continuous shaking. The growth medium used was Luria-Bertani medium (LB-medium) consisting of 1%(w/v) bacto tryptone, 0.5%(w/v) bacto yeast extract and 1%(w/v) NaCl. The LB medium was supplemented with 1.5%(w/v) agar for use in 90cm² petri dishes.

E. coli clones which had been transformed with plasmids containing the ampicillin antibiotic resistance gene were selected on LB agar plates containing 50µg/ml ampicillin. Liquid cultures of transformed clones were also grown in medium supplemented with antibiotic. *E. coli* clones transformed with plasmids containing both the ampicillin resistance gene and the β-galactosidase reporter gene were grown on LB-agar plates supplemented with 50µg/ml ampicillin, 20µl of 200mM IPTG and 20µl of 10%(w/v) X-Gal. Liquid cultures of such clones were grown as above.

All media and solutions, unless otherwise stated, were sterilised by autoclaving for 15 minutes at 121°C. Ampicillin (50mg/ml) and IPTG (200mM) were prepared as stock solutions and sterilised by filtration through 0.22µm syringe filters.

2.19 Cryogenic storage of transformed *E. coli*

E. coli bacterial strains were cryogenically preserved as follows. The *E. coli* liquid culture was diluted with 80%(v/v) sterile glycerol to give a final composition of 15%(v/v) glycerol 85% bacterial culture. Aliquots of 1ml were snap frozen on dry ice and stored at -80°C.

2.20 Preparation of chemically competent *E.coli*

Untransformed *E.coli* cells were streaked onto LB-agar plates in the absence of antibiotic and incubated overnight at 37°C. From this plate a single colony was picked and used to inoculate a 5ml LB medium culture, the culture was incubated overnight at 37°C with continuous shaking. The 5ml culture was used to inoculate 500ml of LB medium which was cultured until it reached a growth density of 1×10^8 cells/ml (equivalent to OD 600 of approximately 0.25 absorbance units). The culture was then cooled on ice for 30 minutes and the bacterial cells were harvested by centrifugation at 10,000 rpm for 10 minutes at 4°C in a Beckman J-2-21 centrifuge equipped with a Ja-14 rotor. The supernatant was discarded and the *E.coli* pellet was re-suspended into 40ml sterile ice cold buffer 1 (100mM RbCl₂, 10mM CaCl₂, 50mM MnCl₂, 15%(v/v) glycerol and 30mM KAc (pH 5.8)). The *E.coli* suspension was left to chill on ice for 5 minutes before being re-centrifuged as before. The supernatant was again discarded and the *E.coli* pellet was re-suspended in 2ml sterile ice cold buffer 2 (10mM RbCl₂, 75mM CaCl₂, 15%(v/v) glycerol, 10mM MOPS (pH 6.5)). The resuspended bacteria were chilled on ice for a further 15 minutes before being snap frozen and stored at -80°C in 250µl aliquots.

2.21 Transformation of chemically competent cells

Care was taken to thaw the competent cells on ice before use so as to minimise cell death. An aliquot of 100µl of competent cells was incubated with either 50ng of supercoiled plasmid DNA or 10µl of ligation reaction. The DNA/competent cell mix was left to incubate on ice for 15 minutes before being heat shocked at 42°C for 90 seconds. After the heat shock step the cells were returned to ice for 2 minutes before being incubated with 800µl LB at 37°C for 1 hour with continuous shaking.

Aliquots of 100-200µl of cells were plated onto LB agar plates supplemented with 50µg/ml ampicillin and other selection reagents as dictated by the plasmid being transformed. Plates were incubated overnight at 37°C and transformed clones were selected and grown in 5ml cultures containing 50µg/ml ampicillin.

2.22 Preparation of plasmid DNA

2.22.1 Small scale DNA preparation

Small scale DNA preparation was performed using the Wizard Plus SV miniprep DNA purification system (Promega) as according to manufacturer's instructions. A total of 3ml of overnight bacterial culture was pelleted by centrifugation at 21000g in an IEC micromase RF centrifuge. The supernatant was discarded and the pellet was re-suspended in 250µl cell resuspension solution (10mM EDTA, 100µg/ml RNase A, 50mM Tris-HCl (pH7.5)) by vortexing. The cells were then lysed by the addition of 250µl of cell lysis solution (0.2M NaOH and 1% SDS), the contents of the tube were mixed by inverting. Lysis was deemed to be complete when the lysate became translucent. Further lysis was terminated by addition of 350µl of neutralisation solution (4.09M guanidine hydrochloride, 0.75% potassium acetate (pH4.2), 2.12M glacial acetic acid, the contents were once again mixed by inverting the tube 4 times. The bacterial lysate was cleared by centrifugation as described earlier. The clear supernatant was centrifuged at 14000g for 1 minute and the flow through from the column was discarded. The SV miniprep spin column was washed twice with first 750µl and then 250µl of column wash solution (60mM potassium acetate, 10mM Tris-HCl (pH 7.5) and 60% ethanol), each time the spin column was centrifuged and the flow through was discarded. The spin column was then allowed to air dry for 5 minutes to allow any residual ethanol to evaporate. The plasmid DNA was eluted from the spin column and then centrifuging as above.

2.22.2 Large scale preparation of plasmid DNA

Large scale DNA preparation for mammalian cell transformation was performed using the Qiagen plasmid maxi kit (Qiagen) as according to manufacturers instructions. A total of 250ml of overnight bacterial culture was pelleted by centrifugation at 10000rpm in a Beckman J2-21 centrifuge equipped with a JA-14 rotor. The supernatant was discarded and the pellet was re-suspended in 10ml of buffer P1 (10mM EDTA, 50mM Tris-HCl (pH8) by pipetting and vortexing. The bacterial resuspension was lysed by the addition of 10ml of buffer P2 (200mM NaOH, 1% SDS) mixing was by gentle inverting. Cell lysis was terminated after 5 minutes by the addition of 10ml of buffer P3 (3M potassium acetate pH 5.5). Chromosomal DNA was allowed to precipitate by incubating the lysate on ice for 20 minutes. The lysate was

cleared by centrifuging at 2000rpm for 1 hour in a Beckman TJ-6 centrifuge. Any remaining particulate matter which had not been sedimented was removed by filtering the supernatant through mira cloth. The supernatant was applied to a Quiagen-tip pre-wetted with 10ml of buffer QBT (750mM NaCl, 0.15% Triton X-100, 15% isopropanol, 50mM MOPS (pH7.0)). The Quiagen tip was then washed with 2x 30ml of buffer QC (1M NaCl, 50mM MOPS (pH7.0), 15% isopropanol). The plasmid DNA was subsequently eluted into a 50ml polycarbonate tube by the addition of 15ml of buffer QF (1.25M NaCl, 50mM Tris-HCl (pH 8.5), 15% isopropanol). The plasmid DNA was precipitated by centrifugation at 16000rpm as above. The supernatant was discarded and the pellet washed with 5ml ice cold 70% ethanol before being dried in a 50°C oven. The pellet was re-suspended in 500µl of nuclease-free water.

2.23 Restriction digestion of DNA

DNA was digested with one or more of the restriction endonucleases listed in **Table 2.2** at a final concentration of 0.1µg/ml in a total reaction volume of not less than 10µl. The DNA and restriction endonucleases were incubated at 30°C for 2 hours in a 1x concentration of the appropriate buffer as referenced from **Tables 2.2** and **2.3**.

2.24 Ethanol precipitation of DNA

DNA was precipitated from solution with 0.25 vol. of 3M sodium acetate (pH 7.0) and 2.5 vol. of absolute ethanol at -20°C for 30 min. The precipitated DNA was pelleted by centrifugation at 21000g for 10 minutes. Sodium acetate was removed by washing the pellet twice with ice cold 70% (v/v) ethanol. The DNA pellet was then air dried at 60°C in an oven before being re-suspended in sterile de-ionised H₂O.

2.25 Quantitative and qualitative analysis of DNA

The concentration and quality of a DNA sample produced by large or small scale plasmid purification was determined by measuring the absorbance at both 260 and 280nm (A₂₆₀, A₂₈₀). The DNA sample was diluted to 1 in 200 in sterile Milli Q H₂O to a final volume of 1ml. The A₂₆₀ reading for the sample was determined on a Shimadzu UV-2101 spectrophotometer. The concentration was calculated assuming that 1 absorbance unit was equivalent to 50µg/ml of double stranded DNA. The purity of the DNA was assessed by measuring the A₂₈₀ in parallel and calculating the ratio of A₂₆₀:A₂₈₀. A ratio of 1.8 was considered sufficiently pure for use. The concentration

Table 2.2 Restriction endonuclease buffer requirements

The restriction endonucleases used in this study are listed in combination with the buffer required from maximal endonuclease efficiency. The composition of the buffers listed in this table are detailed in **Table 2.3**.

Table 2.2

| Enzyme | Buffer |
|----------------------------------|--------|
| <i>Nru I</i> | B |
| <i>Eco RI</i> | H |
| <i>Bam HI / EcoRI</i> | B |
| <i>SmaI / Bgl II</i> | A |
| <i>NcoI / Not I</i> | H |
| <i>Xho I</i> | H |
| <i>Pvu I / Eco RI / Hind III</i> | B |
| <i>Eco RI / Hind III</i> | B |
| <i>Pvu I</i> | II |
| <i>Not I / Hind III</i> | H |
| <i>Not I / Xho I</i> | H |

Table 2.3 Composition of restriction endonuclease buffers

Table 2.3 details the composition of buffers used in restriction digestion of DNA with the restriction endonucleases listed in **Table 2.2**. All values listed represent the final concentration in mM.

Table 2.3

| Reagent | Buffer | | | | | |
|------------------------|--------|-----|-----|-----|-----|-----|
| | A | B | L | M | H | 4 |
| Tris acetate | 33 | - | - | - | - | 20 |
| Tris-HCl | - | 10 | 10 | 10 | 50 | - |
| Magnesium acetate | 10 | - | - | - | - | 10 |
| MgCl ₂ | - | 5 | 10 | 10 | 10 | - |
| Potassium Acetate | 66 | - | - | - | - | 50 |
| NaCl | - | 100 | - | 50 | 100 | - |
| Dithioerythritol (DTE) | - | - | 1 | 1 | 1 | - |
| Dithiothreitol (DTT) | 0.5 | - | - | - | - | 1 |
| 2-mercaptoethanol | - | 1 | - | - | - | - |
| pH at 37°C | 7.9 | 8.0 | 7.5 | 7.5 | 7.5 | - |
| pH at 25°C | - | - | - | - | - | 7.9 |

of the DNA sample was confirmed by imaging on an agarose gel against standards of known concentration.

2.26 Separation of plasmid DNA by electrophoresis

Restriction enzyme digested DNA was routinely analysed and purified by agarose gel electrophoresis. Samples were prepared by the addition of 6 x loading buffer (30% (v/v) glycerol, 0.25% (w/v) bromophenol blue) to give a final concentration of 1 x loading buffer. DNA fragments between the size of 0.5kb and 7kb were separated by electrophoresis through a 1% (w/v) agarose gel containing 1 x TAE buffer (40mM Tris-acetate, 1mM EDTA (pH 8.0)). Smaller DNA fragments of sizes of less than 0.5kb were separated on 1.5-2% (w/v) agarose gels.

Electrophoresis was conducted towards the anode at 70-100mA at room temperature in a horizontal electrophoresis tank containing 1 x TAE buffer. Upon completion of electrophoresis separation DNA fragments were visualised by incubating the agarose gel in a 2.5mg/ml ethidium bromide solution for 10 minutes and then illuminating with UV light. Fragment sizes were assessed by comparison with DNA molecular weight markers of known length.

2.27 Purification of DNA from Agarose gels

Upon electrophoresis and visualisation of DNA samples (as in Section 2.26) DNA fragments of interest were recovered using a modified protocol of the Wizard DNA clean-up system (Promega). The fragment to be purified was excised from the agarose gel and melted in 300µl of 6M NaI at 65°C. The DNA solution was then cooled on ice for 2 minutes before being incubated with 1ml of Wizard DNA clean-up purification resin. The resin was incubated with the DNA solution for 2 minutes, mixing was achieved by constant gentle inversion. At the end of the incubation period the DNA resin mix was applied to a Wizard minicolumn and drawn through under vacuum. The wizard minicolumn was then washed with 2ml of 80% (v/v) isopropanol which was also drawn through under vacuum. The minicolumn was dried of any remaining solvent by centrifugation at 21000g for 2 minutes followed by vacuum drying for a further 5 minutes.

The DNA was eluted from the minicolumn by the application of 25µl of nuclease free water pre-heated to 80°C followed by centrifugation at 21000g for 1 minute. The eluted DNA was quantitated as described in **Section 2.25**.

2.28 Phosphatase treatment of DNA

Restriction enzyme digested vector was treated with calf intestinal alkaline phosphatase (Cip) to prevent re-circularisation during ligation by removing the 5' phosphate. The vector was heat-treated to 70°C for 15 minutes on a hot block (Techne) to denature the ends of the DNA. The DNA was cooled on ice for 2 minutes to prevent re-ligation of the ends of the denatured vector. Phosphatase treatment was performed at 37°C for 5 minutes using 1 unit of Cip in a 1x concentration of the supplied buffer (0.5M Tris (pH 9.3), 10mM MgCl₂, 1mM ZnCl₂ and 10mM spermidine). The de-phosphorylation was repeated one more time before being terminated by heating the sample to 72°C for 10 minutes. The dephosphorylated vector was purified by agarose gel electrophoresis (**Section 2.26**) and gel extraction (**Section 2.27**).

2.29 Ligation of DNA fragments

Ligation of vector and insert DNA was routinely carried out overnight at 4°C in a reaction volume of 10µl containing a 1x concentration of ligation buffer (300mM Tris-HCl (pH7.8), 100mM MgCl₂, 100mM DTT and 10mM ATP), 3 Weiss units of T4 DNA ligase plus vector and insert DNA fragments. Reactions were performed using vector to insert ratios of 1:10 for fragments with blunt ends or 1:3 for fragments with overhanging ends. The vector concentration was kept constant at 100ng / reaction and the insert concentrations were varied for all reactions conducted in this study. Ligated DNA was then transformed as described in **Section 2.21**.

2.30 PCR amplification

DNA amplification by polymerase chain reaction was routinely performed in a reaction volume of 50µl containing 50ng of DNA template, dNTPs (0.2mM each dATP, dCTP, dGTP, dTTP (Pharmacia)), 100pmol each of sense and antisense oligonucleotide primers (as indicated in **Table 2.4** and 1 unit of *Pfu* DNA polymerase (Stratagene) in a 1x concentration of *Pfu* reaction buffer (200mM Tris-HCl (pH8.8), 20mM MgSO₄, 100mM KCl, 100mM (NH₄)₂SO₄, 1%(v/v) Triton X-100 and

1mg/ml nuclease free BSA). The temperature cycling conditions were as in **Table 2.5**. All reactions were performed using a Perkin-Elmer 9600 thermal cycler.

2.31 DNA sequencing

Sequence data was generated at the dedicated sequencing facility at Glaxo-Wellcome Research and Development (Stevenage, UK) using an ABI 377 Prism sequencing machine. The sample to be sequenced was presented as 500ng template and 3.2pmol of oligonucleotide primer as indicated in **Table 2.6** in a total volume of 12 μ l of nuclease-free water.

Table 2.4 Oligonucleotide primers for mutagenesis

Oligonucleotide primers used for mutagenesis of $G_{i\alpha 1}$ cDNA are listed in combination with the specificity of the mutation generated and the nucleotide sequence of the primer.

Table 2.4

| Primer | Mutation generated | Primer sequence (5' to 3') |
|------------------|--|--|
| Common 5' primer | none | AGCT GAA TTC GCC ACC ATG GGC TGC ACA CTG AGC GC |
| 3' primer 1 | Cys ³⁵¹ to Ile, Met, Thr, Asn, Lys, Ser, Arg | ACGT GAA TTC TTA GAA GAG ACC (G/C)NT GTC TTT TAG G |
| 3' primer 2 | Cys ³⁵¹ to Pro, Arg, Leu | ACGT GAA TTC TTA GAA GAG ACC (C/G)(A/G/C)G GTC TTT TAG G |
| 3' primer 3 | Cys ³⁵¹ to Val, Ala, Asp, Glu | ACGT GAA TTC TTA GAA GAG ACC (C/G)(T/G/A)C GTC TTT TAG G |
| 3' primer 4 | Cys ³⁵¹ to Phe, Tyr, Trp, Leu | ACGT GAA TTC TTA GAA GAG ACC (C/G)(C/T/A)A GTC TTT TAG G |
| 3' primer 5 | Cys ³⁵¹ to His | ACGT GAA TTC TTA GAA GAG ACC GTG GTC TTT TAG G |
| 3' primer 6 | Cys ³⁵¹ to Gln | ACGT GAA TTC TTA GAA GAG ACC CTG GTC TTT TAG G |
| 3' primer 7 | Cys ³⁵¹ to Lys | ACGT GAA TTC TTA GAA GAG ACC CTT GTC TTT TAG G |
| 3' primer 8 | Cys ³⁵¹ to Thr | ACGT GAA TTC TTA GAA GAG ACC TGT GTC TTT TAG G |
| 3' primer 9 | Cys ³⁵¹ to Ala | ACGT GAA TTC TTA GAA GAG ACC CGC GTC TTT TAG G |
| 3' primer 10 | Last 6 amino acids of G _{11α} to G _{3α} | CCA CGT GAA TTC TTA TTA GAG TTC ATA TTG CCT TAG GTT ATT CTT TAT |
| 3' primer 11 | Last 6 amino acids of G _{11α} to G _{9α} | CCA CGT GAA TTC TTA GAC GAG ATT ATA TTC TTT TAG GTT ATT CTT TAT |
| 3' primer 12 | Last 6 amino acids of G _{11α} to G _{16α} | CCA CGT GAA TTC TTA GAG GAG ATT AAT TTC ATC TAG GTT ATT CTT TAT |

Table 2.5 Conditions for PCR mutagenesis

Table 2.5 details the temperature cycles used for PCR mutagenesis of $G_{\alpha 1}$ cDNA in combination with the number of amplifications at each cycle.

Table 2.5

| Denaturing (temp./time) | Annealing (temp./time) | Extension (temp./time) | Number of cycles |
|----------------------------|---------------------------|---------------------------|---------------------|
| 95oC/2min | 55oC/45sec | 72oC/3min | 1 |
| 95oC/45sec | 55oC/45sec | 72oC/3min | 30 |
| 95oC/45sec | 55oC/45sec | 72oC/5min | 1 |

Table 2.6 Oligonucleotide primers for sequencing

Oligonucleotide primers used for sequencing in this study are listed in combination with the nucleotide sequence of the primer.

Table 2.6

| Primer | Primer sequence (5' to 3') |
|--------|---------------------------------|
| SP6 | CAT ACG ATT TAG GTG ACA CTA TAG |
| T7 | TAA TAC GAC TCA CTA TAG GGA GA |
| T3 | ATT AAC CCT CAC TAA AGG GA |

Chapter 3

**The coupling efficiency of C351x G_{iα1}
mutants at the α₂A adrenoceptor**

3.1 Introduction

G-proteins are membrane associated heterotrimeric GTP binding proteins which mediate the transduction of a signal from a cell surface receptor to an effector molecule. They are composed of three subunits designated α , β and γ . The α -subunit contains a nucleotide binding pocket into which either GDP or GTP can bind. G-proteins can exist in either an active or inactive conformation, in the inactive conformation the α -subunit contains GDP in the nucleotide binding pocket and is in tight association with the $\beta\gamma$ subunits. Activation occurs through receptor catalysed guanine nucleotide exchange in the α -subunit. The GDP molecule dissociates from the α -subunit and is replaced by GTP; this results in a conformational change in the β subunit binding site and so $\beta\gamma$ dissociation. The GTP bound G α -subunit and the $\beta\gamma$ subunits are then free to interact with and activate downstream effectors. G-protein deactivation occurs when the α -subunit hydrolyses GTP to GDP, resulting in a conformational change back to the inactive state and the re-association of $\beta\gamma$. The rate of hydrolysis varies between the four main classes of α -subunit. Osawa and Weiss (1995) demonstrated that PTX sensitive α -subunits such as $G_{t\alpha}$, $G_{o\alpha}$ and $G_{i\alpha}$ possess a higher intrinsic GTP hydrolysis activity than PTX resistant forms such as $G_{q\alpha}$ and $G_{s\alpha}$. This high catalytic activity is the basis behind assays measuring either the hydrolysis of GTP to GDP (GTPase assay) or receptor catalysed exchange of GDP for the non-hydrolysable GTP analogue GTP γ S ([35 S]GTP γ S binding assay).

The specificity and efficiency of receptor /G-protein coupling is determined by the C-terminal tail of the G_{α} subunit. Alanine scan mutations in the last 7 amino acids in the C-terminal tail of $G_{t\alpha}$ have shown that specific mutations can lead to a loss of receptor coupling or guanine nucleotide exchange and in some extreme cases both functions (Osawa and Weiss, 1995). From this study the leucine residues L344 and L349, which are conserved among all G_{α} subunits, were found to be intolerant to mutation in $G_{t\alpha}$. Substituting alanine at either of these positions resulted in a loss of receptor coupling and nucleotide exchange function (Osawa and Weiss, 1995; Garcia *et al.*, 1995). It could be postulated that because these residues are conserved throughout all G_{α} subunits that they play a vital role in G-protein function. However, mutations of the equivalent residues in $G_{s\alpha}$ (L388A) and $G_{i\alpha}$ (L349A) have shown this not to be the case, these mutants display little or no decrease in guanine nucleotide exchange or

receptor coupling. A similar case exists for the $G_{i\alpha}$ C347 residue, which is conserved in the α_i , α_o and α_t subunits. The C347 residue is one of many potential sites for post-translational modification by bacterial toxins. Pertussis toxin catalysed ADP-ribosylation at this cysteine residue results in a loss of receptor coupling, the molecular mechanism behind this is thought to be due to steric hindrance by the ADP-ribose group. To study the importance of the C347 residue in $G_{i\alpha}$, the residue was mutated to both alanine and the equivalent residue of $G_{q\alpha}$, tyrosine (C347Y). The two mutants displayed profoundly different activities, the C347Y mutation abolished all activity while the C347A mutation only resulted in a slight decrease in coupling efficiency (Osawa and Weiss, 1995). The mutation of the equivalent residue in $G_{i\alpha1}$ to glycine (C351G) (Senogles, 1994) or serine in $G_{i\alpha3}$ (C351S) (Hunt *et al.*, 1994) has been shown to confer resistance to pertussis toxin treatment whilst retaining receptor coupling and nucleotide binding activity. From these findings it seems as if the mutation of the $G_{i\alpha1}$ C351 may provide a general strategy for the generation of PTX resistant mutants. The effects of the $G_{i\alpha}$ C347Y mutation can be reversed by mutating the adjacent amino acids to those of $G_{q\alpha}$ (D346E, C347Y, G348N, F350V) (Osawa and Weiss, 1995). In a similar observation Conklin *et al.* (1993) has shown that replacement of the last 3 C-terminal amino acids in $G_{q\alpha}$ to those of $G_{i\alpha}$ can change the α -subunit's receptor specificity to that of a G_i subunit. Not all residues in the C-terminal tail of $G_{i\alpha}$ are equally susceptible to mutation. The F350 residue in the $G_{i\alpha}$ was shown to be very tolerant to substitution, only the removal or insertion of a single amino acid at this position could abolish nucleotide binding activity (Osawa and Weiss, 1995). In comparison Denker *et al.* (1992) had previously demonstrated that the deletion of 5, 10 and even 14 amino acids from the C-terminus of $G_{o\alpha}$ resulted in no loss of GTP γ S binding, although the 14 amino acid deletion did result in a loss of GDP affinity. This would suggest that the length of the C-terminal tail may play a more important role in $G_{i\alpha}$ than in other α -subunits. These findings would indicate that the specificity of receptor/G-protein interaction is determined by the combined structural and physiochemical properties of the residues in the C-terminal tail as opposed to specific interactions with one or two key residues. It can also be inferred that α -subunits respond differently to mutations in the C-terminal tail, therefore it would be unwise to predict the effects of mutating equivalent residues in different α -subunits.

The most convenient way of analysing G-protein mutants, such as those described above, is by transient transfection. Transient transfection allows the rapid analysis of overexpressed signal transduction components, as well as their mutant variants, in the natural environment of the cell membrane. Unfortunately the presence of endogenous receptor and G-proteins frequently interferes with the output of several biochemical assays used to characterise G-protein function. Since a major source of interference comes from endogenously expressed PTX sensitive G-proteins, it can easily be removed by PTX treatment. Receptor/G-protein interactions can then be assayed through transiently expressed PTX resistant G-protein mutants.

It has already been demonstrated that mutating the cysteine residue 351 in $G_{i\alpha1}$, $G_{i\alpha2}$ or $G_{i\alpha3}$ to glycine or in $G_{i\alpha3}$ to serine (Senogles, 1994; Hunt *et al.*, 1994) generates a pertussis toxin insensitive G-protein mutant capable of coupling to $G_{i\alpha}$ associated G-protein receptors. However the $G_{i\alpha1}$ C³⁵¹G mutant has been shown to couple an order of magnitude less effectively to the $\alpha2A$ adrenoceptor than the wild type thereby producing a lower maximal output (Wise *et al.*, 1997d). In order to use such a strategy in ligand screening it would be advantageous to produce mutants whose coupling efficiency was equal to or greater than that of the wild type. In order to find such mutants it was decided to systematically substitute the cysteine residue at position 351 in $G_{i\alpha1}$ to all other amino acids.

3.2 Results

3.2.1 Generation of C³⁵¹Xaa G_{ict1} mutants by PCR mutagenesis

The cysteine residue 351 in rat G_{ict1} was mutated to all other amino acids by PCR amplification (Section 2.30) of *NruI* linearised (Section 2.23) wild type rat G_{ict1} cDNA in the pBluescript KS(-) (Stratagene) vector. The PCR reaction used 100pmol each of a common 5' forward primer and one of four degenerate 3' reverse primers (Table 2.4). The reaction conditions were as in Table 2.5.

The amplicons were resolved on a 1%(w/v) agarose gel and stained with ethidium bromide as in Section 2.26. DNA corresponding to the 1065bp band (Fig. 3.1) was excised from the gel and purified (Section 2.27) before being ligated into the pCR-Script SK(+) (Stratagene) vector as described in Section 2.29. The ligated DNA products were transformed into the bacterial strain XL1-blue (Stratagene) and grown on agar plates supplemented with ampicillin, X-gal and IPTG (Section 2.18).

Blue/white colour selection was used to select white clones containing recombinant vector, blue clones were deemed to contain empty vector. Twenty-five clones were selected and cultured from each of the transformations (Section 2.18) giving one hundred samples in total. The plasmid DNA was extracted from the cultures by a small scale DNA preparation (Section 2.22.1) and visualised on a 1%(w/v) agarose gel (Fig. 3.2). Two discrete rates of supercoiled plasmid migration were observed, only clones whose DNA migrated the least were analysed for the presence and orientation of the insert. Seventy-six clones out of the 100 arbitrarily chosen were found to be positive for insert.

A restriction digest with the enzymes *NotI* and *NcoI* (Section 2.23) was used to screen the orientation of the insert in the pCR-Script vector. The digestion products were resolved on a 1%(w/v) agarose gel (Fig. 3.3). The presence of two distinct bands indicated that the start codon of the G_{ict1} cDNA was next to the T3 promoter priming site, such clones were sequenced with the opposing T7 primer. The presence of three bands indicated that the start codon was next to the T7 priming site and therefore the T3 primer was to be used for sequencing. Using this orientation data, the 3' end of the G_{ict1} mutant cDNA was sequenced (Section 2.31) by either the T3 or T7 primer (Promega).

The sequencing profiles were translated to determine the amino acid encoded at position 351 in the G-protein mutant. Using this approach 13 out of the 18 required amino acid substitutions were generated. Dedicated 3' reverse primers (Table 2.4) were used to generate the remaining amino acids – glutamine, histidine, leucine, threonine and alanine. One clone was arbitrarily chosen from each mutation to be subcloned into the mammalian expression vector pCDNA3.

The $G_{i\alpha 1}$ mutants were subcloned into the mammalian expression vector pCDNA3 (Invitrogen) according to the following strategy. If the $G_{i\alpha 1}$ mutant cDNA was subcloned into the pCR-Script vector with the start codon next to the T3 promoter priming site the cDNA was excised using the restriction enzymes *NotI* and *XhoI* and subcloned into the pCDNA3 vector digested with the same two enzymes. Clones with the $G_{i\alpha 1}$ cDNA in the opposite orientation were similarly subcloned into pCDNA3 using the restriction enzymes *NotI* and *EcoRI*.

3.2.2 Transient expression of $G_{i\alpha 1}$ point mutants with the porcine α_2A adrenoceptor

The HEK293T cell line was transiently co-transfected (Section 2.8) with the porcine α_2A adrenoceptor cDNA in pCDNA3 in combination with $C^{351}Xaa$ $G_{i\alpha 1}$ mutants in pCDNA3. The transfected cells, with the exception of the wild type $G_{i\alpha 1}$ co-transfection, were treated with 50ng/ml of pertussis toxin 12 hours prior to harvesting (Section 2.7) in order to inactivate the endogenously expressed (PTX sensitive) G-proteins by ADP-ribosylation. At 72 hours post transfection cells were harvested and membranes were prepared as in section (Section 2.9).

3.2.3 Immunodetection of the expression of $G_{i\alpha 1}$ point mutants co-transfected into HEK293T cells with the porcine α_2A adrenoceptor

The expression levels of the $C^{351}Xaa$ $G_{i\alpha 1}$ mutants co-transfected with the porcine α_2A adrenoceptor were compared in the same membrane preparation as that used to generate receptor and GTP γ S binding data. A sample of membrane preparation was resolved on a 10% SDS-PAGE gel as described in Section 2.11. The resolved proteins were subsequently western blotted onto a nitrocellulose membrane (Section 2.12) which was then probed for $G_{i\alpha 1}$ immunoreactivity using the antiserum IIC as detailed in Table 2.1. It can be seen from Fig. 3.4 that all $C^{351}Xaa$ $G_{i\alpha 1}$ cDNAs

transfected were over expressed at levels higher than endogenously expressed $G_{i\alpha 1}$, in cells transfected with receptor alone (lane C-). It was also noted that there was some variation in $G_{i\alpha 1}$ mutant expression between transfections.

3.2.4 The relative expression levels of the porcine α_2A adrenoceptor in HEK293T cells co-transfected with the $C^{351}Xaa$ $G_{i\alpha 1}$ mutants are not significantly different

The expression level of the porcine α_2A adrenoceptor was assayed in HEK293T cells co-transfected with both empty vector and $C^{351}Xaa$ $G_{i\alpha 1}$ cDNA. The receptor binding assay was carried out as described in Section 2.13.1. Analysis of the receptor binding data (Fig. 3.5) shows that the porcine α_2A adrenoceptor was expressed at levels at or above 9pmol/mg in all of the co-transfections assayed. From other studies (Wise *et al.*, 1997d) it can be assumed that variation in receptor expression between transfections was not large enough to affect [^{35}S]GTP γ S binding in this membrane preparation.

3.2.5 The expression level of $C^{351}Xaa$ $G_{i\alpha 1}$ mutants increases upon transfection with increasing amounts of cDNA

The $C^{351}A$ $G_{i\alpha 1}$ mutant was arbitrarily chosen to examine if a relationship existed between the amount of cDNA transfected into the HEK293T cells and the level of G-protein immunodetected with the antiserum I1C. HEK 293T cells were co-transfected with 1.5 μ g of porcine α_2A adrenoceptor and from 0.15 to 1.5 μ g of $C^{351}A$ $G_{i\alpha 1}$ cDNA. The total amount of cDNA transfected (3 μ g) was standardised in each experiment by using empty pCDNA3 vector. The expression level of $C^{351}A$ $G_{i\alpha 1}$ was detected by resolving 25 μ g of membrane preparation on a 10% SDS-PAGE gel followed by western blotting and immunodetection with the I1C antiserum (Table 2.1). From the immunoblot (Fig. 3.6) it can be seen that the expression level of the $C^{351}A$ $G_{i\alpha 1}$ mutant was proportional to the amount of cDNA transfected.

3.2.6 A linear function exists between $C^{351}A$ $G_{i\alpha 1}$ expression and [^{35}S]GTP γ S binding at non-limiting receptor expression levels and maximal agonist concentration

A correlation between $C^{351}A$ $G_{i\alpha 1}$ expression and [^{35}S]GTP γ S binding at non-limiting receptor and agonist concentrations was determined in HEK293T cells co-transfected with porcine α_2A adrenoceptor cDNA (1.5 μ g) and increasing quantities of

C³⁵¹A G_{iα1} cDNA (0.15, 0.3, 0.75 and 1.5 μg). The cells were treated with PTX to remove the endogenous G-protein signalling component 12 hours prior to harvesting and membrane preparation. The level of C³⁵¹A G_{iα1} expression was quantitated by densitometric analysis of a representative immunoblot probed with the G_{iα1} specific antiserum I1C (Fig. 3.6). In parallel a [³⁵S]GTPγS binding assay was performed on the same membrane preparation. By stimulating the cognate receptor with UK14304, an α₂A adrenoceptor agonist, it can be seen in Fig. 3.7 that a linear function describes [³⁵S]GTPγS binding versus the level of G-protein expression.

3.2.7 The relative efficiency of C³⁵¹Xaa G_{iα1} mutants to bind [³⁵S]GTPγS in response to UK14304 stimulation of the α₂A adrenoceptor

The capacity of each of the C³⁵¹Xaa G_{iα1} mutants to bind the non-hydrolysable GTP analogue, [³⁵S]GTPγS, in response to UK14304 stimulation of the α₂A adrenoceptor was measured as detailed in Section 2.15.2. The dose response data for each of the G-protein mutants was plotted using the mathematical package Robosage (Fig. 3.8). In parallel, the expression level of each of the mutants was quantitated by densitometric analysis of a representative immunoblot probed with the G_{iα1} specific antiserum I1C (Section 2.14). The maximal [³⁵S]GTPγS binding value of each of the C³⁵¹Xaa G_{iα1} mutants was then normalised with regard to the level of G-protein expression. The data (Fig. 3.9), now corrected for variation in G-protein expression, depicts the efficacy of the C³⁵¹Xaa G_{iα1} mutants to bind [³⁵S]GTPγS relative to wild type G_{iα1} in response to activation by the α₂A adrenoceptor fully occupied by UK14304.

3.2.8 Demonstration of the capacity of poorly activated C³⁵¹Xaa mutants to bind GTPγS

The capacity of poorly activated mutants to exchange nucleotides was confirmed by limited trypsin digestion of such mutants in both the presence and absence of GTPγS. In this assay only G-proteins in the GTPγS bound form are resistant to complete tryptic digestion. Limited tryptic digestion of these GTPγS bound G-protein subunits results in the production of a 38 KDa fragment which is resistant to further tryptic cleavage. This protected fragment can be detected by the G_{iα1} specific antiserum I1C. The immunoblot in Fig. 3.10 clearly indicates that the poorly activated mutant

Cys³⁵¹Arg is equally capable of binding GTP γ S as some of the more potent mutants such as Cys³⁵¹Phc G_{i α 1}, Cys³⁵¹Ile G_{i α 1} and Cys³⁵¹Gly G_{i α 1}. It should also be noted that the apparent size of the GTP γ S bound, protected fragment was the same for each of the mutants.

3.2.9 A correlation exists between the hydrophobicity of the amino acid at residue ³⁵¹ in G_{i α 1} and the capacity of the mutant to bind [³⁵S]GTP γ S upon UK14304 stimulation

The relationship between the n-octanol/water partition co-efficient of the amino acid at position ³⁵¹ in G_{i α 1} and the ability of the mutant to bind [³⁵S]GTP γ S was determined by plotting the respective values from **Table 3.2** to produce **Fig. 3.11**. It can be seen from **Fig. 3.11** that a strong correlation does indeed exist between the n-octanol/water partition co-efficient of the amino acid at residue ³⁵¹ and the capacity of the mutant to bind [³⁵S]GTP γ S upon stimulation with UK14304. The general rule appears to be the more hydrophobic the residue at position ³⁵¹ the greater the capacity of the mutant to bind [³⁵S]GTP γ S. The correlation between the n-octanol/water partition co-efficient and [³⁵S]GTP γ S loading capacity gave an even better correlation when the amino acid mutants alanine, threonine and glycine were eliminated from the analysis.

3.2.10 The correlation between the hydrophobicity of the amino acid at position ³⁵¹ in the G_{i α 1} subunit and the pEC₅₀ derived from UK14304 stimulation.

The relationship between pEC₅₀ derived from UK14304 stimulated [³⁵S]GTP γ S binding and the hydrophobicity of the amino acid at residue ³⁵¹ was examined by plotting the octanol/water partition co-efficient as a function of the pEC₅₀ values in **Table 3.2** to produce **Fig. 3.12**. Analysis of **Fig. 3.12** shows that a close correlation exists between the hydrophobicity of the residue at position ³⁵¹ and the pEC₅₀ derived from UK14304 stimulation. The general rule appears to be the more hydrophobic the residue, the lower the pEC₅₀ value.

3.2.11 The C³⁵¹Xaa G_{1α1} mutants do not have different affinities for GDP.

The guanine nucleotide affinity of the two G_{1α1} mutants C³⁵¹H and C³⁵¹I was compared to that of the wild type by assaying maximal agonist stimulated [³⁵S]GTPγS binding in the presence of increasing concentrations of the competitor GDP.

HEK 293 Large T cells were co-transfected with the porcine α₂A adrenoceptor and a combination of wild type Cys³⁵¹G_{1α1} or one of the two mutants Cys³⁵¹Ile G_{1α1} and Cys³⁵¹His G_{1α1}. Cells transfected with receptor alone were used as control cells. Cells co-transfected with one of the pertussis toxin resistant mutants were PTX treated 12 hours prior to harvesting (Section 2.6.1) and membrane preparation. A membrane sample from each of the co-transfections was used to assay UK14304 stimulated binding of [³⁵S]GTPγS in the presence of increasing amounts of the competitor GDP. The capacity of each of the mutants to bind [³⁵S]GTPγS was plotted against the concentration of competitor, GDP, in the assay (Fig. 3.13). It can be seen from Fig. 3.13 that the [³⁵S]GTPγS binding capacity of each of the G_{1α1} mutants decreased proportionally with increasing GDP concentration.

3.2.12 The capacity of the C³⁵¹Xaa G_{1α1} mutants to be activated remains linear for up to 40 minutes

Hek 293 Large T cells were co-transfected with the porcine α₂A adrenoceptor and either wild type Cys³⁵¹G_{1α1} or one of the two mutants Cys³⁵¹Ile G_{1α1} or Cys³⁵¹His G_{1α1}. Cells transfected with receptor alone were used as a negative control (C-). Transfections involving the pertussis toxin resistant mutants Cys³⁵¹His G_{1α1} and Cys³⁵¹Ile G_{1α1} were PTX treated (Section 2.6.1) 12 hours prior to harvesting (Section 2.7) and membrane preparation (Section 2.9), other transfections were not treated. A maximal concentration of the α₂A adrenoceptoragonist, UK14304 (10 μM), was used to stimulate [³⁵S]GTPγS binding in membranes from each of the co-transfections. The binding assay was stopped at 0, 5, 10, 20, 30 and 40 minutes after the addition of [³⁵S]GTPγS by filtration through GF/C filters (Section 2.15.2). The agonist stimulated [³⁵S]GTPγS binding of each of the mutants was plotted against the duration of the assay (Fig. 3.14).

3.3 Discussion

The resistance of mutant G_{α} subunits to post-translational modification by bacterial toxins is an effect which has frequently been exploited to investigate the selectivity of receptor/G-protein interactions (Senogles, 1994; Hunt *et al.*, 1994). Pertussis toxin catalysed ADP ribosylation at the cysteine 351 residue of rat $G_{i\alpha 1}$ has been shown to substantially disrupt the interaction between an ADP-ribosylated G-protein and its cognate receptor. It is thought that steric hindrance by the ADP-ribose moiety is the molecular mechanism behind this loss of protein-protein interaction. From the data generated by this study it can be postulated that charge may also be a major contributory factor in this effect. Previous pertussis toxin resistant $G_{i\alpha}$ mutants substituted the cysteine residue 351 with "conservative" amino acids such as glycine (Senogles, 1994) or serine (Hunt *et al.*, 1994). Although such residues have been shown to confer resistance to post translational modification by PTX, they have also been shown to negatively affect coupling efficiency when compared to the wild type $G_{i\alpha 1}$ subunit (Wise *et al.*, 1997d).

In spite of this, little has been done to quantitate the physiochemical properties of the amino acid at position 351 and its affect on receptor G-protein coupling. It should also be noted that no one has ever comprehensively analysed the affects of substituting the cysteine residue 351 to all other amino acids. Since current G-protein assays utilise either agonist stimulated binding of the radiolabelled non-hydrolysable GTP analogue [^{35}S]GTP γ S or the hydrolysis of γ [^{32}P]GTP by G-protein α -subunits, it is clearly of relevance to know what the effects of a particular mutation may have upon the sensitivity of an assay or the apparent affinity of various ligands.

Osawa and Weiss (1995) extensively mutated the C-terminal tail of $G_{i\alpha}$ with unexpected results. They found that the $G_{i\alpha}$ subunit was severely affected by mutations which had little or no effect in $G_{i\alpha}$ (Senogles, 1994; Hunt *et al.*, 1994) or $G_{o\alpha}$ (Denker *et al.*, 1992). From these findings it was concluded that it would be restrictive to select "a priori" the mutations to be substituted to C351. For this reason it was decided that a more comprehensive approach to study the functionality of the cysteine residue at position 351 would be to systematically mutate the residue to all other amino acids. The ability of each of the mutants to be activated by the porcine $\alpha_2\text{A}$ adrenoceptor was then assayed by the [^{35}S]GTP γ S binding assay (Fig. 3.8). From the data in Figures 3.8 and

3.9 it can be seen that the G-protein mutants displayed a gradient of responses. It should also be noted that the naturally occurring wild type G-protein was not the most effectively activated by the porcine α_2A adrenoceptor. It could be argued that such discrepancies are due to variations in receptor G-protein expression resulting from poor transfection efficiencies. In order to evaluate the influences of these factors, the expression level of the α_2A adrenoceptor was quantitated along with that of the G-proteins. In each of the co-transfections the receptor level (**Fig. 3.5**) was determined to be at 9pmol/mg or higher. Previous studies (Wise *et al.*, 1997d) have demonstrated that small variations in receptor expression above 9pmol/mg would not be sufficient to explain the range of [35 S]GTP γ S binding capacities seen in **Figures. 3.8** and **3.9**. The relative expression of each of the C 351 Xaa G $_{i\alpha 1}$ mutants was determined by an immunoblot using G $_{i\alpha 1}$ specific antisera (**Fig. 3.4**). Most of the G-protein mutants were overexpressed at levels comparable to that of the overexpressed wild type. However, there was some variation in expression and such discrepancies could partially account for the differences in efficacy. To address this issue a linear relationship was determined between C 351 A G $_{i\alpha 1}$ expression (**Fig. 3.6**) and maximal [35 S]GTP γ S binding. It can be seen from (**Fig. 3.7**) that a linear function does exist between G-protein expression and [35 S]GTP γ S binding at a fixed receptor expression level and maximal agonist stimulation. This relationship justified the normalisation of the [35 S]GTP γ S binding data in **Fig. 3.8** by densitometric analysis of **Fig. 3.4** to produce a rank order of mutant effectiveness corrected for variation in expression of the G $_{i\alpha 1}$ subunit (**Fig. 3.9**).

Osawa and Weiss(1995) and Garcia *et al.*(1995) had previously demonstrated that mutations in the C-terminus of G $_{i\alpha}$ could affect both the nucleotide binding capacity of the α -subunit as well as receptor coupling. Binding data generated by the [35 S]GTP γ S assay used a single concentration of both GDP and GTP γ S, it could be argued that any reduction in receptor/G-protein coupling could be due to either a loss or diminished ability of the α -subunit to exchange nucleotides. Using limited trypsin digestion it was demonstrated that poorly activated mutants have retained the capacity to exchange nucleotides. The C351R G $_{i\alpha 1}$ mutant was previously shown to be incapable of binding [35 S]GTP γ S upon agonist stimulation of the porcine α_2A adrenoceptor. From (**Fig. 3.10**) it can be seen that the same poorly activated mutant is capable of exchanging nucleotides as indicated by the presence of the immunodetectable product of incomplete tryptic digestion. It was demonstrated that other mutants were equally capable of

exchanging nucleotides, as indicated by the capacity of the GTP γ S bound α -subunit to be resistant to complete tryptic cleavage (**Fig. 3.10**).

The experiments above demonstrated the ability of the $G_{i\alpha 1}$ mutants to exchange nucleotides, but they still have not demonstrated that the mutants do not possess different affinities for nucleotides. This issue was addressed by assaying maximal agonist stimulated [35 S]GTP γ S binding in increasing concentrations of the competitor GDP. The wild type α -subunit was assayed along with two arbitrarily chosen mutants containing a charged residue ($C^{351}H$ $G_{i\alpha 1}$) and a strongly hydrophobic ($C^{351}I$ $G_{i\alpha 1}$) residue at position 351. It can be seen from **Fig. 3.13** that the relative [35 S]GTP γ S binding capacity between the mutants remained unchanged throughout the range of GDP concentrations. From this data it can be concluded that the $C^{351}Xaa$ $G_{i\alpha 1}$ mutants do not display different nucleotide affinities when compared to wild type $G_{i\alpha 1}$.

It is possible that a mutation at residue 351 could result in a conformational change in the nucleotide binding pocket leading to either an increase or decrease in the rate of nucleotide exchange. To determine whether the guanine nucleotide exchange rate had indeed been altered the binding capacities of the above two mutants was assayed at various time intervals (**Fig. 3.14**). From this data it can be seen that the nucleotide exchange rate between mutants remains unchanged for up to 40 minutes.

After correcting for any discrepancies in $G_{i\alpha 1}$ subunit expression it may be assumed that any remaining variation in the capacity of agonist occupied GPCR to activate the G-protein is due to the differential properties of the residue at position 351. A relationship was sought between the physiochemical and functional properties of residue 351. After analysing parameters such as hydrophobicity, hydrophilicity, size descriptors and volume to surface areas, it was noted that the partition co-efficient octanol/water (Pliska *et al.*, 1981) gave the best correlation both with pEC_{50} and [35 S]GTP γ S binding. The correlation between the octanol/water partition co-efficient of the residue at position 351 and mutant efficacy, represented as a dimensionless descriptor relative to wild type GTP γ S binding capacity, was plotted from the values in **Table 3.2** to produce **Fig. 3.11**. The elimination of methionine and proline from the equation results in a very strong correlation ($r=0.92$, **Fig. 3.11**) between [35 S]GTP γ S binding and the hydrophobicity of residue 351. The correlation co-efficient becomes close to 1 ($r=0.99$, **Fig. 3.11**) if the values for alanine, threonine and glycine (filled

circles) are also omitted from the equation. Possible explanations why the residues methionine and proline fail to conform to the general trend are as follows. In the case of methionine it is possible that the residue was present as methionine sulfoxide, this would greatly alter its hydrophobic character and so the octanol/water partition co-efficient value. As for the effects observed for proline, in mutation studies, Osawa & Weiss(1995) have shown that substitution of the glycine residue at position 348 to proline in the C-terminal tail of $G_{1\alpha}$ results in a decrease in receptor binding and to a lesser effect on nucleotide binding. This effect is thought to be due to a loss of flexibility in the C-terminal tail resulting from the "kink" introduced into the peptide backbone by proline. Since the equivalent glycine residue lies at position 352 in $G_{1\alpha1}$ it could be assumed that the loss in function is due to a similar loss of flexibility. The pEC_{50} values of the mutants were also analysed with regard to the physiochemical properties of the amino acid at residue 351. Once again the pEC_{50} values (Table 3.2) correlated well with the octanol/water partition co-efficient giving a correlation co-efficient of $r=0.90$ (Fig. 3.12).

The findings of this mutational study would seem to corroborate data generated by previous investigations (Senogles, 1994; Hunt *et al.*, 1994; Osawa and Weiss, 1995). While extensive data has shown that the residue Cys³⁵¹ in $G_{1\alpha1}$ and the equivalent residues in other PTX sensitive $G\alpha$ subunits are not the only determinants for receptor interaction, the data generated indicates that the previous C³⁵¹ $G_{1\alpha1}$ mutants were far from ideal. The mutants generated herein displayed a spectrum of responses, some surprisingly showing greater maximal stimulation than wild type. These responses seem to correlate well with the hydrophobic character of the residue at position 351. More hydrophobic residues such as isoleucine seemed to confer greater coupling efficiency than more hydrophilic ones such as aspartate. It would also seem as if a charge at position 351 is a greater negative factor than size. This is consistent with evidence that protein-protein contact surfaces tend to be of a hydrophobic nature. It should also be noted though that an increase in hydrophobic character could affect solubility and so dissociation from the receptor. The observed negative implication of a charge at this position may play an important role in the mode of action of ADP-ribosylation on G-protein receptor association.

The data generated from these experiments has a practical use in the development of screens for novel ligands as well as in the characterisation of GPCRs. In

screening systems it is advantageous to maximise the sensitivity of an assay, this allows the use of less protein and thereby reduces cost. In the characterisation of GPCRs it is necessary to know how a particular PTX mutant will alter EC_{50} values. This information needs to be taken into account when assaying the potency of a ligand using an assay developed with PTX mutants. Future work could involve characterising the specificity of $\beta\gamma$ subunit interaction with each of the G-protein mutants. It would also be interesting to see if the mutation has altered the capacity of the mutant to interact with secondary effectors. Finally it would be useful to examine if the relative effectiveness of the mutants is receptor specific.

Figure 3.1 PCR amplification of C³⁵¹Xaa G_{iα1} mutants of rat G_{iα1}

Rat C³⁵¹Xaa G_{iα1} mutants were generated by PCR amplification of rat G_{iα1} cDNA with one of four degenerate primers. The PCR reaction products from amplification with **Primer1**(lane1), **Primer2**(lane2), **Primer3**(lane3) and **Primer4**(lane4) were resolved on a 1% agarose gel (100μl/lane) and stained with ethidium bromide. The 1065bp DNA fragment (lanes 1-4) corresponds to the expected size of the amplified G_{iα1} mutant.

Figure 3.1

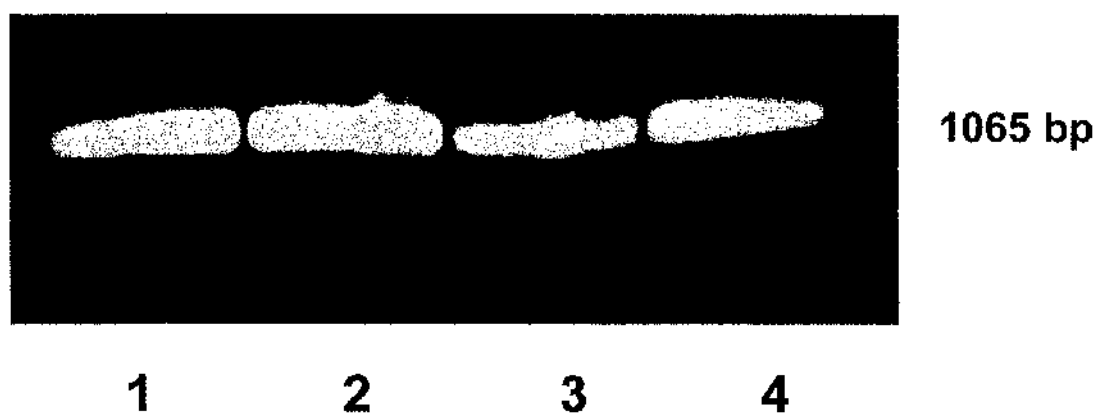
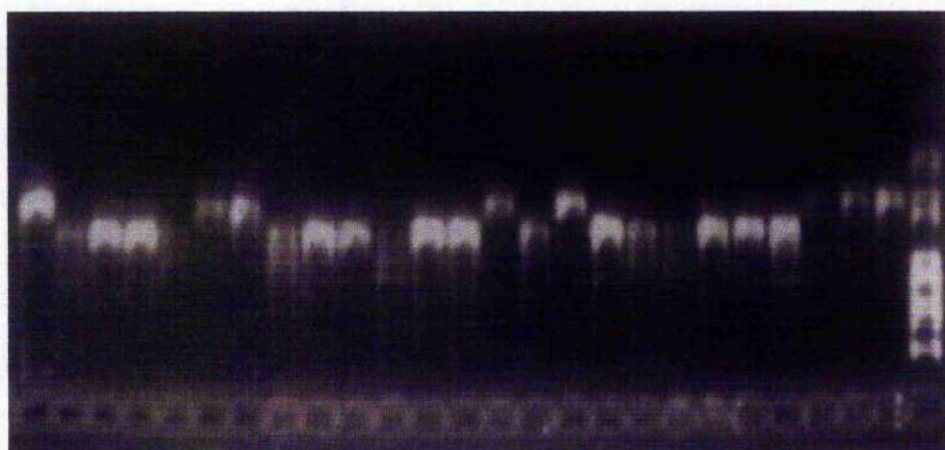


Figure 3.2 Analysis of clones transformed with the C³⁵¹Xaa G_{1α1} in the pCR-Script vector

DNA was isolated from clones thought to be transformed with mutant G_{1α1} cDNA in the pCR-Script vector. A small sample of supercoiled DNA (2 μl) was resolved on a 1% agarose gel stained with ethidium bromide. Clones were selected on the basis of DNA migration, the vector containing the insert (lanes 2, 3, 4, 5, 8, 9, 10, 11, 12, 13, 15, 17, 18, 19, 20, 21 and 22) migrated less than empty vector alone (lanes 1, 6, 7, 14, 16, 23, 24 and 25). A 1Kb ladder provided size markers (lane 26).

Figure 3.2



1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26

Figure 3.3 Analysis of ³⁵¹Xaa G₁₀₁ cDNA orientation in the pCR-Script vector by restriction digestion

Plasmid DNA was isolated from clones transformed with mutant G₁₀₁ cDNA in the pCR-Script vector. Samples were restriction digested with the enzymes *NotI* and *NcoI* (Section 2.23) and resolved on a 1% agarose gel. When the start codon of the G₁₀₁ mutant was next to the T3 promoter priming site in the host vector, the plasmid was cleaved to yield fragments of 11, 260 and 3755 base pairs (lanes 1, 4, 5, 8, 11, 13 and 14). Cleavage of the vector with the insert in the opposite orientation, with the start codon distal to the T3 priming site, yielded fragments of 11, 805 and 3210 base pairs (lanes 2, 3, 6, 7, 9, 10, 12, 15, 16, 17). Lane 18 was loaded with 1Kb molecular weight markers.

Figure 3.3

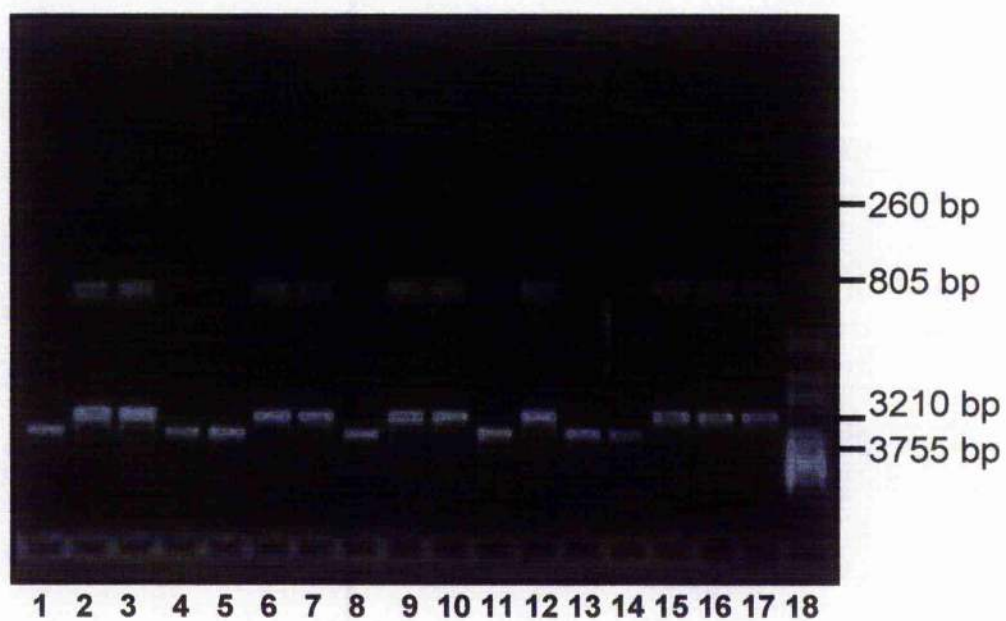


Figure 3.4 The expression of C³⁵¹Xaa mutants transiently co-transfected into HEK293T cells with the porcine α 2A adrenoceptor

Membranes were prepared from HEK293T cells co-transfected with the α 2A adrenoceptor and a combination of G_{i α 1} mutants. Samples (25 μ g) were resolved on a 10% SDS-PAGE gel and subsequently probed for G_{i α 1} immunoreactivity with the antiserum IIC (**Table 2.1**). The residue at position 351 is identified by the conventional three letter code. Membranes prepared from HEK293T cells transfected with receptor alone (lanes C-) were used to compare endogenous G_{i α 1} expression.

Figure 3.4

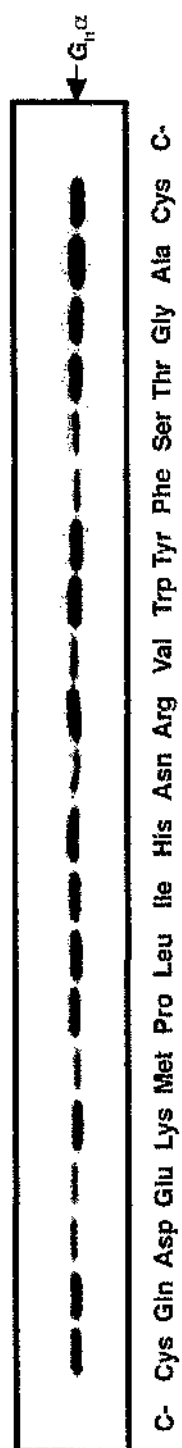


Figure 3.5 The expression of the porcine α_2A adrenoceptor co-transfected into HEK293T cells in combination with the C³⁵¹Xaa G_{i α 1} mutants

Membranes were prepared from HEK393T cells transfected with either the α_2A adrenergic receptor alone (A-) or in combination with one of the C³⁵¹Xaa G_{i α 1} mutants. With the exception of the ³⁵¹C G_{i α 1} co-transfection, cells were treated with 50ng/ml PTX prior to harvesting. The level of porcine α_2A adrenoceptor was assayed in each of the membrane samples as detailed in **Section 2.15.1**. The B.max value for each transfection is listed in **Table 3.1**, the identity of the mutant is denoted by the conventional three letter code. The α_2A adrenoceptor transfected alone is represented as (A-). The results shown are the means \pm S.D. of triplicate measurements for a single experiment. The data represents a typical experiment.

Figure 3.5

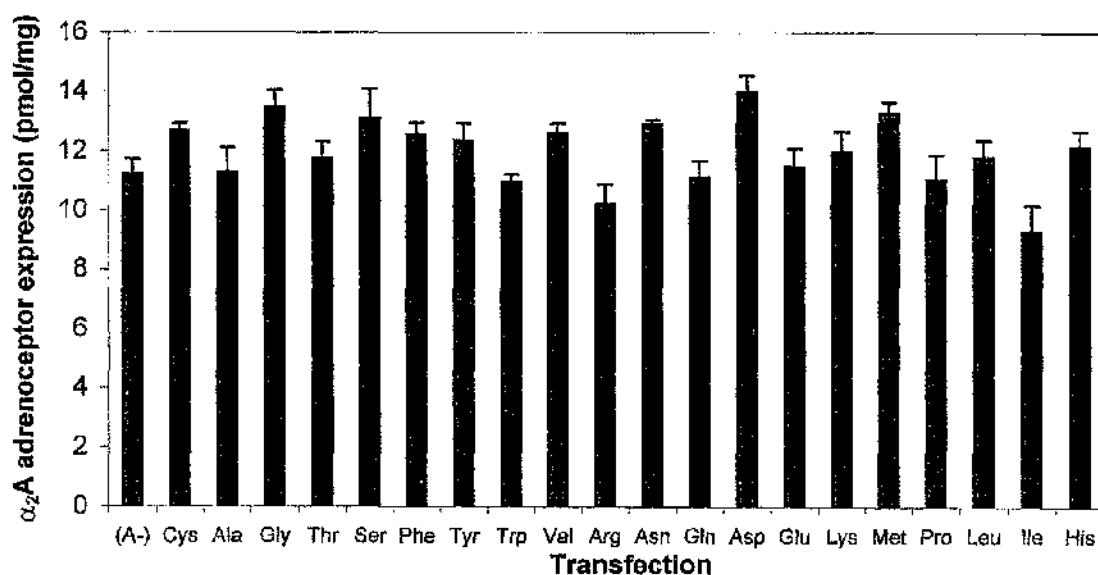


Table 3.1 B.max values of α_2A adrenoceptor co-transfection

| Transfection | α_2A expression B.max | Transfection | α_2A expression B.max |
|--------------|------------------------------|--------------|------------------------------|
| (A-) | 11.21 | Asn | 12.91 |
| Cys | 12.67 | Gln | 11.1 |
| Ala | 11.28 | Asp | 13.99 |
| Gly | 13.48 | Glu | 11.47 |
| Thr | 11.74 | Lys | 11.99 |
| Ser | 13.1 | Met | 13.3 |
| Phe | 12.53 | Pro | 11.02 |
| Tyr | 12.33 | Leu | 11.78 |
| Trp | 10.93 | Ile | 9.28 |
| Val | 12.59 | His | 12.13 |
| Arg | 10.19 | | |

Figure 3.6 Transient transfection of increasing amounts of C³⁵¹A G_{iα1} cDNA into HEK293T cells results in higher levels of C³⁵¹A G_{iα1} expression.

Membranes were prepared from HEK293T cells co-transfected with a constant amount of porcine α₂A adrenoceptor cDNA (1.5μg) in combination with increasing quantities of C³⁵¹A G_{iα1} cDNA (0.15, 0.3, 0.75 and 1.5μg). A sample of membrane preparation was resolved on a 10% SDS-PAGE gel, which was subsequently probed for G_{iα1} immunoreactivity with the antiserum I1C (**Table 2.1**). The quantity of C³⁵¹A G_{iα1} cDNA used in each transfection is listed below the respective lane.

Figure 3.6

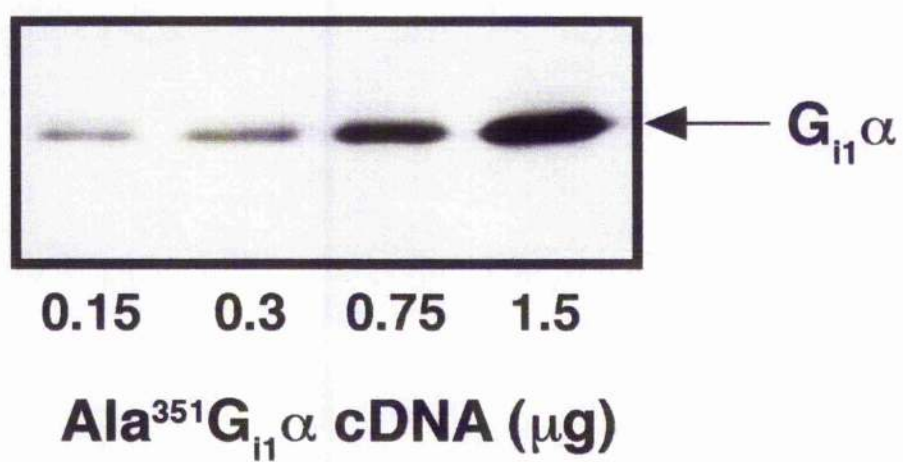


Figure 3.7 A linear relationship exists between C³⁵¹A G_{iα1} expression and [³⁵S]GTPγS binding at a fixed receptor level and maximal agonist concentration

Membranes were isolated from HEK293T cells co-transfected with a constant amount of α₂A adrenoceptor cDNA (1.5 μg) and increasing quantities of C³⁵¹A G_{iα1} cDNA (0.15, 0.3, 0.75 and 1.5 μg). Cells were treated with PTX 12 hours prior to harvesting. UK14304 stimulated (10 μM) [³⁵S]GTPγS binding was assayed in each of the membrane preparations. The expression levels of C³⁵¹A G_{iα1} were quantitated in the same membrane sample by densitometric analysis of a representative immunoblot probed with the G_{iα1} specific antiserum I1C (Fig. 3.6). The results from the [³⁵S]GTPγS assay was plotted as a function of the G_{iα1} immunoreactivity detected in each of the membrane samples. The relationship between G-protein expression and [³⁵S]GTPγS binding can be described by a linear equation whose experimental points correlate with a correlation co-efficient of $r=0.988$. The data shown represents the means of triplicate measurements from the same experiment.

Figure 3.7

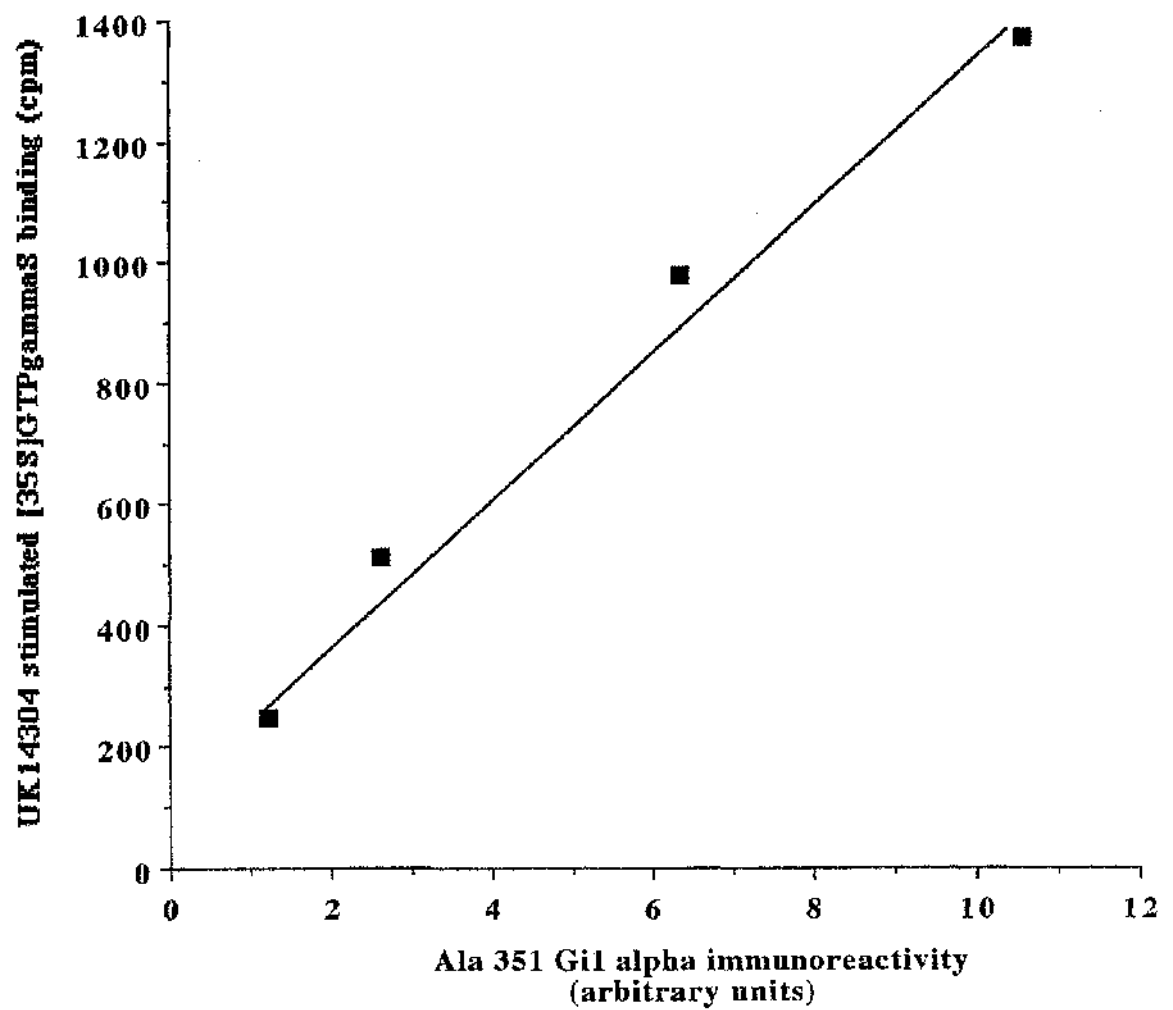


Figure 3.8 The relative coupling efficiency of the $G_{i\alpha 1}$ mutants with the porcine α_2A adrenoceptor upon UK14304 stimulation

The coupling efficiency of the various mutants was assayed by UK14304 stimulated [35 S]GTP γ S binding in membranes prepared from HEK293T cells co-transfected with the porcine α_2A adrenoceptor and a combination of C 351 Xaa $G_{i\alpha 1}$ mutants. All transfections, with the exception of those including the wild type G-protein, were pre-treated with 50ng/ml PTX 12 hours prior to harvesting and membrane preparation. Each data point in **Fig. 3.8** represents the average of duplicate measurements of a single experiment. The identity of the mutant is denoted by the standard three letter code. The data represents a typical experiment.

Figure 3.8

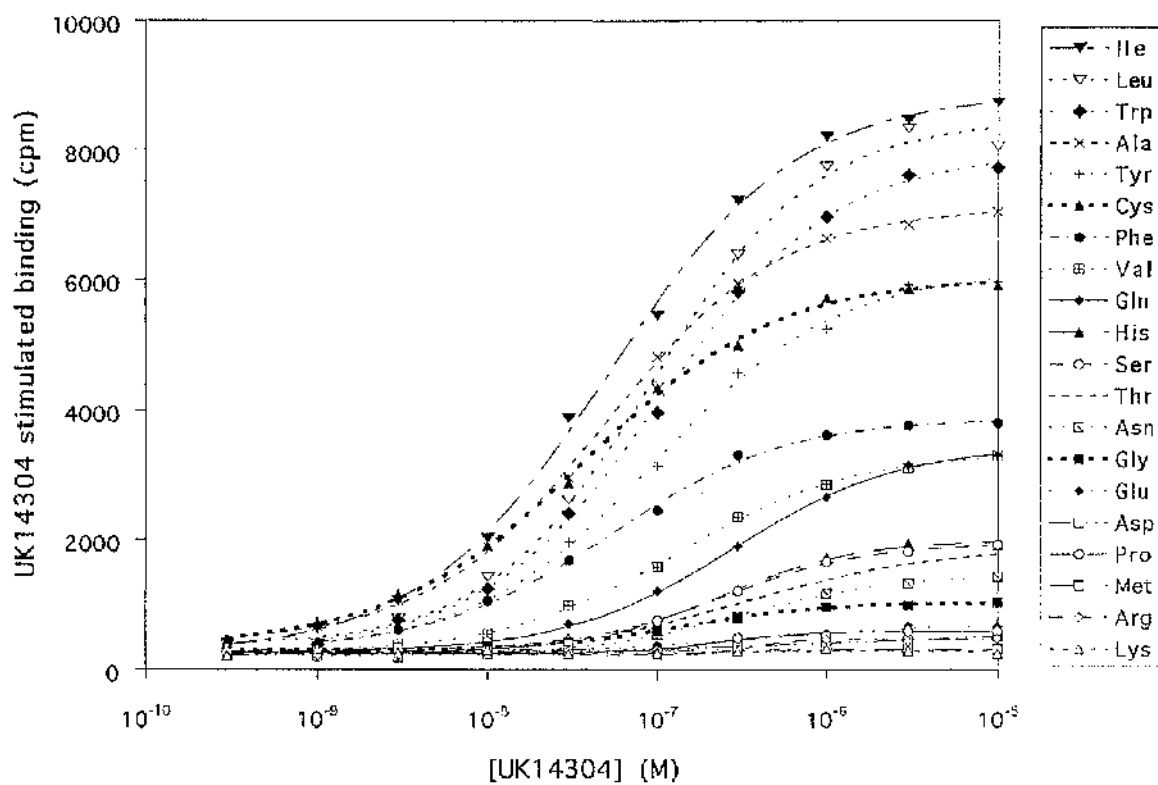


Figure 3.9 A comparison of the efficacy of C³⁵¹Xaa G_{iα1} mutants to bind [³⁵S]GTPγS at maximal agonist concentrations

The capacity of maximal concentrations of UK14304 (10 μM) to stimulate [³⁵S]GTPγS binding was assayed in membranes prepared from HEK293T cells co-transfected with a combination of porcine α2A adrenoceptor and G-protein mutants. The level of G_{iα1} mutant expression was quantitated in the same membrane preparation by densitometric analysis of an immunoblot probed for G_{iα1} immunoreactivity by the antiserum IIC. The efficacy of the mutants was normalised with regard to variation in G_{iα1} subunit expression. Results are presented as percentage of wild type ³⁵¹C G_{iα1} stimulation. The identity of the residue at position 351 is represented using a single letter amino acid symbol.

Figure 3.9

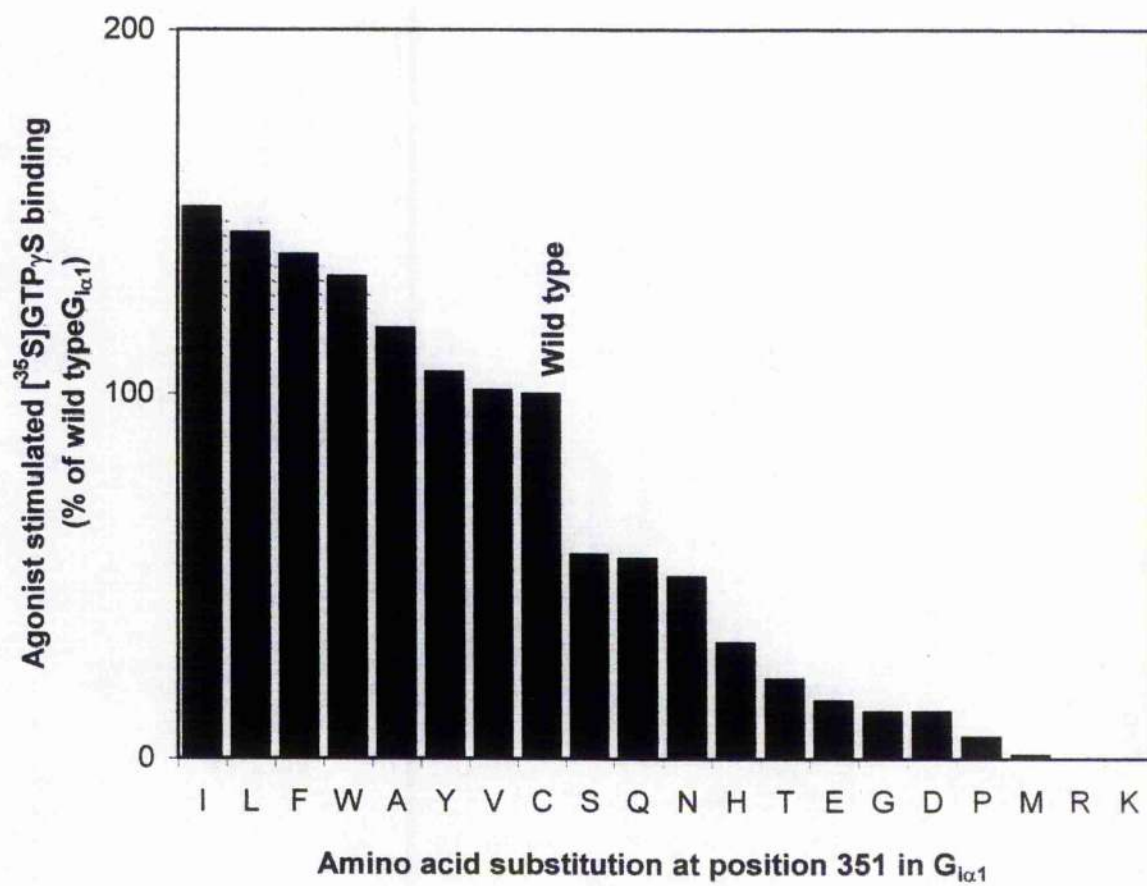


Figure 3.10 An immunoblot demonstrating the capacity of poorly activated C³⁵¹Xaa G_{iα1} mutants to exchange nucleotide

Membranes were prepared from HEK293T cells transfected with either empty pCDNA3 vector (lanes 1-3) or one of the following C³⁵¹Xaa G_{iα1} subunits- ³⁵¹Cys (lanes 4-6), Cys³⁵¹Gly (lanes 7-9), Cys³⁵¹Arg (lanes 10-12), Cys³⁵¹Phe (lanes 13-15) and Cys³⁵¹Ile (lanes 16-18). The capacity of the G-protein to exchange nucleotides was assayed by limited tryptic digestion (Section 2.17) of a membrane sample (100 μg) in the absence (lanes 3, 6, 9, 12, 15 and 18) or presence of GTPγS (lanes 2, 5, 8, 11, 14 and 17). Untreated membrane samples were then resolved on a 10% SDS-PAGE gel and probed for G_{iα1} immunoreactivity with the antiserum 11C (Table 2.1). Fig. 3.10 is a representative immunoblot.

Figure 3.10

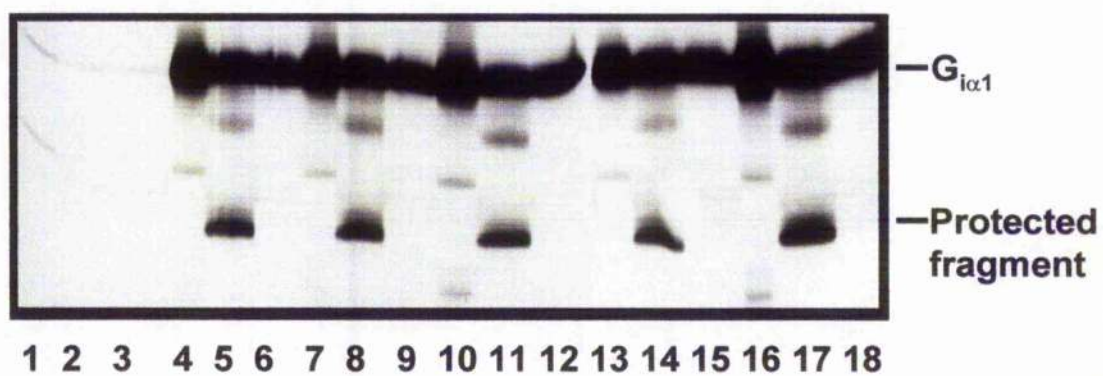


Figure 3.11 The correlation between the hydrophobicity of the amino acid at residue 351 and G-protein mutant coupling efficiency at maximal agonist concentration

Membranes were isolated from HEK293T cells co-transfected with the porcine α_2A adrenoceptor in combination with C³⁵¹Xaa G_{i α 1} mutants. A sample of membrane preparation was used to assay [³⁵S]GTP γ S binding in each of the transfections upon stimulation with maximal agonist concentrations (10 μ M UK14304). The coupling efficiency of the mutants was normalised with regard to variation in G-protein expression and presented as the percentage of binding relative to the wild type G_{i α 1} subunit (Table 3.2). This binding index was plotted as a function of the log(octanol/water) partition co-efficient of the amino acid at residue 351 (Table 3.2). When the methionine and proline mutants were excluded from the analysis, the relationship between the hydrophobicity of amino acid 351 and its effect on efficiency could be described by the linear equation $GTP\gamma S = 1.237(\pm 0.617) + 0.522(\pm 0.054) \log p(octanol/water)$. The figures in parenthesis in the above equation denote the 95% confidence intervals. The experimental points correlate with a correlation co-efficient of $r=0.924$, where the number of forms of G_{i α 1} (n)=18 and standard deviation (s)=0.213. Elimination of alanine, threonine and glycine (filled circles) gave a better correlation between the two factors ($r=0.989$).

Figure 3.11

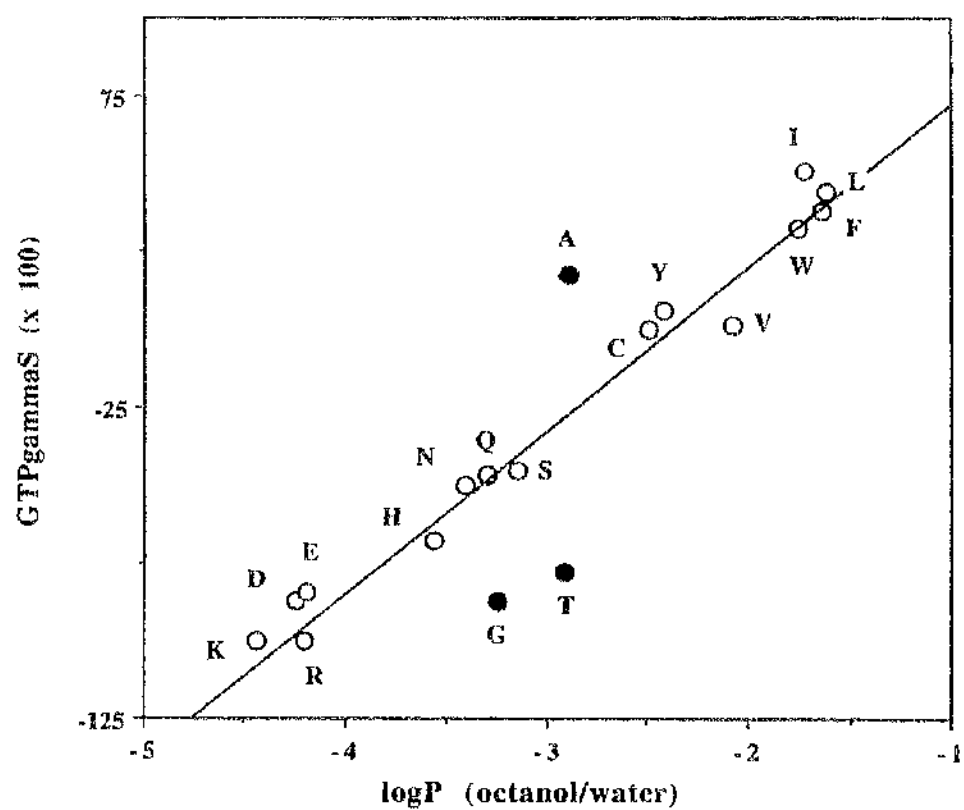


Figure 3.12 The correlation between the hydrophobicity of the amino acid at residue 351 and the pEC₅₀ value derived by UK14304 stimulation

Membranes were isolated from HEK293T cells co-transfected with the porcine α_2A adrenoceptor in combination with C³⁵¹Xaa G₁₀₁ mutants. A sample of membrane was used to assay [³⁵S]GTP γ S binding in each of the transfection conditions at a range of agonist concentrations (10⁻⁵ to 10⁻¹⁰ M UK14304). pEC₅₀ values were calculated from the binding data using the mathematical package Robosage (Table 3.2) and plotted as a function of hydrophobicity of the amino acid at position 351 (values taken from Table 3.2). The relationship between the hydrophobicity of residue 351 and its influence on pEC₅₀ was described by the linear equation $pEC_{50} = 8.246(\pm 0.133) + 0.351(\pm 0.055) \log P(\text{octanol/water})$, where the number of forms of G₁₀₁ (n)=14 and standard deviation (s)=0.129. The figures in parenthesis in the above equation correspond to the 95% confidence intervals. The experimental points correlated with a correlation co-efficient of r=0.90. Omitting the glycine mutant from the analysis, as indicated by the filled circle, resulted in a tighter correlation with the correlation co-efficient increasing to r=0.916.

Figure 3.12

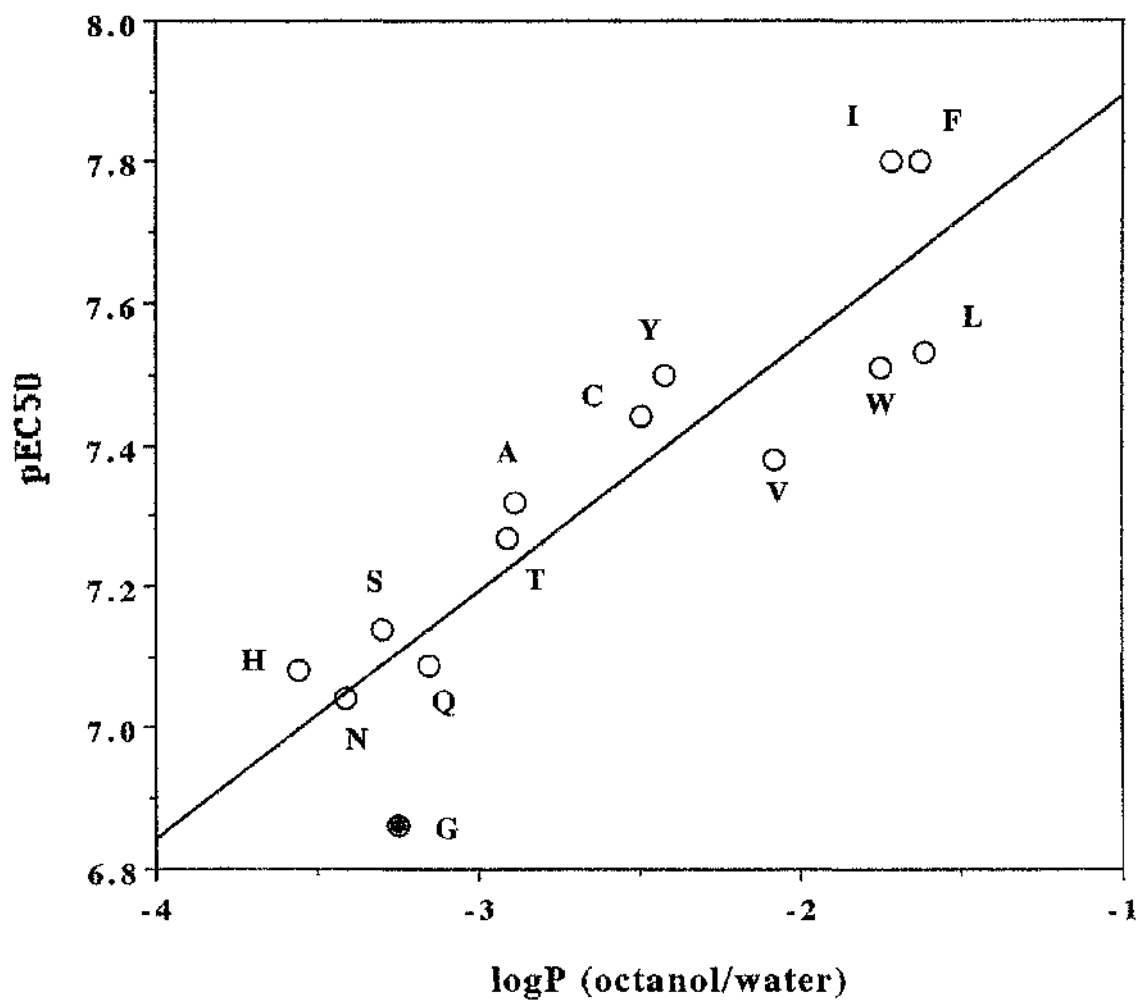


Figure 3.13 No difference was observed in the nucleotide binding affinity between C³⁵¹Xaa G_{iα1} mutants

Membranes were prepared from HEK293T cells which were co-transfected with the porcine α_2A adrenoceptor in combination with either Cys³⁵¹His G_{iα1}, Cys³⁵¹Ile G_{iα1}, wild type G_{iα1} or empty pCDNA3 vector (C-). Cells co-transfected with the Cys³⁵¹His and Cys³⁵¹Ile G_{iα1} mutants were PTX treated with 50ng/ml PTX 12 hours prior to harvesting. A sample of membrane was used to assay [³⁵S]GTPγS binding at maximal agonist concentration (10 μM UK14304) in a modified assay mix containing increasing quantities of the competitor nucleotide GDP (0, 1, 2, 4, 10, 16, 32 and 64 μM). The [³⁵S]GTPγS binding values were plotted against concentration of competitor (GDP). The [³⁵S]GTPγS binding data was not normalised with regard to G-protein expression. The values shown are the means ± S.D. of triplicate measurements in a representative experiment of three performed.

Figure 3.13

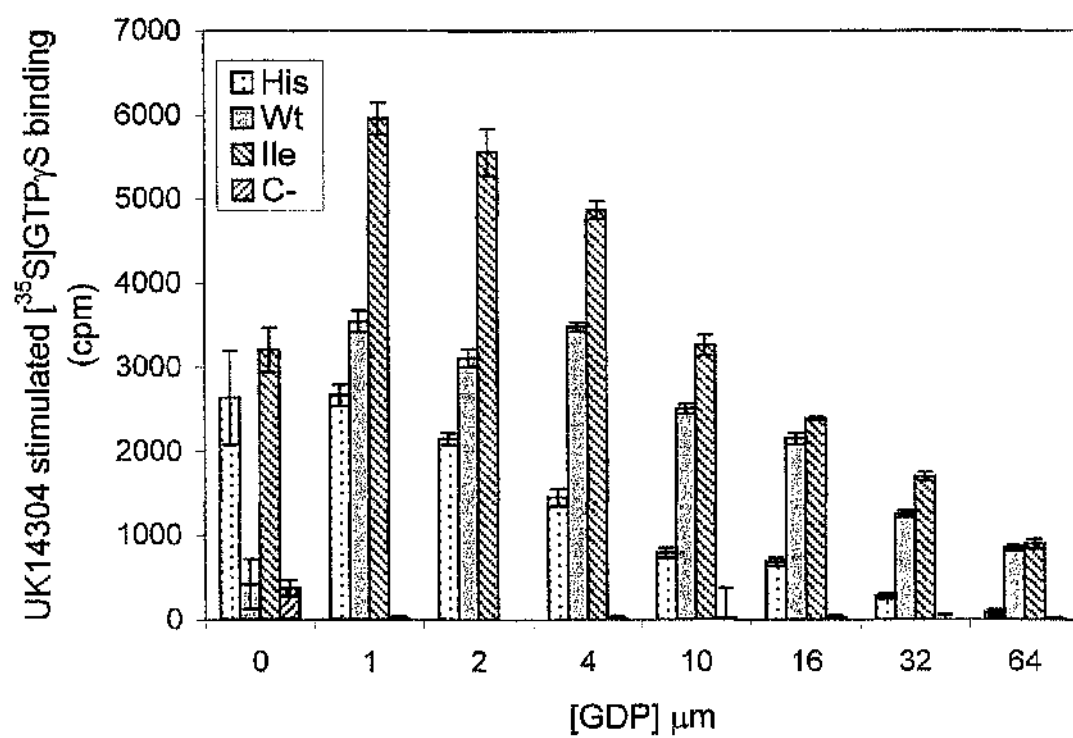


Figure 3.14 The nucleotide exchange rate of the various C³⁵¹Xaa G_{iα1} mutants remains proportional for up to 40 minutes.

Membranes were prepared from HEK293T cells co-transfected with the porcine α_2A adrenoceptor in combination with either Cys³⁵¹His G_{iα1}, Cys³⁵¹Ile G_{iα1}, wild type G_{iα1} or empty pCDNA3 vector (C-). Cells co-transfected with Cys³⁵¹Ile and Cys³⁵¹His G_{iα1} were PTX treated 12 hours prior to harvesting. A sample of membrane was used to assay [³⁵S]GTP γ S binding at maximal UK14304 concentration (10 μ M) over a period of 40 minutes. Reactions were stopped by filtration (as described in **Section 2.15.2**) at 0, 5, 10, 20, 30 and 40 minutes after the addition of [³⁵S]GTP γ S. The [³⁵S]GTP γ S binding values were plotted in function of time, the data presented is not normalised with regard to variation in G-protein expression. The values shown are the means \pm S.D. of triplicate measurements in a representative experiment of three performed.

Figure 3.14

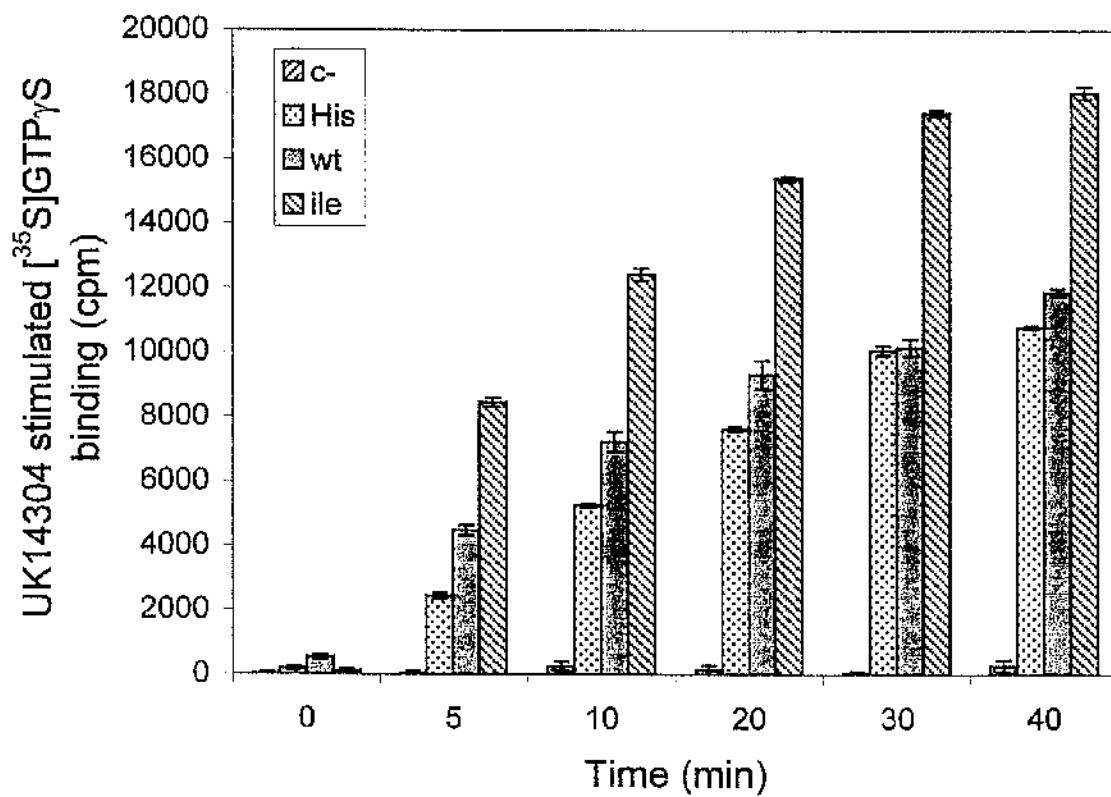


Table 3.2 A table summarising the order of EC₅₀, partition co-efficient (octanol/water) and [³⁵S]GTPγS binding capacity of each of the C³⁵¹Xaa G_{ia1} mutants with regard to the porcine α₂A adrenoceptor and the agonist UK14304.

The [³⁵S]GTPγS binding capacity index for each of the mutants was calculated relative to the binding capacity of the wild type Cys³⁵¹G_{ia1} G-protein subunit using the following equation-

$$[\text{³⁵S}]\text{GTP}\gamma\text{S capacity} = \frac{(\text{Cpm mutant} - \text{Cpm wild type})}{\text{Cpm wild type}}$$

The variation in the level of mutant G_{ia1} expression was taken into account as described in **Fig. 3.5**. The EC₅₀ values were derived from the dose response data in **Fig. 3.8** using the mathematical computer package Robosage. The (octanol/water) partition co-efficient of each of the amino acid residues at position ³⁵¹ were taken from the findings by Pliska *et al* (1981).

Table 3.2

| Amino Acid ³⁵¹ | GTP _γ S Index | EC ₅₀ (M) | log P (octanol/water) |
|---------------------------|-----------------------------|--|--------------------------|
| I | 0.51 | $1.6 \times 10^{-8} \pm 8.2 \times 10^{-10}$ | -1.72 |
| L | 0.44 | $2.9 \times 10^{-8} \pm 2.5 \times 10^{-9}$ | -1.61 |
| F | 0.38 | $1.6 \times 10^{-8} \pm 1.9 \times 10^{-9}$ | -1.63 |
| W | 0.32 | $3.1 \times 10^{-8} \pm 1.6 \times 10^{-9}$ | -1.75 |
| A | 0.18 | $4.8 \times 10^{-8} \pm 2.9 \times 10^{-9}$ | -2.89 |
| Y | 0.06 | $3.2 \times 10^{-8} \pm 2.3 \times 10^{-9}$ | -2.42 |
| V | 0.01 | $4.2 \times 10^{-8} \pm 1.6 \times 10^{-9}$ | -2.08 |
| C | 0.00 | $3.7 \times 10^{-8} \pm 3.2 \times 10^{-9}$ | -2.49 |
| S | -0.44 | $7.3 \times 10^{-8} \pm 7.5 \times 10^{-9}$ | -3.30 |
| Q | -0.45 | $8.2 \times 10^{-8} \pm 5.7 \times 10^{-9}$ | -3.15 |
| N | -0.50 | $9.1 \times 10^{-8} \pm 9.1 \times 10^{-9}$ | -3.41 |
| H | -0.68 | $8.4 \times 10^{-8} \pm 1.1 \times 10^{-8}$ | -3.56 |
| T | -0.78 | $5.4 \times 10^{-8} \pm 2.9 \times 10^{-8}$ | -2.91 |
| E | -0.84 | $7.8 \times 10^{-8} \pm 3.5 \times 10^{-8}$ | -4.19 |
| G | -0.87 | $1.4 \times 10^{-8} \pm 9.9 \times 10^{-9}$ | -3.25 |
| D | -0.87 | | -4.25 |
| P | -0.94 | | -2.50 |
| M | -0.99 | | -1.84 |
| R | -1.00 | | -4.20 |
| K | -1.00 | | -4.44 |

Chapter 4

The role of the G-protein C-terminus in defining the specificity of receptor coupling

4.1 Introduction

Heterotrimeric GTP binding proteins (G-proteins) mediate the transduction of a signal from cell surface receptors, characterised by seven transmembrane repeats, to secondary messenger producing enzymes or ion channels. G-proteins are composed of three subunits designated α , β , γ . The α -subunit is folded to form a guanine nucleotide binding pocket which can accommodate either GDP or GTP. G-proteins can exist in either active or inactive conformations, in the inactive conformation the α -subunit contains GDP in the nucleotide binding pocket and is in tight association with the $\beta\gamma$ subunits. Activation occurs through receptor-mediated guanine nucleotide exchange in the α -subunit, which results in a lower affinity for GDP in the nucleotide binding pocket. Consequently, the GDP molecule in the α -subunit nucleotide binding pocket is replaced by GTP, which is in the cell at a ten times higher molar concentration than GDP. This guanine nucleotide exchange event causes a conformational change in the β subunit binding site resulting in $\beta\gamma$ subunit dissociation. The GTP bound α -subunit and the $\beta\gamma$ subunits are then free to interact with downstream effector molecules. G-protein de-activation is regulated by the hydrolysis of GTP to GDP by the catalytic activity of the α -subunit. This results in a conformational change in the α -subunit, which allows $\beta\gamma$ subunit reassociation and so reversion to the inactive heterotrimeric state.

G-protein α -subunits couple rather selectively to membrane bound receptors and activate specific effector molecules. Receptor selectivity is thought to be determined by the structure of the α -subunit C-terminus, while determinants for effector recognition are more scattered. These recognition sites, in part, have been mapped by the use of chimeric G-proteins and further characterised by co-crystallography with effector molecules. Chimeric G-proteins are hybrids of the primary structures of two or more α -subunits. The signal output of a chimera is determined by the enzymatic and transducing properties of the relative α -subunit components. It has been demonstrated that short substitutions in the α -subunit C-terminal tail often preserve effector specificity, while switching receptor selectivity (Voyno-Yasentskaya *et al.*, 1994a; Komatsuzaki *et al.*, 1997; Conklin *et al.*, 1993). The substitution of as little as three amino acids in the C-terminal tail of $G_{q\alpha}$ for those of $G_{i\alpha 2}$ is sufficient to switch the selectivity of the chimera to G_i -coupled receptors (Conklin *et al.*, 1993). Such a substitution resulted in a switch of receptor coupling specificity but did not prevent the

chimera ($G_{q\alpha}/G_{i\alpha2}$) from interacting with its natural effector, phospholipase C. In similar studies, involving 5 amino acid substitutions in the C-terminal tail, Voynoyasentskaya *et al.* (1994a) have demonstrated that a $G_{13\alpha}/G_{z\alpha}$ chimera can activate $Na^+ - H^+$ exchange upon agonist stimulation of the dopamine D_2 receptor. In addition Komatsuzaki *et al.* (1997) have shown that upon agonist stimulation of the SSTR3 somatostatin receptor, the chimeras $G_{s\alpha}/G_{i\alpha(1/2)}$, $G_{s\alpha}/G_{14\alpha}$ and $G_{s\alpha}/G_{16\alpha}$ can activate the Gs effector adenylyl cyclase.

The efficacy of receptor/chimera interactions appears to be related to the length of the C-terminal substitution. Conklin *et al.* (1993) demonstrated that increasing the length of amino acid substitutions in the C-terminal tail of $G_{q\alpha}$ for the corresponding sequence of $G_{i\alpha2}$, first conferred an increase in receptor coupling efficacy, but then resulted in a gradual loss of receptor/Gi coupling. From this data it could be inferred that additional substitutions in the C-terminal tail of the α -subunit generated conformational changes which result in uncoupling of the receptor from the chimera. Conformational changes may also affect other functions associated with the α -subunit. Woon *et al.* (1989) demonstrated that substituting the C-terminal 38 amino acids of $G_{s\alpha}$ with those from $G_{i\alpha2}$, resulted in the constitutive activity of the $G_{s\alpha}$ moiety, as assayed by the production of the secondary messenger cAMP. From this it may be inferred that conformational changes in the α -subunit can result in either an increase in affinity for GTP or a reduction or loss of GTPase activity. In a similar observation Osawa *et al.* (1990) demonstrated that a chimera, created by Masters *et al.* (1988), featuring both the receptor and effector putative recognition sites in the C-terminal portion, is not only constitutively active, but also interacts with effectors cognate to the C-terminal portion of the chimera. This chimera was composed of the 60% N-terminal region of $G_{i\alpha2}$ and the 40% C-terminal region of $G_{s\alpha}$. Masters *et al.* (1988) have shown that while the above chimera is constitutively active, agonist stimulation of a co-transfected β_2 adrenergic receptor can further stimulate adenylyl cyclase in a dose-dependent manner. From these results it can be inferred that while limited substitutions in the C-terminus may result in conformational changes detrimental to chimera activity, more extensive chimerism may re-stabilise a functional conformation and therefore biological activity.

The hypothesis that the conformational structure of the α -subunit is altered by substitutions in the N or C-termini is supported by the findings of Osawa *et al.* (1990),

who used CTX catalysed ADP-ribosylation to assess the conformation of $G_{s\alpha}/G_{i\alpha2}$ chimeras. Cholera toxin was previously shown to catalyse ADP-ribosylation of the Arg 201 residue in $G_{s\alpha}$, but not in a $G_{s\alpha}$ chimera composed of the last 38 amino acids of $G_{i\alpha2}$. The insensitivity of this $G_{s\alpha}/G_{i\alpha2}$ chimera is thought to be related to a conformational change in the α -subunit, which prevents CTX access to the catalytic core in order to ADP-ribosylate Arg 201. Osawa *et al.* (1990) also demonstrated that by substituting 54 amino acids in the N-terminus of the chimera to those of $G_{i\alpha2}$ or reducing the number of substitutions in the C-terminus to 8 amino acids, restored sensitivity to CTX catalysed ADP-ribosylation. However, a restricted substitution of only 54 amino acids in the N-terminus of $G_{s\alpha}$ failed to confer sensitivity to CTX. From this data it would appear that substitutions in both the N and C-termini of a chimera are prone to confer conformational changes in the α -subunit with repercussions on the catalytic domain, the nature of which can be quite laborious to understand. The limited trypsin digestion assay is a useful method of determining the active conformation of α -subunits, as only α -subunits in the GTP bound form are resistant to full tryptic digestion. The presence of the non-hydrolysable GTP analogue, GTP γ S, in the nucleotide binding pocket stabilises the α -subunit in an active conformation in which many of the tryptic cleavage sites are inaccessible to the actions of trypsin. These conformations are different for both $G_{i\alpha2}$ and $G_{o\alpha}$, resulting in different tryptic cleavage patterns. The idea that substitutions in the N-terminus of the chimera can influence the conformation of the rest of the protein is supported by limited tryptic digestion data on $G_{i\alpha2}/G_{o\alpha}$ chimeras. Denker *et al.* (1995) demonstrated that the tryptic protection patterns produced by GTP γ S protected chimeras composed of the 60% N-terminal region of either $G_{i\alpha2}$ or $G_{o\alpha}$ and the 40% C-terminal region of the opposing α -subunit were the same as those of the N-terminal donor G-protein. From these findings it can be assumed that the sequence of the N-terminus is a major determinant on the active, GTP bound, conformation of $G_{i\alpha2}/G_{o\alpha}$ and $G_{o\alpha}/G_{i\alpha2}$ chimeras.

Komatsuzaki *et al.* (1997) systematically substituted the carboxyl-terminal 5 amino acids of $G_{s\alpha}$ for those of all other α -subunits and demonstrated how G-protein chimeras can be used to assay receptor specificity. However, a problem arises with this approach in respect of the use of secondary messenger production to measure receptor/chimera interactions. The experiment was flawed as the molecular origin of the secondary messenger measured could be due to direct activation of the effector by the

chimera or by $\beta\gamma$ subunits dissociated as a result of receptor activation of either the chimera or endogenous G-proteins cognate to the receptor being assayed. A more direct method of determining receptor/chimera interactions would have been to assay the rate of receptor catalysed guanine nucleotide exchange. However, in the experiment conducted by Komatsuzaki *et al.* (1997) this was not feasible due to the low guanine nucleotide exchange rate present in $G_{s\alpha}$.

Receptor-catalysed guanine nucleotide exchange is the basis of a number of assays, measuring either receptor-catalysed exchange of GDP for the non-hydrolysable GTP analogue [^{35}S]GTP γ S ([^{35}S]GTP γ S binding assay) or the hydrolysis of γ -phosphate labelled GTP to GDP (GTPase assay). In order to produce a chimera, whose activity could be measured by the above assays, the chimera would require a high guanine nucleotide exchange rate in combination with good coupling efficacy. Since the guanine nucleotide exchange rate varies between the four main classes of α -subunit (Osawa and Weiss, 1995), only the subclass of PTX sensitive α -subunits such as α_t , α_o and α_i display a nucleotide exchange rate high enough for use in the above assays.

Osawa and Weiss (1995) have previously demonstrated that $G_{i\alpha 1}$ not only possess a high guanine nucleotide exchange rate, but is also tolerant to mutations in the extreme C-terminus. The above findings suggest that $G_{i\alpha 1}$ would make an excellent candidate as the N-terminal donor for the G-protein chimera. It was therefore decided to substitute the last 6 amino acids of $G_{i\alpha 1}$ to those of $G_{s\alpha}$, $G_{q\alpha}$ and $G_{16\alpha}$. This decision was made based on the findings of Conklin *et al.* (1993) who demonstrated that optimal coupling of the $G_{q\alpha}/G_{i\alpha 2}$ chimera was obtained by substituting between 5 and 9 amino acids in the C-terminus, these chimeras also displayed activation of the effector molecule cognate to the N-terminal donor α -subunit. Larger C-terminal substitutions were not deemed suitable, as they have been shown to confer constitutive activity (Woon *et al.*, 1989) and to switch effector selectivity (Osawa *et al.*, 1990). The coupling efficacy of the chimeras was then assayed with a panel of GPCRs.

4.2 Results

4.2.1 Generation of $G_{i\alpha 1}$ chimeras by PCR mutagenesis

The last 6 amino acids of rat $G_{i\alpha 1}$ were mutated to those of $G_{q\alpha}$, $G_{s\alpha}$ and $G_{16\alpha}$ by PCR amplification with mutagenic primers. The pertussis toxin insensitive mutant Cys³⁵¹Asp $G_{i\alpha 1}$ in the pCDNA3 vector (Invitrogen) was used as the template. The PCR reaction used respectively 50 pmol of a common 5' forward primer, annealing on the $G_{i\alpha 1}$ DNA sequence, and one of three dedicated 3' reverse primers, encoding the hybrid $G_{i1\alpha}G_{x\alpha}$ sequence (Table 2.4). The reaction conditions were as in Table 2.5.

The amplicons were resolved on a 1%(w/v) agarose gel and stained with ethidium bromide as in Section 2.26. The expected 1065 bp band (Fig. 4.1) was excised from the gel, purified and ligated into *EcoRV* linearised, alkaline phosphatase treated pCDNA3. The ligation reaction was used to transform the DH5 α bacterial strain, which was subsequently grown on agar plates under ampicillin selection (Section 2.18).

Five clones were arbitrarily chosen from each of the transformations and cultured as in section (Section 2.18). Plasmid DNA was extracted from the cultures by small scale DNA preparation (Section 2.21) and digested with, the restriction enzyme, *EcoRI* (Section 2.23). The restriction digests were resolved on a 1% agarose gel and stained with ethidium bromide (Fig. 4.2). The presence of a DNA fragment with an apparent molecular weight of 1065 bp, corresponding to the expected size of the $G_{i1\alpha}G_{x\alpha}$ chimera, indicated recombinant clones, while a single fragment of 5.4 Kb indicated re-ligated vector.

A restriction digestion with the enzyme *NcoI* was used to screen the orientation of the insert in the pCDNA3 vector in DNA extracted from recombinant clones. The digested products were resolved and visualised on a 1% agarose gel stained with ethidium bromide (Fig. 4.3). The presence of a 1831bp DNA fragment indicated that the start codon of the $G_{i1\alpha}G_{x\alpha}$ chimera was next to the T7 promoter priming site in the pCDNA3 vector. This is the orientation required for transient expression of the subcloned cDNA in mammalian cells. The presence of two bands of 1145bp and 1026bp indicated that the start codon was distal to the T7 promoter site and therefore not in suitable orientation for transient expression. Only DNA samples containing the $G_{i1\alpha}G_{x\alpha}$ chimera in the correct orientation were chosen for sequencing.

The 3' end of the $G_{i1\alpha}G_{x\alpha}$ chimeras were sequenced using a primer annealing on the SP6 promoter priming site (Section 2.31) in the pCDNA3 vector. The sequencing profiles were translated to confirm the identities of the last 6 amino acids in the C-terminal tail of each of the chimeras.

4.2.2 Transient expression of $G_{i1\alpha}G_{x\alpha}$ chimeras with G-protein coupled receptors

The HEK 293T cell line was transiently co-transfected (Section 2.8) with 1.5 μ g GPCR cDNA in the pCDNA3 vector in combination with either 1.5 μ g $G_{i1\alpha}G_{x\alpha}$ chimera in pCDNA3, rat $G_{i\alpha1}$ in pCDNA3 or with empty pCDNA3 vector. The cells transfected with $G_{i1\alpha}G_{x\alpha}$ chimera were treated with 50 ng/ml of pertussis toxin 12 hours prior to harvesting (Section 2.7) in order to inactivate, by ADP-ribosylation, endogenously expressed PTX-sensitive G-proteins. At 72 hours after transfection the cells were harvested and membranes were prepared as in Section 2.9.

4.2.3 Immunodetection of $G_{i1\alpha}G_{x\alpha}$ chimera expression in HEK293T cells

The expression of the $G_{i1\alpha}G_{x\alpha}$ chimeras were detected by immunoblotting with antisera raised against an internal epitope of $G_{i\alpha1}$ (I1C) and with the C-terminus of $G_{i\alpha1}$ and $G_{i\alpha2}$ (SG1). HEK 293T cells were co-transfected with 1.5 μ g of $G_{i1\alpha}G_{x\alpha}$ chimera cDNA in pCDNA3 in combination with 1.5 μ g of empty pCDNA3 vector to standardise the total amount of DNA to 3 μ g. Following membrane preparation samples (25 μ g) were denatured in Laemmli buffer and resolved in duplicate on 8% SDS-PAGE gels (Section 2.11). The resolved proteins were subsequently western blotted onto nitrocellulose membranes (Section 2.12), which were probed for $G_{i\alpha1}$ and $G_{i\alpha1/2}$ immunoreactivity with the antisera I1C and SG1 respectively (Table 2.1). It can be seen from Figure 4.4(b) that chimera expression was not detectable by antiserum SG1, raised against the C-terminus of $G_{i\alpha1}$ and $G_{i\alpha2}$. Membrane samples probed with this antiserum displayed the same immunogenicity as the mock-transfected cells (lane C-). A membrane sample of rat brain cortex (25 μ g), rich in $G_{i\alpha1}$, was used as a positive control. In contrast, chimera expression was detected on the duplicate blot by the antiserum raised against the internal epitope of $G_{i\alpha1}$ (I1C). It can also be seen from Figure 4.4(a) that chimeras were over-expressed at levels higher than endogenously expressed $G_{i\alpha1}$ in the mock transfected cells (lane C-).

4.2.4 Immunological characterisation of $G_{i1\alpha}G_{x\alpha}$ chimeras expressed in HEK293T cells

The $G_{i1\alpha}G_{x\alpha}$ chimeras were characterised by immunodetection with antisera raised against an internal epitope in the chimera in combination with immunodetection of the C-terminal donor sequence. HEK 293T cells were transiently transfected with either $G_{i1\alpha}G_{x\alpha}$ chimera cDNA or with empty pCDNA3 vector. Membranes were prepared and samples (25 μ g) were resolved in triplicate on 10% SDS-PAGE gels as in Section 2.11. The resolved proteins were western blotted onto nitrocellulose and probed with antiserum directed against the internal epitope of $G_{i\alpha1}$ (I1C) in combination with antisera raised against the C-terminal residues of $G_{s\alpha}$ (CS3), $G_{q\alpha}$ (CQ5) and $G_{16\alpha}$ (SB1) as detailed in Table 2.1. It can be seen from Figures 4.5(a), 4.5(b) and 4.5(c) that each of the chimeras displayed immunoreactivity with antiserum raised against an internal epitope of $G_{i\alpha1}$ (I1C) as well as with antisera directed against the donor C-terminal residues. In each instance the immunoreactive bands co-migrated and were in excess of endogenously expressed α -subunits.

4.2.5 Characterisation of chimeric $G_{i1\alpha}G_{x\alpha}$ receptor specificity by agonist stimulated [35 S]GTP γ S binding

The receptor specificity of the $G_{i1\alpha}G_{x\alpha}$ chimeras was assayed by the efficacy of the chimera to bind the non-hydrolysable GTP analogue, [35 S]GTP γ S, in response to agonist stimulation of a panel of GPCRs. The receptors chosen for this study are as follows – muscarinic M1, P_2Y_2 , P_2Y_4 , P_2Y_6 , β_2 adrenergic, V_2 vasopressin, adenosine A2a, serotonin 5HT5a, serotonin 5HT5b, TRH, α 2A adrenergic, oxytocin, glutamate mGluR1a and glutamate mGluR5a. HEK 293T cells were co-transfected with one of the above receptors in combination with $G_{i1\alpha}G_{x\alpha}$ chimera, $G_{i\alpha1}$ or empty pCDNA3 vector. Cells co-transfected with the $G_{i1\alpha}G_{x\alpha}$ chimeras were treated with PTX to remove the endogenous G-protein signalling component 12 hours prior to harvesting. Agonist stimulated [35 S]GTP γ S binding was assayed in membrane preparations from each of the co-transfections as detailed in Section 2.15.2, the concentration response data was subsequently plotted using the mathematical package robosage. It can be seen that agonist stimulation of the P_2Y_4 (Fig 4.6) and TRH (Fig. 4.7) receptors catalysed guanine nucleotide exchange in the $G_{i1\alpha}G_{q\alpha}$ chimera. Similarly, agonist stimulation of the β_2 adrenergic (Fig. 4.8) and V_2 vasopressin (Fig. 4.9) receptors stimulated

[³⁵S]GTPγS binding in the G_{i1α}G_{sα} chimera. Despite the diversity of receptors assayed, none were seen to catalyse [³⁵S]GTPγS binding in the G_{i1α}G_{16α} chimera.

4.2.6 Evidence for the expression of receptors incapable of activating G_{i1α}G_{xα} chimeras

Some of the receptors assayed failed to induce agonist stimulated [³⁵S]GTPγS binding in any of the chimeras. In these cases receptor expression was demonstrated in membrane preparations by agonist stimulated [³⁵S]GTPγS binding. In **Figure 4.10** it can be seen that agonist stimulation of the mGluR5a receptor in the G_{iα1} wild type co-transfection resulted in [³⁵S]GTPγS binding, while agonist stimulation of the chimera co-transfections or receptor alone failed to elicit such a response. Similar observations were seen for the mGluR1a, α₂A adrenoceptor, 5HT_{5a}, adenosine A_{2A}, P₂Y₂ and P₂Y₆ receptors (data not shown)

4.2.7 The expression of G_{i1α}G_{xα} chimeras co-transfected into HEK293T cells in combination with a bank of G-protein coupled receptors

The same membrane preparation as that used to generate [³⁵S]GTPγS binding data was used to assay the relative expression levels of the co-transfected G-proteins. A sample of membrane preparation was denatured in Laemmli buffer and resolved on a 10% SDS-PAGE gel (**Section 2.11**). The resolved proteins were subsequently western blotted onto nitrocellulose membrane (**Section 2.12**) which was probed for immunoreactivity against an internal epitope of G_{iα1} using the antiserum IIC as described in **Table 2.1**. It can be seen from **Figures 4.11** and **4.12** that all G-protein cDNAs co-transfected with the β₂ adrenergic, V₂ vasopressin and the mGluR5a glutamate receptors were overexpressed at levels higher than endogenously expressed G_{iα1} in cells transfected with receptor alone. It was also noted that although there was some variation in expression between chimeras co-transfected in combination with the different receptors, there was no variation in expression within a receptor co-transfection. The same observation was seen for the rest of the receptor/chimera co-transfections.

4.2.8 Demonstration of the capacity of the non-activated $G_{i1\alpha}G_{16\alpha}$ chimera to bind $GTP\gamma S$

The capacity of the non-activated $G_{i1\alpha}G_{16\alpha}$ chimera to exchange nucleotides was confirmed by limited trypsin digestion in both the presence and absence of $GTP\gamma S$. In this assay the exchange of GDP for GTP in the chimeric α -subunit stabilises a conformation which is resistant to complete tryptic digestion. Limited tryptic digestion of $GTP\gamma S$ bound G-protein α -subunits results in the production of a 38kDa fragment, which is resistant to further tryptic cleavage. The protected fragment can be detected by the $G_{i\alpha1}$ specific antiserum IIC. The immunoblot in **Figure 4.13** clearly indicates that the non-activated chimera $G_{i1\alpha}G_{16\alpha}$ is equally capable of binding $GTP\gamma S$ as the other chimeras. It should also be noted that the tryptic protection pattern for each of the chimeras was the same as that of wild type $G_{i\alpha1}$.

4.2.9 The efficacy of the $G_{i1\alpha}G_{s\alpha}$ chimera to bind [^{35}S]GTP γS in response to maximal agonist stimulation of the human IP prostanoid receptor

The capacity of the $G_{i1\alpha}G_{s\alpha}$ chimera to bind the non-hydrolysable GTP analogue, [^{35}S]GTP γS , in response to maximal iloprost stimulation of the FlagTM-tagged human IP prostanoid receptor was assayed as described in **Section 2.15.2**. Clone 13 cells, HEK 293 cells stably expressing the Flag-tagged human IP prostanoid receptor at 2.5 pmol/mg, were transfected to express the $G_{i1\alpha}G_{s\alpha}$ chimera or mock transfected with empty pCDNA3 vector. Membranes were prepared from each of the transfections along with parental HEK293 cells and samples were used to assay iloprost stimulated (10 μM) [^{35}S]GTP γS binding. It can be seen from **Figure 4.14** that agonist stimulated [^{35}S]GTP γS binding was much greater in membrane preparations from the $G_{i1\alpha}G_{s\alpha}$ transfection than HEK293 cells or clone 13 cells alone. It should also be noted that a degree of agonist stimulated [^{35}S]GTP γS binding was detected in membrane preparations from clone 13 cells, but not in parental HEK293 membranes

4.2.10 The $G_{i1\alpha}G_{s\alpha}$ chimera is resistant to post translational modification by the bacterial toxins PTX and CTX

Agonist stimulated [^{35}S]GTP γS binding was used to assay $G_{i1\alpha}G_{s\alpha}$ chimera sensitivity to post translational modification by both PTX and CTX. Clone 13 cells were transfected with $G_{i\alpha1}$, $G_{s\alpha}$, $G_{i1\alpha}G_{s\alpha}$ or empty pCDNA3 vector. In parallel the cells were

treated with vehicle or a combination of 200 ng/ml CTX and 25 µg/ml PTX to remove the $G_{s\alpha}$ and $G_{i\alpha}$ signalling component 16 hours prior to harvesting. Membrane preparations from each of the transfections were used to assay both basal and iloprost (1 µM) stimulated [35 S]GTPγS binding. It can be seen from **Figure 4.15** that [35 S]GTPγS binding in membrane preparations from the $G_{i1\alpha}G_{s\alpha}$ transfection were least affected by bacterial toxin treatment. It should also be noted that the loss of [35 S]GTPγS binding in $G_{i1\alpha}G_{s\alpha}$ transfected cells was equivalent to that seen in mock transfected cells.

4.2.11 The relative efficacy of the $G_{i1\alpha}G_{s\alpha}$ chimera to catalyse the hydrolysis of [32 P]GTP in response to iloprost stimulation of the IP prostanoid receptor

Clone 13 cells, stably transfected with the FlagTM-tagged human IP prostanoid receptor, were used to assay the efficacy of agonist stimulated [32 P]GTP hydrolysis by the $G_{i1\alpha}G_{s\alpha}$ chimera in relation to both endogenous and overexpressed $G_{i\alpha1}$ and $G_{s\alpha}$. Clone 13 cells were either mock transfected or transiently transfected to express wild type $G_{i\alpha1}$, $G_{s\alpha}$ or $G_{i1\alpha}G_{s\alpha}$ chimera. Membranes were prepared from each of the transfections, along with parental HEK293 cells as a negative control, were used to assay maximal agonist stimulated (conc) [32 P]GTP hydrolysis. It can be seen in **Figure 4.16** that the rate of [32 P]GTP hydrolysis in membranes prepared from the $G_{i1\alpha}G_{s\alpha}$ transfections was significantly greater than that seen in any of the other transfections. It was also noted that the overexpression of either $G_{i\alpha1}$ or $G_{s\alpha}$ gave little or no increase in the rate of [32 P]GTP hydrolysis over mock-transfected cells. Membranes from parental HEK293 cells failed to respond to agonist stimulation, indicating that all effects were mediated through the stably expressed human IP-prostanoid receptor.

4.2.12 The relative efficacy of agonist stimulated [32 P]GTP hydrolysis by the $G_{i1\alpha}G_{s\alpha}$ chimera is not related to the expression level

The expression levels of $G_{i\alpha1}$ and $G_{i1\alpha}G_{s\alpha}$ chimera were compared in the same membrane preparations as used to generate the high affinity [32 P]GTP hydrolysis data. Membrane samples (10µg) from clone 13 cells transfected with $G_{i\alpha1}$, $G_{s\alpha}$, or $G_{i1\alpha}G_{s\alpha}$ were denatured in Laemmli buffer and resolved on a 10% SDS-PAGE gel (**Section 2.11**). The resolved proteins were subsequently western blotted onto nitrocellulose membrane (**Section 2.12**) which was then probed for immunoreactivity to an internal

epitope of $G_{i\alpha 1}$ with the antiserum IIC as described in **Table 2.1**. It can be seen from **Figure 4.17** that both $G_{i\alpha 1}$ and $G_{11\alpha}G_{s\alpha}$ were expressed at roughly equivalent levels. It was also noted that both G-proteins were detected at levels substantially higher than those of endogenously expressed $G_{i\alpha 1}$.

4.3 Discussion

Conservative G-protein chimeras, containing only a few amino acid substitutions in the C-terminus, can be used as adapter molecules allowing agonist stimulated activation of an effector molecule not normally cognate to the receptor (Voyno-Yasentskaya *et al.*, 1994a; Conklin *et al.*, 1993; Komatsuzaki *et al.*, 1997). This property has been exploited to investigate the selectivity of receptor/G-protein interactions (Komatsuzaki *et al.*, 1997). However, a problem with this approach lies in the use of effector molecule activation to assay receptor/chimera interactions as well as receptor/G-protein selectivity. It is often laborious to prove that increases in secondary messenger are solely the result of chimera/effector interactions, as frequently the activity of the effector can integrate the signal output from $\beta\gamma$ subunits dissociated from either the chimera or from endogenous G-proteins cognate to the receptor. A less ambiguous method of determining receptor/chimera interactions would be to assay receptor catalysed guanine nucleotide exchange, either by agonist stimulated binding of [^{35}S]GTP γS or by agonist stimulated hydrolysis of [^{32}P]GTP. At present these assays are best suited to characterising receptors coupled G-proteins that are the target of PTX. PTX-sensitive G-proteins, such as Gi , Go and Gt , display a substantially higher guanine nucleotide exchange rate than other classes of α -subunit. Therefore, non PTX-sensitive α -subunits are not suited to the above assays as they give a poor signal to noise ratio. To summarise, it would be extremely advantageous to develop a universal ligand screening strategy, in which receptor stimulation by any newly synthesised molecule, with agonistic interest, could be characterised by the output of a single non-ambiguous biochemical assay.

Although 5 amino acids substitutions in the extreme carboxyl terminus of an α -subunit are sufficient to switch receptor selectivity, as various studies have shown (Voyno-Yasentskaya *et al.*, 1994a; Komatsuzaki *et al.*, 1997; Conklin *et al.*, 1993), this switch has no discernible effect on effector selectivity. Conklin *et al.* (1993) have shown that chimera efficacy can be increased by substituting up to 9 amino acids in the C-terminus of $\text{G}_{\text{q}\alpha}$, but substitutions beyond this point are incompatible in terms of receptor coupling. Based on these findings, in order to produce a generic ligand screening strategy whose output could be measured by guanine nucleotide exchange based biochemical assays, it was decided to substitute the 6 extreme C-terminal amino acids of $\text{G}_{\text{ia}1}$ with those of $\text{G}_{\text{q}\alpha}$, G_{sa} and $\text{G}_{16\alpha}$. Each of the chimeras was then

immunologically characterised with antisera raised against an internal epitope of $G_{i\alpha 1}$ (Fig. 4.4(a)) or the extreme C-termini of $G_{i\alpha 1}/G_{i\alpha 2}$ (Fig. 4.4(b)), $G_{s\alpha}$ (Fig. 4.5(b)), $G_{q\alpha}$ (Fig. 4.5(a)) and $G_{16\alpha}$ (Fig. 4.5(c)). It can be seen from Figure 4.4(a) that all of the chimeras displayed immunoreactivity to the antiserum I1C directed against the internal epitope of $G_{i\alpha 1}$, but failed to display immunoreactivity to antiserum SG1, directed to the extreme C-terminus of $G_{i\alpha 1}$ and $G_{i\alpha 2}$. As expected, the immunoreactive bands co-migrated with wild type $G_{i\alpha 1}$, which determined the final molecular mass of the chimeras. It could be argued that the loss of SG1 immunoreactivity was due to the production of a truncated $G_{i\alpha 1}$ G-protein, as opposed to the generation of a chimera. While the co-migration of the chimera with the wild type would suggest that both polypeptides are of the same molecular mass, a more comprehensive method of demonstrating full length translation would be to immunologically characterise the sequence of the extreme C-terminus. Using this strategy, each of the chimeras was probed for immunoreactivity against both the extreme C-terminus of the chimera and the internal epitope of $G_{i\alpha 1}$. It can be seen from Figures 4.5(a), 4.5(b) and 4.5(c) that each of the chimeras displayed immunoreactivity to antisera directed against the internal epitope of $G_{i\alpha 1}$ and the extreme C-terminus of either $G_{s\alpha}$, $G_{q\alpha}$ or $G_{16\alpha}$. It should also be noted that the immunoreactive bands, in each instance, co-migrated with wild type $G_{i\alpha 1}$. From this data it can be concluded that each of the chimera cDNA constructs expressed a full-length chimera composed of the first 349 residues of $G_{i\alpha 1}$ and the last 6 residues of $G_{s\alpha}$, $G_{q\alpha}$ or $G_{16\alpha}$.

The chimeras were then assayed for receptor catalysed [35 S]GTP γ S binding, in order to assess functional coupling. It can be seen from Figures 4.6 and 4.7 that agonist stimulation of the P_2Y_4 , TRH and oxytocin receptors resulted in enhanced [35 S]GTP γ S binding of the $G_{i1\alpha}G_{q\alpha}$ chimera. Similarly, agonist stimulation of the V_2 vasopressin (Fig. 4.9) and the β_2 adrenergic (Fig. 4.8) receptor catalysed guanine nucleotide exchange in the $G_{i1\alpha}G_{s\alpha}$ chimera. However, none of the receptors assayed in this study stimulated GDP/[35 S]GTP γ S exchange in the $G_{i1\alpha}G_{16\alpha}$ chimera. All of the above receptors displayed a high level of selectivity in coupling, in so far as the receptor was not seen to stimulate guanine nucleotide exchange in any of the other G-proteins assayed.

Previous studies have shown that the level of G-protein expression can influence [35 S]GTP γ S binding (Wise *et al.*, 1997a), it could therefore be argued that the apparent selectivity in receptor/chimera coupling was due to a variation in chimera expression resulting from different transfection efficiencies between groups. To determine if this was the case the relative expression levels of the chimeras were determined by immunodetection with antiserum 11C, directed against the internal epitope of G $_{i\alpha 1}$, in the same membrane preparation as that used to generate the [35 S]GTP γ S binding data. It can be seen from **Figure 4.11** that in the β_2 adrenergic and V $_2$ vasopressin receptor co-transfections the expression levels of the chimeras were equivalent to those of transfected G $_{i\alpha 1}$ and significantly higher than endogenous G $_{i\alpha 1}$, which is not detectable. It should also be noted that although there was variation in chimera expression between the transfected receptors, there was no variation in expression within a particular receptor co-transfection. Similar observations were made for all other receptor co-transfections in this study. From these findings it can be concluded that the apparent specificity of chimera activation was not due to a variation in chimera expression.

Many of the receptors assayed in this study failed to activate any of the chimeras. It was previously demonstrated that all of the chimeras were expressed at equivalent levels, it could therefore be assumed that the lack of chimera activation was not due to a variation in chimera expression. Based on the study by Wise *et al.*, (1997a), who demonstrated that variations in receptor expression can influence [35 S]GTP γ S binding, it would have been prudent to assay receptor expression to determine if this was negatively affecting [35 S]GTP γ S output. Unfortunately, the lack of suitable radiolabelled ligands restricted this readily available simple control; although in a few cases receptor expression was indirectly demonstrated by agonist enhanced binding of [35 S]GTP γ S in the wild type G $_{i\alpha 1}$ co-transfection. It can be seen, for instance, in **Figure 4.10** that agonist stimulation of the mGluR5a receptor promoted [35 S]GTP γ S binding in wild type G $_{i\alpha 1}$, but not in any of the other chimeras. The same membrane preparation was used to assess the relative expression levels of G $_{i\alpha 1}$ and each of the chimeras. From **Figure 4.12**, it can be seen that all of the G-proteins were expressed at equivalent levels. It can therefore be assumed that the selectivity of receptor coupling was not due to a variation of chimera expression. Throughout the study the chimera expression levels were remarkably consistent within a receptor co-transfection. From this finding, it could be assumed that the levels of receptor expression would be equally consistent

between a set of co-transfections. Therefore, agonist enhanced binding of [35 S]GTP γ S in the G $_{i\alpha 1}$ co-transfection would lead us to believe that the receptor was expressed at equivalent levels throughout the co-transfections. It may therefore be concluded, in such cases, that the lack of chimera activation is solely due to the receptor incompatibility for the chimera.

Osawa and Weiss (1995) and Garcia *et al.* (1995) have demonstrated that mutations in the C-terminus of G $_{\alpha}$ can affect the capacity of nucleotide binding, as well as receptor coupling. From these observations, it could be argued that the absence of agonist stimulated [35 S]GTP γ S binding in the G $_{i1\alpha}$ G $_{16\alpha}$ chimera could be associated with either a loss or diminished ability of the chimeric α -subunit to exchange nucleotides. To determine whether this was the case, the G $_{i1\alpha}$ G $_{16\alpha}$ chimera was assayed by limited trypsin digestion in the presence and absence of the non-hydrolysable GTP analogue, GTP γ S. From **Figure 4.13** it can be seen that the G $_{i1\alpha}$ G $_{16\alpha}$ chimera is capable of exchanging nucleotides as indicated by the immunodetectable products of incomplete tryptic digestion. The G $_{i1\alpha}$ G $_{sz\alpha}$ and G $_{i1\alpha}$ G $_{qa\alpha}$ chimeras, known to undergo activation by the GPCRs assayed in this study, were also shown to be able to exchange nucleotides. (**Fig. 4.13**). From this data it can be concluded that the apparent inability of the G $_{i1\alpha}$ G $_{16\alpha}$ chimera to be activated by any of the receptors assayed, is not due to a loss of guanine nucleotide exchange. In **Figure 4.13** it was also noted that all of the G $_{i1\alpha}$ G $_{nx\alpha}$ chimeras produced the same tryptic protection pattern as wild type G $_{i\alpha 1}$. Denker *et al.* (1995) have shown that the N-terminal regions of Gi/Go and Go/Gi chimeras can influence the conformation of the peptide, which in turn influences the tryptic protection pattern. The findings of this study corroborate those of Denker *et al.* (1995) and it may be assumed that since all three chimeras produced the same tryptic protection pattern that they all share a common conformational structure.

Osawa *et al.* (1990) have previously demonstrated that the loss of bacterial toxin ADP-ribosylation sites in the G-protein chimera can confer insensitivity to bacterial toxin treatment. While both G $_{i\alpha 1}$ and G $_{sz\alpha}$ are susceptible to ADP ribosylation by the respective bacterial toxins PTX and CTX, the ADP-ribosylation sites lie in different regions on the α -subunit primary sequence. PTX ribosylates the cysteine 351 residue, which in the GDP complexed α -subunit faces the core of the protein, while CTX acts upon the arginine 201 residue located in the guanine nucleotide binding pocket. Post-

translational modification of either of these G-proteins results in a loss of receptor catalysed guanine nucleotide exchange. Fortunately, $G_{i1\alpha}G_{s\alpha}$ chimera is composed of the first 349 residues of $G_{i\alpha1}$ and the last 6 residues of $G_{s\alpha}$ and therefore contains neither of the above ADP-ribosylation sites. The loss of these ADP-ribosylation sites should result in insensitivity to both PTX and CTX. To determine whether this was the case, the $G_{i1\alpha}G_{s\alpha}$ chimera was characterised in detail along with the human IP prostanoid receptor. The ability of the $G_{i1\alpha}G_{s\alpha}$ chimera to be activated by the human IP prostanoid receptor was demonstrated by both agonist enhanced binding of [35 S]GTP γ S and high affinity GTPase activity. It can be seen from **Figure 4.14** that co-expression of the $G_{i1\alpha}G_{s\alpha}$ chimera and the IP prostanoid receptor greatly enhanced agonist stimulated [35 S]GTP γ S binding compared to expression of receptor alone. No increase in [35 S]GTP γ S binding was observed in membranes prepared from parental HEK293 cells. From these findings, it can be concluded that agonist stimulation of the $G_{i1\alpha}G_{s\alpha}$ chimera was mediated by the IP prostanoid receptor. The rate of GTP hydrolysis should be higher in the $G_{i1\alpha}G_{s\alpha}$ chimera than in the $G_{s\alpha}$ subunit as the rate is controlled by the G_i moiety. To investigate this hypothesis $G_{i\alpha1}$, $G_{s\alpha}$ and the $G_{i1\alpha}G_{s\alpha}$ chimera were co-expressed with the IP prostanoid receptor and assayed for agonist enhanced GTPase activity. It can be seen from **Figure 4.16** that agonist stimulated GTPase activity was approximately 3 times higher in membranes containing the $G_{i1\alpha}G_{s\alpha}$ chimera than in membranes overexpressing either $G_{i\alpha1}$ or $G_{s\alpha}$. No increase in high affinity GTPase activity was seen in membranes prepared from parental HEK 293 cells. From this data it can be concluded that the 6 amino acid substitution in the C-terminus of the $G_{i1\alpha}G_{s\alpha}$ chimera is sufficient to switch the receptor specificity of $G_{i\alpha1}$ while retaining the G-protein's high guanine nucleotide exchange rate.

To investigate whether the $G_{i1\alpha}G_{s\alpha}$ chimera was a substrate for either PTX or CTX, agonist stimulated [35 S]GTP γ S binding was assayed in membranes prepared from cells treated *in vivo* with either a combination of PTX and CTX or vehicle. It can be seen from the data in **Figure 4.15** that treatment with bacterial toxins abolished agonist stimulated [35 S]GTP γ S binding in membranes prepared from cells transfected to express $G_{s\alpha}$, $G_{i\alpha1}$ or receptor alone, but did not abolish agonist enhanced binding in the $G_{i1\alpha}G_{s\alpha}$ chimera. The small loss of [35 S]GTP γ S binding in the $G_{i1\alpha}G_{s\alpha}$ chimera upon toxin treatment was equal to the reduction seen in membranes prepared from cells transfected

with receptor alone. From this data it may be concluded that the $G_{i1\alpha}G_{s\alpha}$ chimera is insensitive to both PTX and CTX and that the observed drop in [^{35}S]GTP γ S binding was due to the ADP-ribosylation of endogenously expressed $G_{s\alpha}$. Since all of the above data was generated in membranes prepared from a cell line stably expressing the human IP prostanoid receptor, the level of receptor expression is identical between transfections. It can therefore be concluded, that the [^{35}S]GTP γ S binding data was not affected by variations in receptor expression. However, the G-proteins were transiently transfected and so may have been expressed at different levels in each of the transfections. In order to evaluate what influence the level of α -subunit expression may have had on agonist stimulated [^{35}S]GTP γ S binding, the same membrane preparation was used to determine the relative expression levels of each of the transiently transfected α -subunits by immunoblotting with antisera raised against $G_{i\alpha1}$ and $G_{s\alpha}$. From **Figure 4.17** it can be seen that while $G_{i\alpha1}$ expression is equivalent to that of the $G_{i1\alpha}G_{s\alpha}$ chimera, the expression of $G_{i\alpha1}$ was four times greater than that of $G_{s\alpha}$. When the α -subunit expression levels are related to the [^{35}S]GTP γ S data in **Figure 4.15** it can be seen that while $G_{i\alpha1}$ expression may have been four times greater than that of $G_{s\alpha}$, over expression of either α -subunit had no effect upon agonist stimulated [^{35}S]GTP γ S binding. In contrast, transient transfection of the $G_{i1\alpha}G_{s\alpha}$ chimera greatly elevated agonist stimulated [^{35}S]GTP γ S binding even though it was expressed at equivalent levels to $G_{i\alpha1}$. From this data, it can be concluded that the observed insensitivity of $G_{i1\alpha}G_{s\alpha}$ to both PTX and CTX was not due to a variation in receptor or G-protein expression.

The data generated herein has shown that while a 6 amino acid substitution in the extreme C-terminus of $G_{i\alpha1}$ for $G_{s\alpha}$ or $G_{q\alpha}$ is sufficient to alter receptor selectivity, it has no measurable effect on the conformation of the α -subunit or its capacity to exchange nucleotide. It can also be concluded that the C-terminus does not appear to be the only determinant for receptor interaction, in fact, many of the receptors assayed in this study failed to activate any of the chimeras. In this respect, receptor recognition could have been influenced by the $G_{i\alpha1}$ like conformation of the α -subunit or by the absence of recognition sequences located more N-terminally in the C-terminal donor α -subunit.

The G-protein chimeras created in this study have several advantages over previous chimeras that used secondary messenger production to assay receptor/chimera coupling and agonist potency. A problem arises with this approach in respect that the output of secondary messenger assays is prone to being affected by free $\beta\gamma$ subunits dissociated through receptor catalysed activation of the chimera or endogenous G-proteins cognate to the receptor being assayed. A more direct way of determining receptor/chimera interaction is through guanine nucleotide exchange-based assay. However, due to the low guanine nucleotide exchange rate in previously generated chimeras, this has not been a feasible approach. In contrast, the high catalytic activity conferred by the G_i moiety in the $G_{i1\alpha}G_{s\alpha}$ chimeras allows receptor/chimera coupling to be measured by guanine nucleotide-based assays. Therefore, the chimeras generated in this study provide a more accurate means of assaying potentially new agonists and antagonists than was previously available. In addition, the loss of the PTX and the CTX ADP-ribosylation sites in the $G_{i1\alpha}G_{s\alpha}$ chimera has conferred insensitivity to these bacterial toxins, while retaining the high guanine nucleotide exchange rate associated with PTX sensitive G-proteins. This property has proved to be particularly useful in selectively evaluating the signal output from the $G_{i1\alpha}G_{s\alpha}$ chimera when PTX and CTX are used to uncouple endogenously expressed $G_{s\alpha}$ subunits and remove noise generated by PTX sensitive G-proteins. In conclusion, the chimeras generated herein, provide a useful tool to screen ligands for certain receptors not normally linked to PTX-sensitive G-proteins.

Bacterial toxin treatment reduced agonist stimulated [^{35}S]GTP γ S binding in the $G_{i1\alpha}G_{s\alpha}$ chimera when compared to non-treated control. This observation would suggest that receptor/chimera interactions are not saturated and therefore coupling efficacy may be improved. Future work could involve identifying further receptor chimera recognition sites by systematically substituting regions of the α -subunits in order to increase receptor coupling efficacy.

Figure 4.1 PCR amplification of $G_{i1\alpha}G_{x\alpha}$ chimeras of rat $G_{i\alpha1}$

$G_{i1\alpha}G_{x\alpha}$ chimeras were generated by PCR amplification of the PTX insensitive $C^{351}D$ $G_{i\alpha1}$ mutant with one of three hybrid primers. The PCR reaction products from amplification with primer 12 (lane 2), primer 13 (lane 3) and primer 14 (lane 4) were resolved on a 1%(w/v) agarose gel (50 μ l/lane) and stained with ethidium bromide. The 1065bp DNA fragment (lanes 2-4) corresponds to the expected size of the amplified $G_{i1\alpha}G_{x\alpha}$ chimera. A 1Kb ladder provided size markers (lane 1).

Figure 4.1

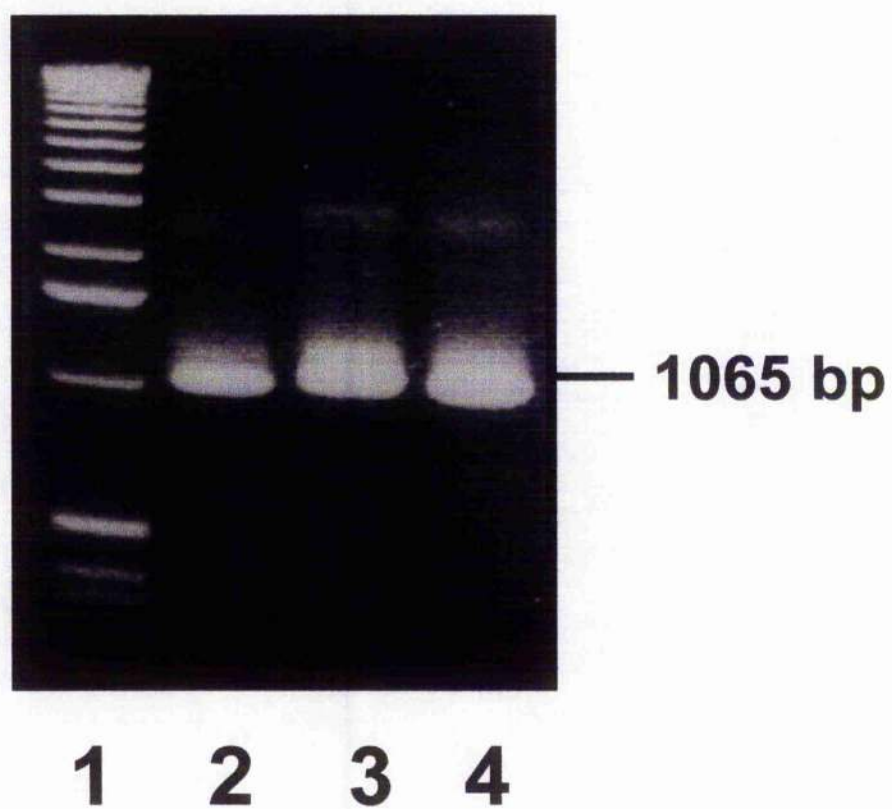


Figure 4.2 Analysis of clones transformed with $G_{i1\alpha}G_{x\alpha}$ chimera cDNAs in the pCDNA3 vector

DNA was isolated from clones thought to be transformed with $G_{i1\alpha}G_{x\alpha}$ chimera cDNA in the pCDNA3 vector. Samples (2 μ l) were digested with the restriction enzyme *Eco*R1 (Section 2.23) and resolved on a 1% (w/v) agarose gel. The 1065bp band (lanes 2, 3 and 5) corresponds to the expected size of the cleaved insert and indicated recombinant clones. The single 5.4 Kb DNA fragment (lanes 4 and 6) corresponds to the expected size of the pCDNA3 vector and indicated clones containing empty vector.

Figure 4.2



Figure 4.3 Analysis of $G_{i1\alpha}G_{x\alpha}$ chimera cDNA orientation in the pCDNA3 vector by restriction digestion

Plasmid DNA was isolated from clones transformed with $G_{i1\alpha}G_{x\alpha}$ chimera cDNA in the pCDNA3 vector. Samples (2 μ l) were restriction digested with the enzyme *NcoI* (Section 2.23) and resolved on a 1%(w/v) agarose gel. When the start codon of the $G_{i\alpha1}$ mutant was next to the T7 promoter priming site in the pCDNA3 vector, the plasmid was cleaved to yield fragments of 340, 260, 735, 1831 and 3299bp (lanes 5, 6, 7 and 8). Cleavage of the vector with the insert in the opposite orientation, with the start codon distal to the T7 priming site, yielded fragments of 260, 735, 1026, 1145 and 3299bp (lanes 2, 3 and 4). A 1Kb ladder provided size markers (lane 1).

Figure 4.3

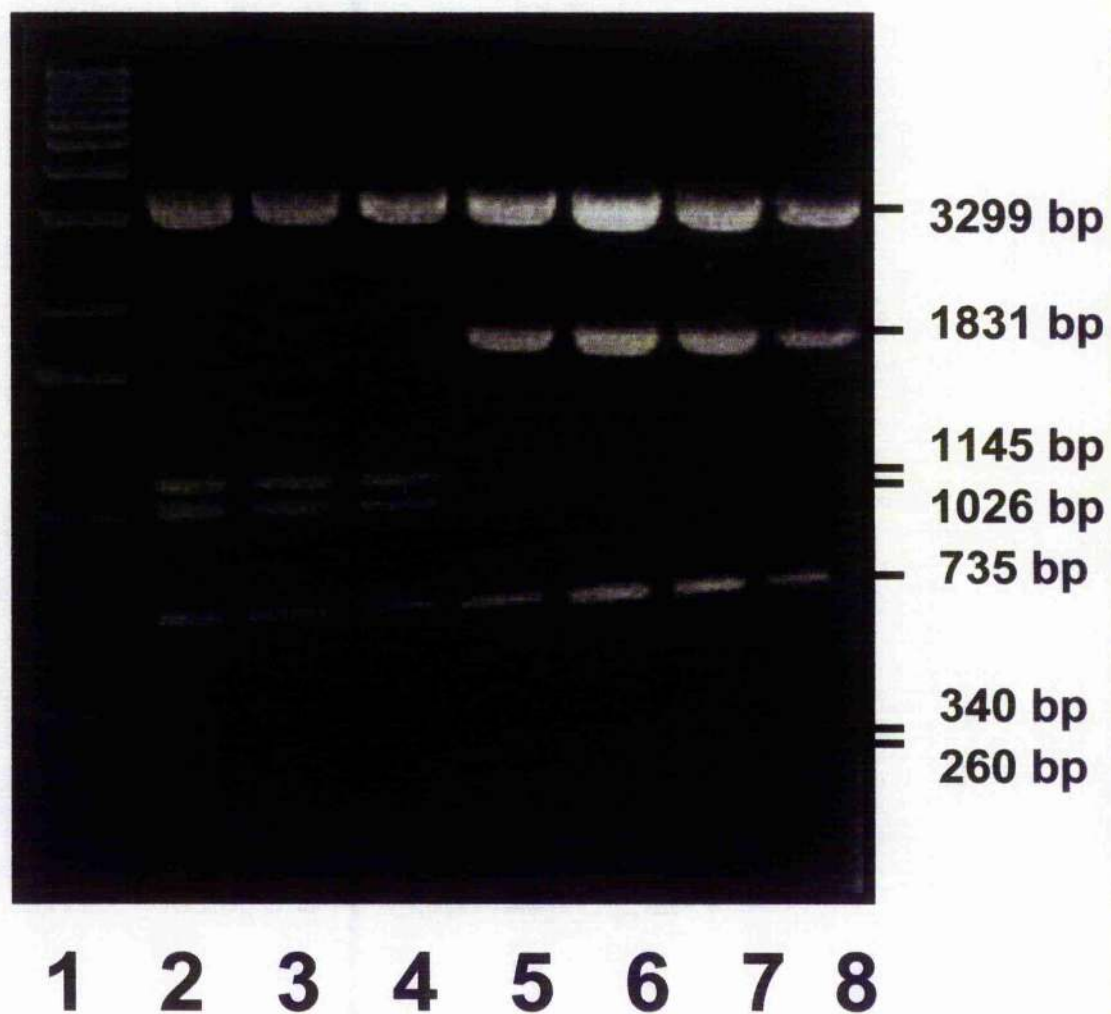


Figure 4.4 Immunological characterisation of the $G_{i1\alpha}G_{x\alpha}$ chimeras

Membranes were prepared from HEK293T cells transiently transfected to express $G_{i1\alpha}G_{s\alpha}$ (lane 1), $G_{i1\alpha}G_{q\alpha}$ (lane 2) or $G_{i1\alpha}G_{16\alpha}$ (lane 3). Samples of membrane preparation (25 μ g) were resolved in duplicate on 10% SDS-PAGE gels, and immunoblotted with antiserum I1C (internal epitope of $G_{i\alpha1}$)(**Fig. 4.4(a)**) or antiserum SG1 (C-terminus of $G_{i\alpha1}$ and $G_{i\alpha2}$)(**Fig. 4.4(b)**). Membranes prepared from mock transfected HEK293T cells (lane 4) were used to compare endogenous $G_{i\alpha1}$ expression. A membrane sample (25 μ g) of rat brain cortex, rich in $G_{i\alpha1}$, was used as a positive control (lane 5).

Figure 4.4

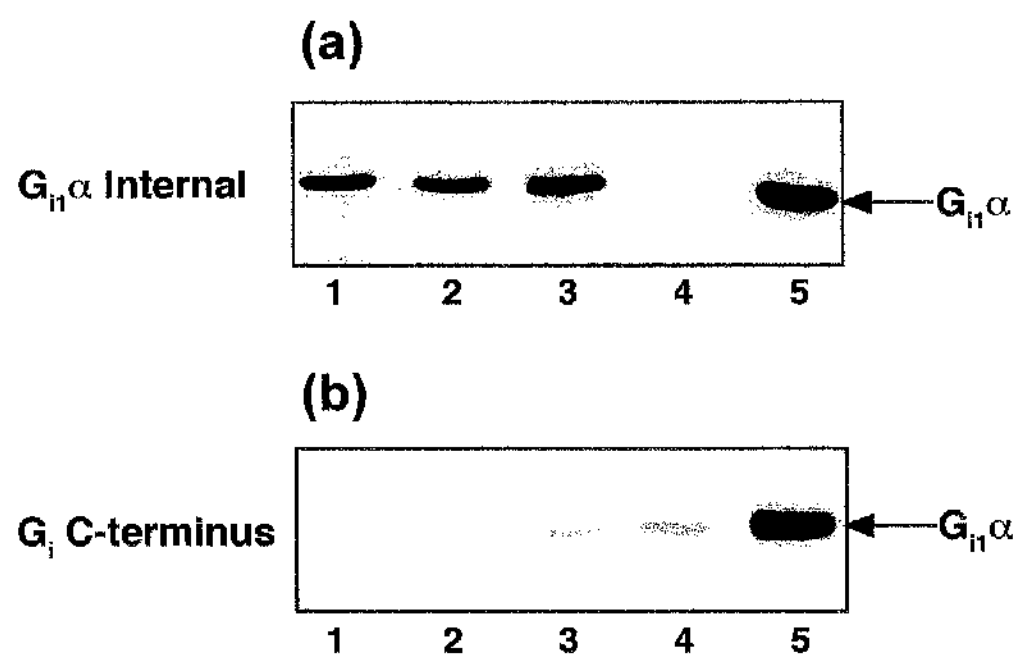


Figure 4.5 Immunological characterisation of the C-terminal sequence of the $G_{i1\alpha}G_{x\alpha}$ chimeras

HEK293T cells were either mock transfected (lanes 1) or transiently transfected to express $G_{i1\alpha}G_{q\alpha}$ (lanes 2), $G_{i1\alpha}G_{s\alpha}$ (lanes 3) or $G_{i1\alpha}G_{16\alpha}$ (lanes 4). Membrane samples (25 μ g) prepared from these cells were resolved by SDS-PAGE and immunoblotted with antiserum I1C (internal epitope of $G_{i\alpha1}$) (left panels A, B and C) in combination with antiserum CQ1 (C-terminus of $G_{q\alpha}$) (right panel A), CS1 (C-terminus of $G_{s\alpha}$) (right panel B) and SB1 (C-terminus of $G_{16\alpha}$) (right panel C).

Figure 4.5

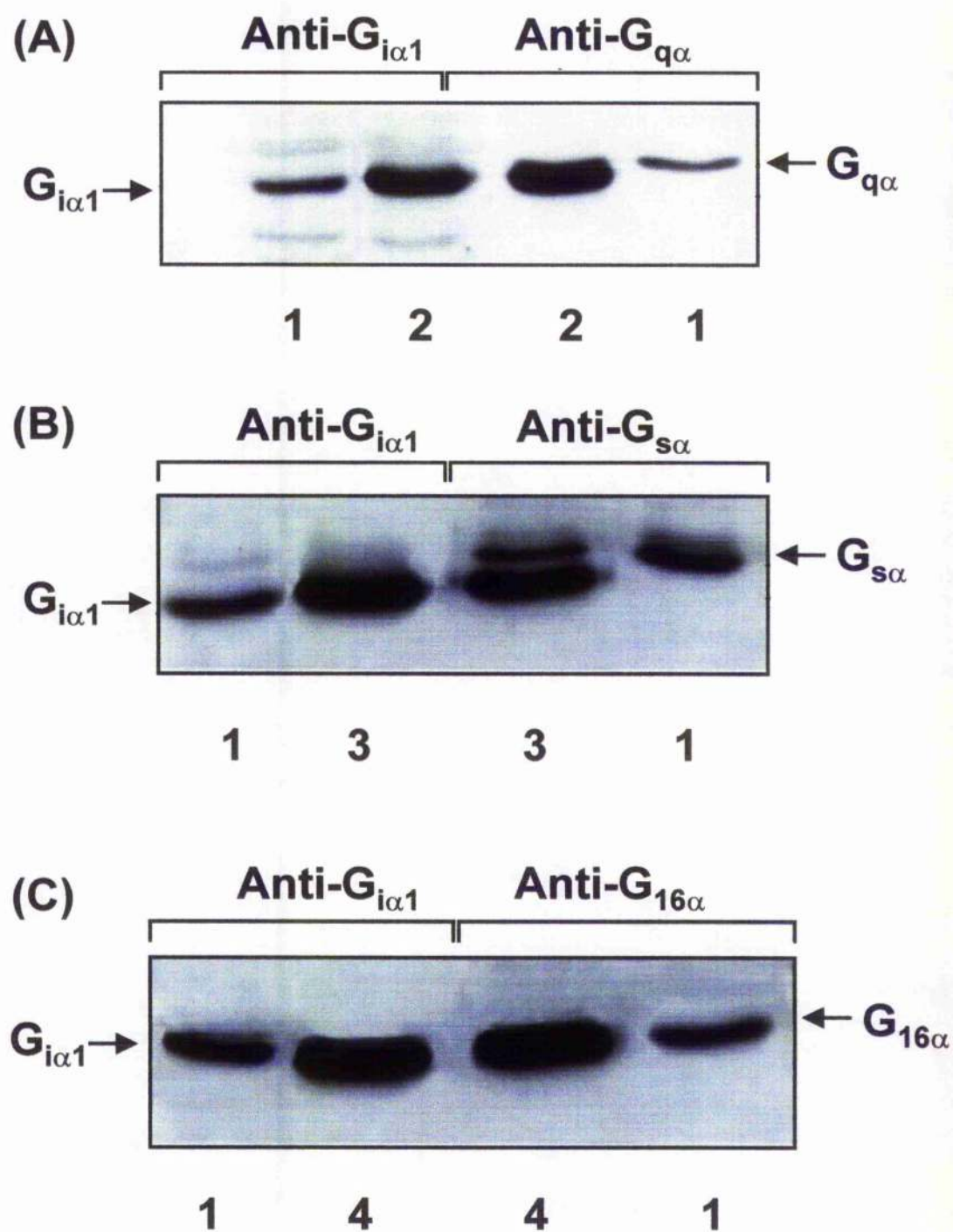


Figure 4.6 The relative coupling efficiency of the $G_{i1\alpha}G_{x\alpha}$ chimeras with the P_2Y_4 purinergic receptor upon stimulation with UTP

Membranes were prepared from HEK293T cells co-transfected to express the P_2Y_4 purinergic receptor in combination with $G_{i\alpha1}$ wild type (2), $G_{i1\alpha}G_{q\alpha}$ (3), $G_{i1\alpha}G_{16\alpha}$ (4) or receptor alone (1). All transfections, with the exception of those including the wild type G-protein or receptor alone, were pre-treated with 50 ng/ml PTX 12 hours prior to harvesting and membrane preparation. UTP stimulated [35 S]GTP γ S binding was assayed in the membrane preparations as detailed in **Section 2.15.2**. Each data point represents the average of duplicate measurements of a single experiment. The data shown represents UTP stimulated [35 S]GTP γ S binding above basal and was taken from a representative experiment.

Figure 4.6

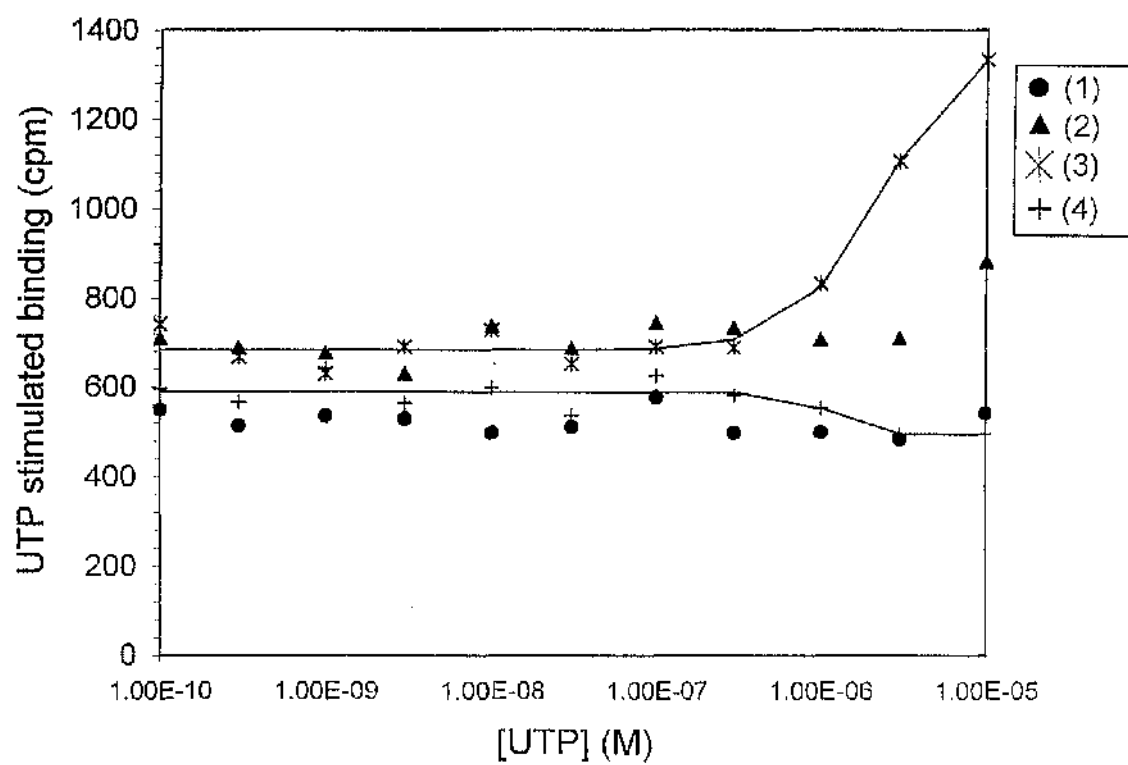


Figure 4.7 The relative coupling efficiency of the $G_{i1\alpha}G_{x\alpha}$ chimeras with the TRH receptor upon stimulation with TRH

Membranes were prepared from HEK293T cells co-transfected to express the rat TRH receptor in combination with $G_{i\alpha1}$ wild type (2), $G_{i1\alpha}G_{q\alpha}$ (3), $G_{i1\alpha}G_{16\alpha}$ (4) or receptor alone (1). All transfections, with the exception of those including the wild type G-protein or receptor alone, were pre-treated with 50 ng/ml PTX 12 hours prior to harvesting and membrane preparation. TRH stimulated [35 S]GTP γ S binding was assayed in the membrane preparations as detailed in Section 2.15.2. Each data point represents the average of duplicate measurements of a single experiment. The data shown represents TRH stimulated [35 S]GTP γ S binding above basal and was taken from a representative experiment.

Figure 4.7

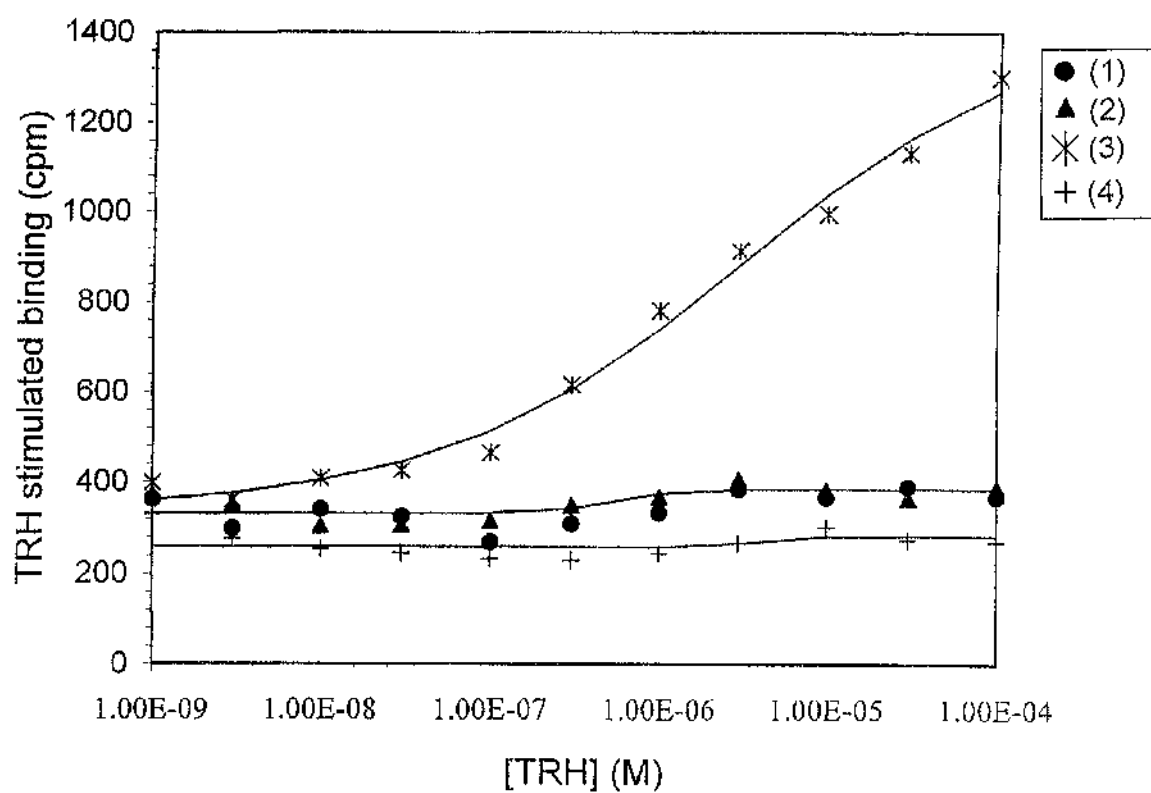


Figure 4.8 The relative coupling efficiency of the $G_{i1\alpha}G_{x\alpha}$ chimeras with the β_2 adrenergic receptor upon stimulation with isoprenaline

Membranes were prepared from HEK293T cells co-transfected to express the β_2 adrenergic receptor in combination with $G_{i\alpha1}$ wild type (2), $G_{i1\alpha}G_{i16\alpha}$ (3), $G_{i1\alpha}G_{s\alpha}$ (4), or receptor alone (1). All transfections, with the exception of those including the wild type G-protein or receptor alone, were pre-treated with 50 ng/ml PTX 12 hours prior to harvesting and membrane preparation. Isoprenaline stimulated [35 S]GTP γ S binding was assayed in the membrane preparations as detailed in **Section 2.15.2**. Each data point represents the average of duplicate measurements of a single experiment. The data shown represents Isoprenaline stimulated [35 S]GTP γ S binding above basal and was taken from a representative experiment.

Figure 4.8

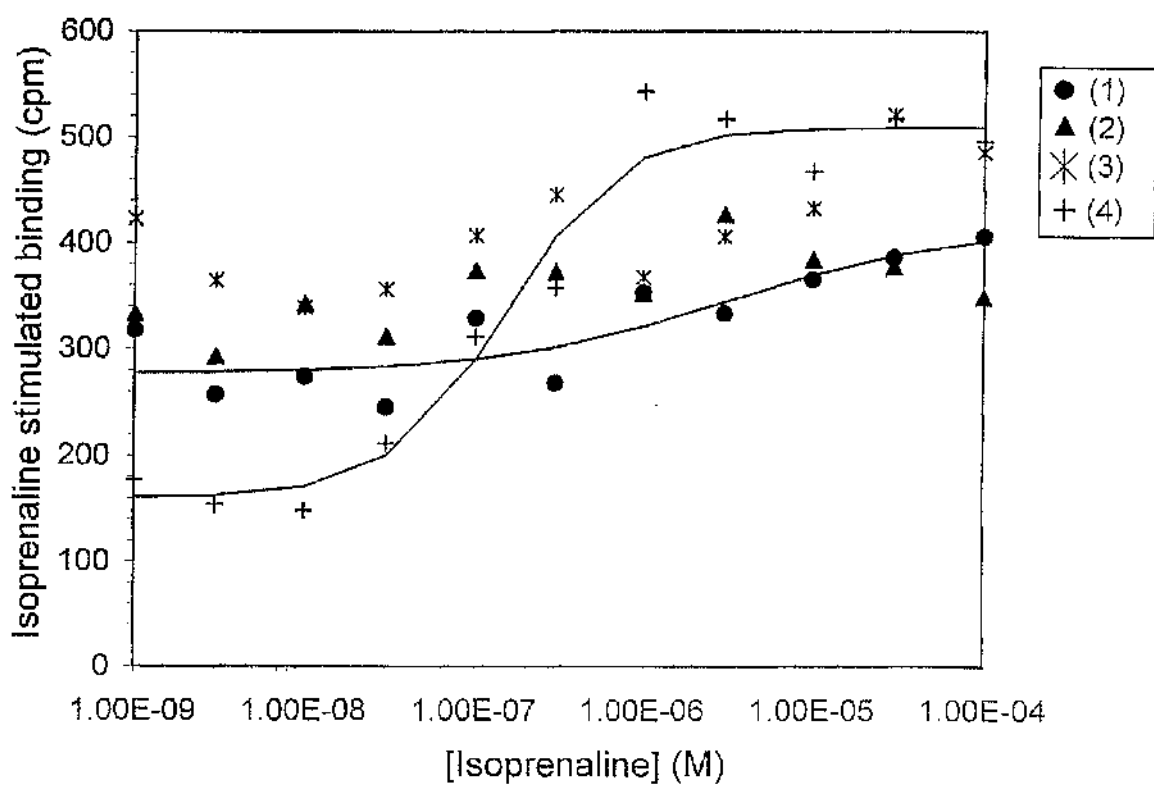


Figure 4.9 The relative coupling efficiency of the $G_{i1\alpha}G_{x\alpha}$ chimeras with the V_2 vasopressin receptor upon stimulation with vasopressin

Membranes were prepared from HEK293T cells co-transfected to express the V_2 vasopressin receptor in combination with $G_{i\alpha1}$ wild type (2), $G_{i1\alpha}G_{16\alpha}$ (3), $G_{i1\alpha}G_{s\alpha}$ (4) or receptor alone (1). All transfections, with the exception of those including the wild type G-protein or receptor alone, were pre-treated with 50 ng/ml PTX 12 hours prior to harvesting and membrane preparation. Vasopressin stimulated [35 S]GTP γ S binding was assayed in the membrane preparations as detailed in **Section 2.15.2**. Each data point represents the average of duplicate measurements of a single experiment. The data shown represents Vasopressin stimulated [35 S]GTP γ S binding above basal and was taken from a representative experiment.

Figure 4.9

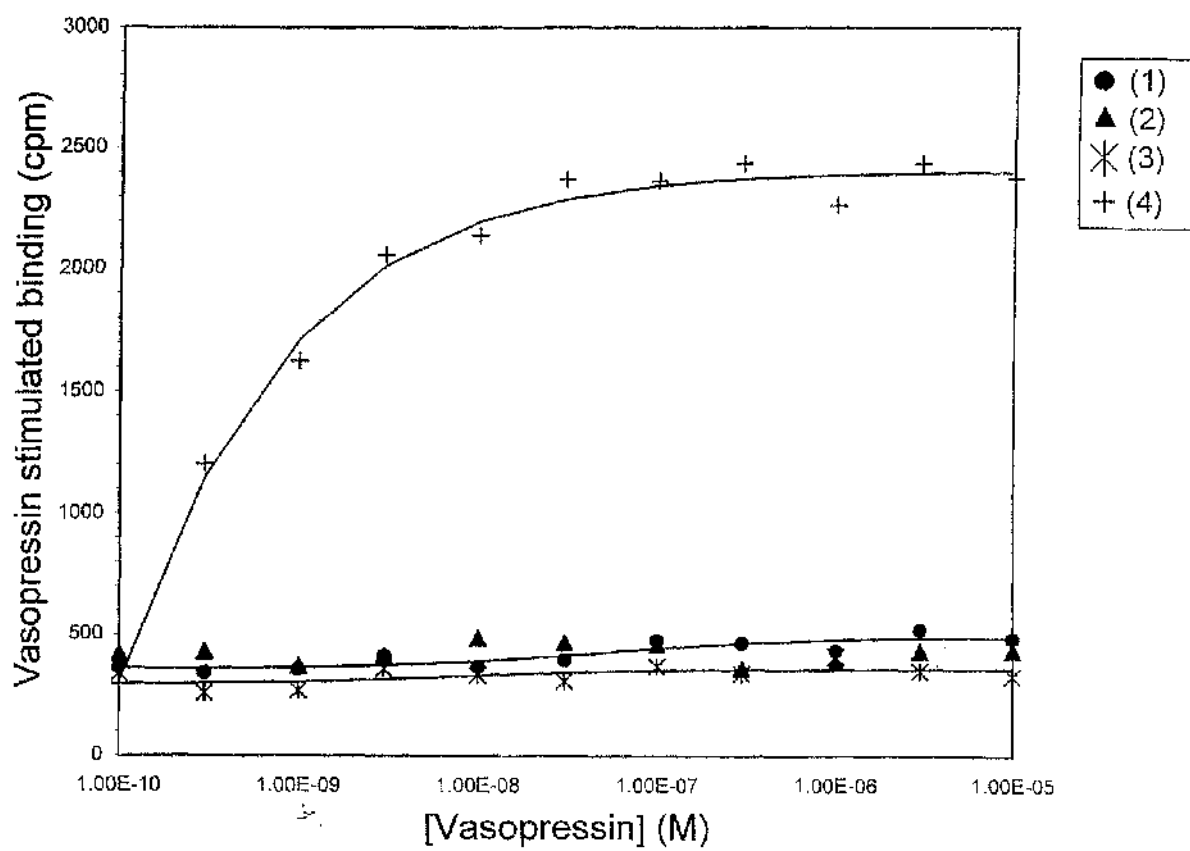


Figure 4.10 The relative coupling efficiency of the $G_{i1\alpha}$ - $G_{x\alpha}$ chimeras with the glutamate mGluR5a receptor upon stimulation with glutamate

Membranes were prepared from HEK293T cells co-transfected to express the glutamate mGluR5a receptor in combination with $G_{i\alpha1}$ wild type (2), $G_{i1\alpha}$ - $G_{q\alpha}$ (3), $G_{i1\alpha}$ - $G_{16\alpha}$ (4) or receptor alone (1). All transfections, with the exception of those including the wild type G-protein or receptor alone, were pre-treated with 50 ng/ml PTX 12 hours prior to harvesting and membrane preparation. Glutamate stimulated [35 S]GTP γ S binding was assayed in the membrane preparations as detailed in **Section 2.15.2**. Each data point represents the average of duplicate measurements of a single experiment. The data shown represents glutamate stimulated [35 S]GTP γ S binding above basal and was taken from a representative experiment.

Figure 4.10

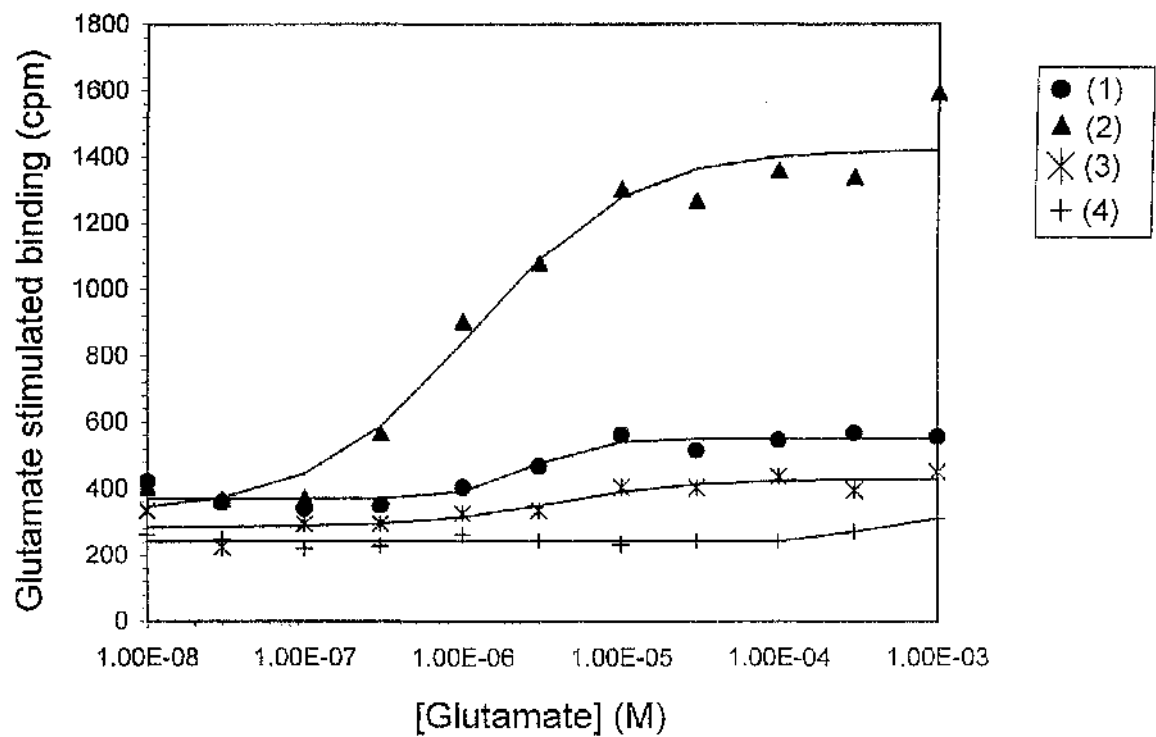


Figure 4.11 The expression of $G_{i1\alpha}G_{x\alpha}$ chimeras transiently co-transfected into HEK293T cells in combination with the β_2 adrenergic and the V_2 vasopressin receptors

Membranes were isolated from HEK293T cells co-transfected with the β_2 adrenergic receptor (lanes 2-4) or the V_2 vasopressin receptor (lanes 5-7) in combination with either $G_{i\alpha 1}$ wild type (lanes 2 and 5), $G_{i1\alpha}G_{i6\alpha}$ (lanes 3 and 6) or $G_{i1\alpha}G_{s\alpha}$ (lanes 4 and 7). Samples (25 μ g) were resolved on a 10% SDS-PAGE gel and immunoblotted with antiserum I1C (Table 2.1). Membranes prepared from mock transfected HEK293T cells (lane 1) were used to compare endogenous $G_{i\alpha 1}$ expression.

Figure 4.11

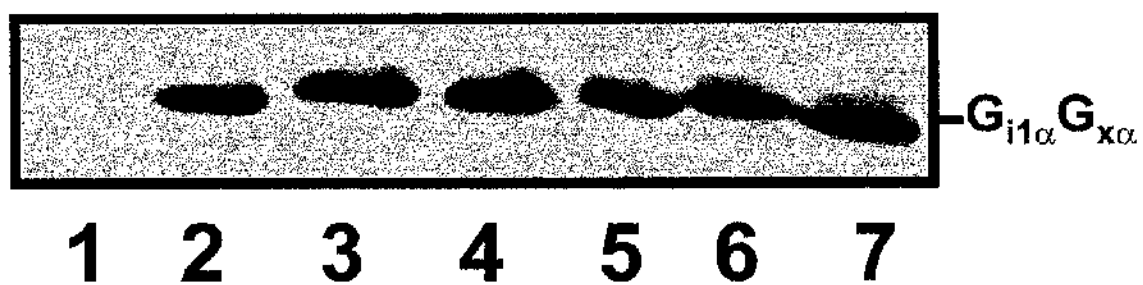


Figure 4.12 The expression of $G_{i1\alpha}$ - $G_{x\alpha}$ chimeras transiently co-transfected into HEK293T cells in combination with the glutamate mGluR5a receptor

Membranes were prepared from HEK293T cells co-transfected with the glutamate mGluR5a receptor in combination with either $G_{i\alpha1}$ wild type (lane 2), $G_{i1\alpha}G_{q\alpha}$ (lane 3) or $G_{i1\alpha}G_{16\alpha}$ (lane 4). Samples (25 μ g) were resolved on a 10% SDS-PAGE gel and immunoblotted with antiserum 11C, raised against the internal epitope of $G_{i\alpha1}$. Membranes prepared from HEK293T cells transfected with receptor alone (lane 1) were used to compare endogenous $G_{i\alpha1}$ expression.

Figure 4.12

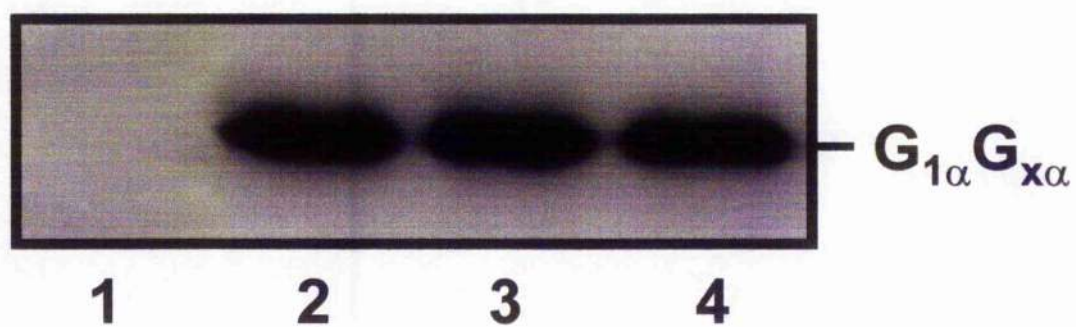


Figure 4.13 An immunoblot demonstrating the capacity of $G_{i1\alpha}G_{x\alpha}$ chimeras to exchange nucleotides

Membranes were prepared from HEK293T cells transfected with either empty pCDNA3 vector (lanes 1-3), wild type $G_{i\alpha1}$ (lanes 13-15) or one of the following chimeras- $G_{i1\alpha}G_{s\alpha}$ (lanes 4-6), $G_{i1\alpha}G_{q\alpha}$ (lanes 7-9) and $G_{i1\alpha}G_{16\alpha}$ (lanes 10-12). The capacity of the G-proteins to exchange nucleotides was assayed by limited tryptic digestion (Section 2.17) of a membrane samples (100 μ g) in the absence (lanes 3, 6, 9, 12 and 15) or presence of GTP γ S (lanes 2, 5, 8, 11 and 14). Untreated membrane samples were used as a negative control (lanes 1, 4, 7, 10 and 13). Membrane samples were then resolved on a 10% SDS-PAGE gel and probed for $G_{i\alpha1}$ immunoreactivity with the antiserum I1C (Table 2.1). The figure shown is a representative immunoblot.

Figure 4.13

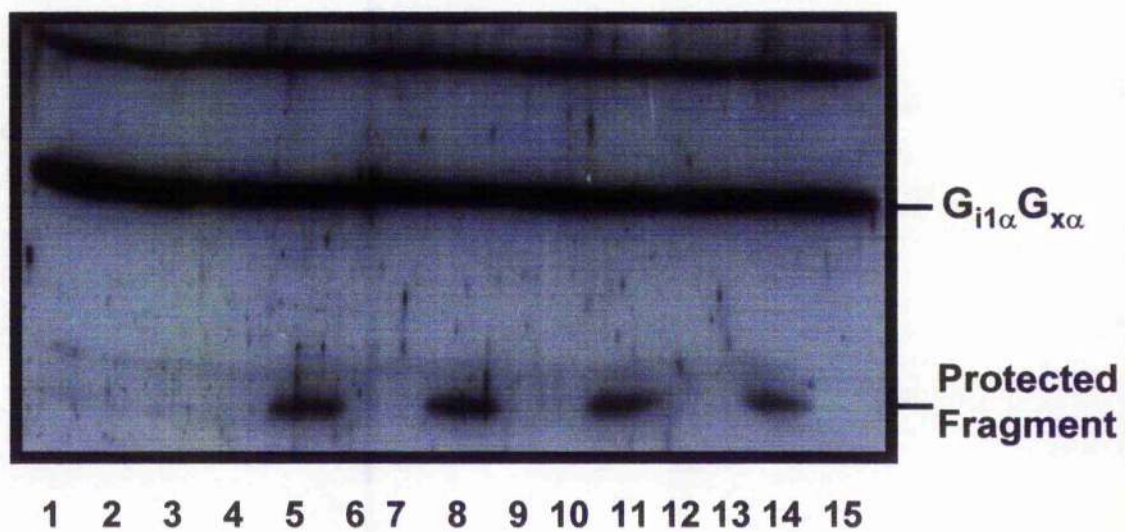


Figure 4.14 The expression of $G_{i1\alpha}G_{s\alpha}$ enhances iloprost stimulated [^{35}S]GTP γ S binding in cells stably expressing the human IP prostanoid receptor

Iloprost stimulated (10 μM) [^{35}S]GTP γ S binding was assayed in membranes prepared from parental HEK293T cells (1), clone 13 cells, stably expressing the FlagTM-tagged human IP prostanoid receptor (2), and clone 13 cells transiently transfected to express $G_{i1\alpha}G_{s\alpha}$ (3). The results shown represent non-specific (dots), basal (lines) and 10 μM iloprost stimulated (filled) binding of [^{35}S]GTP γ S in a typical experiment. All data points are means \pm S.D. of triplicate measurements from a single experiment.

Figure 4.14

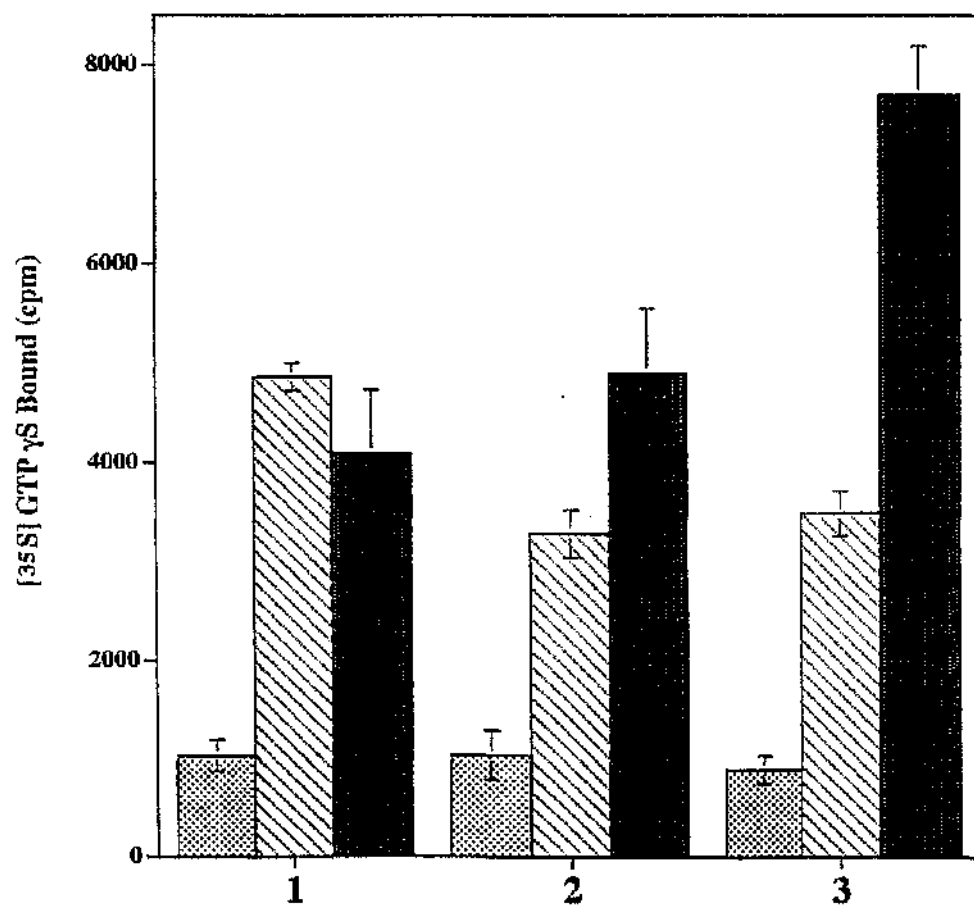


Figure 4.15 The effects of cholera and pertussis toxin on iloprost stimulated [35 S]GTP γ S binding

Iloprost stimulated (1 μ M) [35 S]GTP γ S binding was assayed in membranes isolated from clone 13 cells mock transfected (1) or transiently transfected to express G $_{i\alpha 1}$ (2), G $_{s\alpha}$ (3) or G $_{i1\alpha}$ G $_{s\alpha}$ (4). The cells were treated with either a combination of PTX (25ng/ml) and CTX (200ng/ml) (lines) or vehicle (dotted bars), 16 hours prior to harvesting. The results shown represent iloprost stimulation above basal and are the means \pm S.D. of triplicate measurements from a single experiment. The data represents a typical experiment.

Figure 4.15

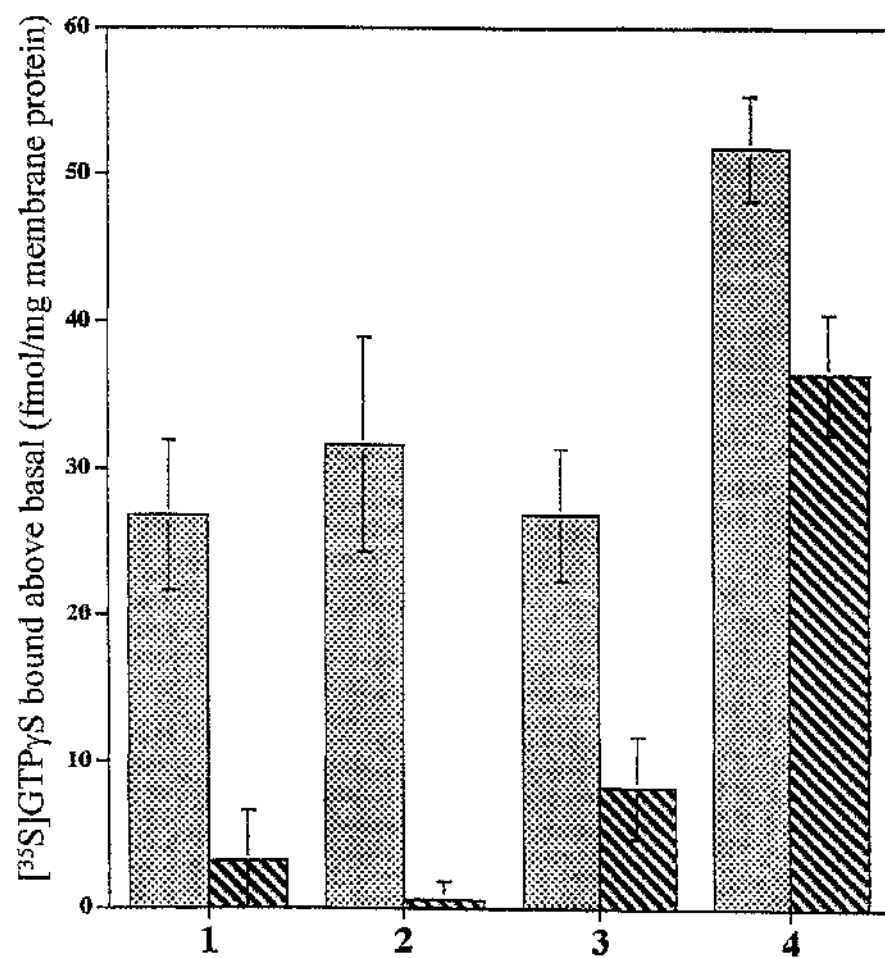


Figure 4.16 The high affinity GTPase activity of the $G_{i1\alpha}G_{s\alpha}$ chimera upon iloprost stimulation of the iP prostanoid receptor

The coupling efficacy of the $G_{i1\alpha}G_{s\alpha}$ chimera was assayed by iloprost stimulated (1 μ M) high affinity GTPase activity in membranes prepared from clone 13 cells mock transfected (1) or transiently transfected to express either $G_{s\alpha}$ (2), $G_{i\alpha1}$ (3) or $G_{i1\alpha}G_{s\alpha}$ (4). Parental HEK293 cells, transiently transfected to express $G_{i1\alpha}G_{s\alpha}$ (5), were used as a negative control. The results shown represent iloprost stimulation above basal and are the means \pm S.D. of triplicate measurements from a single experiment. The data represents a typical experiment.

Figure 4.16

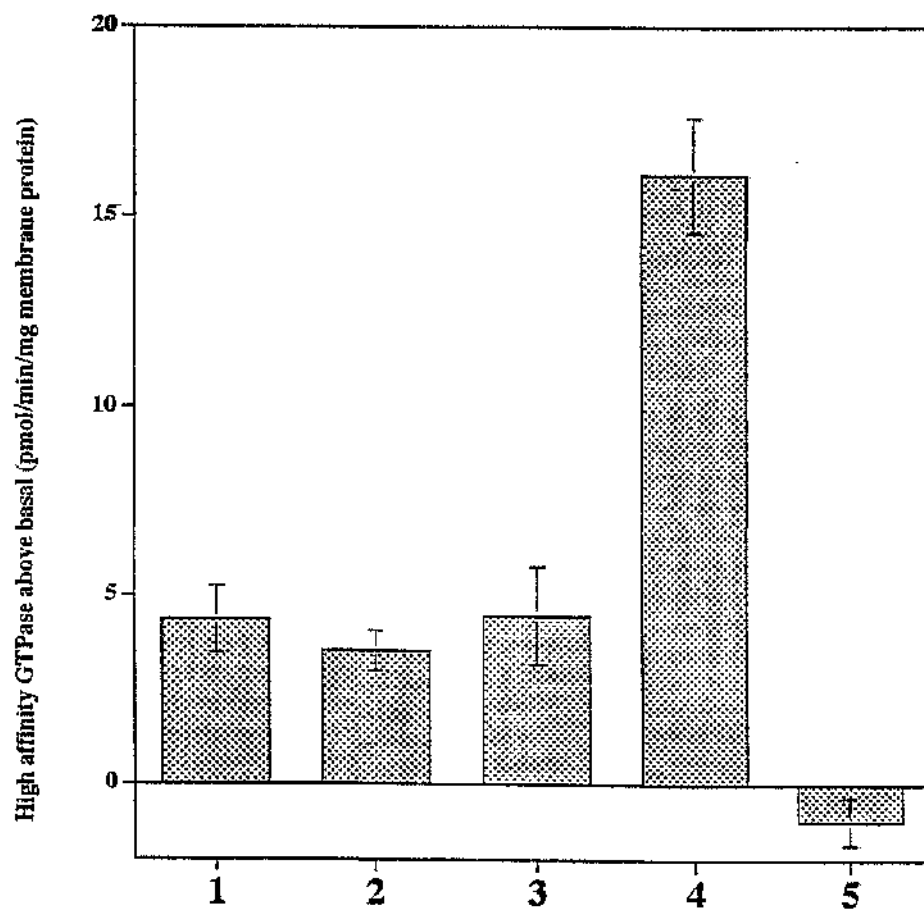
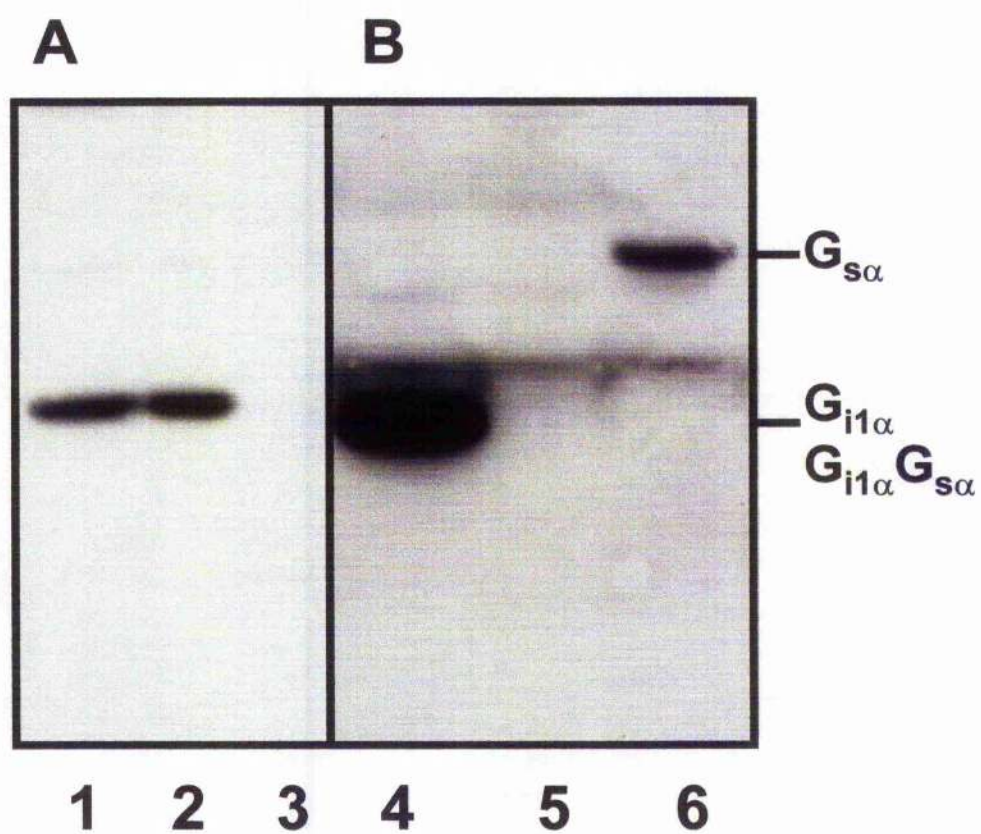


Figure 4.17 The relative expression of the $G_{i1\alpha}G_{s\alpha}$ chimera transiently transfected into clone 13 cells

Membranes were prepared from clone 13 cells transiently transfected to express $G_{i\alpha1}$ (lanes 1 and 5), $G_{i1\alpha}G_{s\alpha}$ (lanes 2 and 4) or $G_{s\alpha}$ (lanes 3 and 6). Samples (25 μ g) were resolved in duplicate on 10% SDS-PAGE gels which were subsequently immunoblotted with antiserum IIC, directed against the internal epitope of $G_{i\alpha1}$ (panel A) and antiserum CS1 (panel B) raised against the C-terminus of $G_{s\alpha}$ (**Table 2.1**).

Figure 4.17



Chapter 5

**The modulatory effects of substituting
residue C351 in $G_{i\alpha 1}$ on agonist intrinsic
activity at an α_2A -C351x $G_{i\alpha 1}$ fusion
construct**

5.1 Introduction

Agonist activation of a G-protein coupled receptor (GPCR) triggers the transduction of a chemical, photon or mechanical signal to secondary messenger producing enzymes or ion channels following the stimulation of heterotrimeric GTP binding proteins. All G-protein coupled receptors are characterised by a highly conserved primary structure, which includes an extracellular N-terminal segment, seven putative transmembrane repeats, which form the transmembrane core, connected by three extracellular loops, three cytoplasmic loops, and an intracellular C-terminal segment. G-protein coupled receptors exist in either inactive (R), active (R*) or desensitised (R*-P) conformations. Agonist binding causes a conformational change that stabilises the receptor in the active (R*) conformation. This conformational change is in turn transmitted to a heterotrimeric GTP binding protein (G-protein).

G-proteins mediate the transduction of extracellular signals from GPCRs to secondary effectors. G-proteins are composed of three subunits designated α , β and γ . The α -subunit is characterised by a guanine nucleotide binding pocket, common to many guanine nucleotide exchanging proteins, into which either GDP or GTP can bind. G-proteins exist in either inactive (GDP bound) or active (GTP bound) conformations. In the inactive conformation the α -subunit contains GDP in the nucleotide binding pocket and is in a high affinity complex with the $\beta\gamma$ subunits. These $\beta\gamma$ subunits are thought to play a pivotal role in stabilising receptor/G-protein interactions. Activation occurs through receptor catalysed guanine nucleotide exchange in the α -subunit. The conformational change in the agonist bound receptor (R*) is transmitted to the G-protein α -subunit. This conformational change in the α -subunit results in a lower affinity for $\beta\gamma$ subunits, increased affinity for Mg^{++} and decreased affinity for GDP. Consequently, the GDP molecule dissociates and is replaced by a Mg^{++} GTP complex. The GTP bound α -subunit and $\beta\gamma$ subunits are then free to interact with downstream effector molecules. G-protein de-activation is regulated by the hydrolysis of GTP to GDP, the rate of which is determined by the intrinsic catalytic activity of the α -subunit and by ancillary proteins such as regulators of G-protein signalling (RGS). This results in a conformational change in the α -subunit, which allows $\beta\gamma$ subunit re-association and therefore reversion back to the inactive heterotrimeric state.

GPCRs and their associated G-proteins are expressed in the cell as separate components. These single components have the capacity to interact with or be regulated by other molecules, thereby generating signal transduction cascades. These signal transduction cascades create the potential to elicit a diverse range of responses from a single stimulus. However, this "cross talk" between the various components can be problematical in characterising a single aspect, for example agonist efficacy, which is extremely dependent on the relative expression of the individual components. To restrict and simplify the model, Bertin *et al.* (1994) constructed a fusion protein consisting of the β_2 adrenoceptor covalently linked to the G_{sa} G-protein. This fusion was designed to maintain a 1:1 ratio of receptor to G-protein expression and to limit receptor interactions with other G-proteins, so forcing a receptor to channel its signal through a pre-determined G-protein. Despite this, the characterisation of this and other fusion constructs has shown the design by Bertin *et al.* (1994) to be only partially effective in limiting receptor signalling through non-covalently linked G-proteins

The act of covalently linking a receptor to a G-protein has been shown to enhance agonist stimulated receptor/G-protein output when compared to the separately expressed components. Seifert *et al.* (1998a) have demonstrated that a higher percentage of β_2 adrenoceptors in a β_2 - G_{sa} fusion protein are stabilised in the high affinity state for agonist, when compared to separately expressed β_2 adrenoceptors. The percentage of β_2 - G_{sa} fusion receptors in the high affinity state was reduced by the addition of the non-hydrolysable GTP analogue, GTP γ S, which uncouples the G-protein from the receptor. Under these conditions, the fusion protein had the same percentage of receptors in the high affinity state as the non-linked receptor. These findings would suggest that the close proximity of the G-protein to the receptor in the fusion protein favours a "pre-coupled" state that stabilises the receptor/G-protein fusion in the high affinity state for the agonist. It has been postulated that the covalent link between the receptor and the G-protein may play a role in stabilising the ternary complex in a similar fashion to the $\beta\gamma$ subunits. This hypothesis is supported by the findings of Seifert *et al.* (1998a) who demonstrated that while the presence of $\beta\gamma$ subunits increased the percentage of separately expressed β_2 adrenoceptors in the high affinity state, it made little impact in the β_2 - G_{sa} fusion. This observation can not be attributed to a loss of $\beta\gamma$ subunit binding in the fusion protein. The bacterial toxin, cholera toxin (CTX), can only efficiently ADP-ribosylate the G_{sa} subunit at residue Arg 201 when the G-protein is present in its

heterotrimeric state. Bertin *et al.* (1994) have demonstrated that CTX catalysed ADP-ribosylation of the G_{sa} moiety in the β_2 - G_{sa} fusion protein is dependent on the presence of $\beta\gamma$ subunits. Similarly, Wise *et al.* (1997b) have shown that co-expression of $\beta\gamma$ subunits with a fusion protein constructed from the α_2A adrenoceptor and the PTX insensitive G-protein mutant, C351G $G_{i\alpha 1}$ (α_2A -C351G $G_{i\alpha 1}$) enhanced the signal output upon agonist treatment. Despite the fact that the covalent link has been shown to stabilise the receptor in the high affinity state, no change in the pharmacological profile of a receptor has been noted (Wise *et al.*, 1997c; Burt *et al.*, 1998). The covalent link between the β_2 receptor and the G_{sa} G-protein has also been shown to confer a greater capacity to activate the secondary effector, adenylyl cyclase, when compared to the freely interacting components (Bertin *et al.*, 1994; Seifert *et al.*, 1998a; Wenzel-Seifert *et al.*, 1998). This increase in the capacity of the β_2 - G_{sa} fusion to activate adenylyl cyclase could be due to a stabilisation of the ternary complex by the covalent link. Seifert *et al.* (1998b) have also demonstrated that the β_2 adrenoceptor in the β_2 - G_{sa} fusion construct has properties of a constitutively active receptor. From this it can be inferred that the direct physical link may bias stabilisation of the receptor in the active (R^*) conformation in the absence of agonist.

While Bertin *et al.* (1994) hoped that the covalent link between the receptor and G-protein would unequivocally prevent cross talk between different signal transduction pathways, it would appear that the different receptor/G-protein fusions vary in their capacity to interact with other components of the signal transduction cascade. It has been demonstrated that the β_2 - G_{sa} fusion protein can activate the downstream effector, adenylyl cyclase, in an agonist dependent manner (Bertin *et al.*, 1994; Wenzel-Seifert *et al.*, 1998). However, the α_2A -C351G $G_{i\alpha 1}$ fusion protein shows no such capacity to directly interact with the same downstream effector (Burt *et al.*, 1998). Since the C-terminal tail of the α_2A adrenoceptor (20aa) is significantly shorter than that of the β_2 adrenoceptor (84aa), it could be argued that the difference in function between the fusions was due to the size of the C-terminal tail in the α_2A -C351G $G_{i\alpha 1}$ fusion construct. To investigate the relationship between the length of the receptor C-terminal tail in the fusion protein and its effect on downstream effector activation, Wenzel-Seifert *et al.* (1988) deleted 26 and 70 amino acids from the extreme C-terminus of the β_2 adrenoceptor in the β_2 - G_{sa} fusion protein. Contrary to what would be expected from the α_2A -C351G $G_{i\alpha 1}$ model, reducing the length of the receptor C-terminal tail in the β_2 -

$G_{s\alpha}$ fusion increased the capacity of agonist to activate the secondary effector adenylyl cyclase. These findings are of particular interest as the 70 amino acid deletion reduces the C-terminal tail length to less than that of the α_2A adrenoceptor in the α_2A -C351G $G_{i\alpha 1}$ fusion. The difference in the capacity of the two fusions to interact with adenylyl cyclase could be explained by the following hypothesis. The covalent link between the receptor and the G-protein stabilises both signal transduction components in a conformation in which various molecular determinants involved in signal transduction component recognition and interaction may become obscured. This hypothesis can also be used to explain other features peculiar to the fusion proteins, but not the separately expressed components. Bertin *et al.* (1994) have demonstrated that the β_2 - $G_{s\alpha}$ fusion protein is less prone to desensitisation than the freely interacting β_2 adrenoceptor. This effect may be due to the fusion being stabilised, by the covalent link, in a conformation which blocks access to molecular determinants involved in receptor desensitisation. In another observation, Sautel and Milligan (1998) have shown that the α_2A -C351G $G_{i\alpha 1}$ fusion protein couples to free G-proteins more selectively than separately expressed receptor. The α_2A adrenoceptor couples predominantly to $G_{i\alpha}$ subunits, but can also couple to $G_{s\alpha}$ G-proteins. The α_2A -C351G $G_{i\alpha 1}$ can still couple to free $G_{i\alpha}$ subunits, but has lost the capacity to interact with $G_{s\alpha}$ subunits. From this observation, it can be inferred that the G-protein in the fusion may limit access to some, but not all molecular determinants involved in G-protein coupling.

Transient transfection provides a convenient method of overexpressing signal transduction components in order to assess agonist efficacy. However, in co-transfectional studies, quantitation and consistent reproduction of transfection efficiencies is often problematic. This is especially true with regard to the transient transfection of G-proteins where there is no accurate way of measuring G-protein expression levels. The variation in transfection efficiency and so expression can have serious implications in the assessment of agonist efficacy. Agonist efficacy measurements are very susceptible to variations in the ratio of signal transduction component expression. Reconstituted systems containing known quantities of purified signal transduction components provide a more reproducible system for measuring agonist efficacy. However, due to the difficulties involved in purifying sufficient quantities of functional receptors and G-proteins this is often not a viable option. Many of the above problems can be solved by covalently linking the receptor to the G-protein

and then measuring agonist efficacy with G-protein based biochemical assays. The use of the fusion protein has many advantages over co-transfection of the individual components. Fusion protein expression ensures a 1:1 stoichiometric ratio of receptor to G-protein expression which is impossible with current co-transfectional techniques. The covalent link between the two components also ensures cellular co-localisation. Finally, the fusion protein provides an accurate means of quantitating G-protein expression through receptor binding assays. This information can be used to accurately assess the catalytic activity of a particular G-protein.

Carr *et al.*, (1998) have previously demonstrated that agonist efficacy at the α_2A -C351G $G_{i\alpha 1}$ fusion protein is lower than in a similar construct containing wild type $G_{i\alpha 1}$ sequence. This fusion construct has also been shown to couple to endogenously expressed $G_{i\alpha}$ subunits in stably transfected cells Burt *et al.*, (1998). This endogenous G-protein coupling affects the 1:1 stoichiometric ratio of receptor:G-protein expression, so confounding agonist potency measurements. While endogenous G-protein coupling can be abolished in the fusion protein containing the PTX insensitive G-protein, so restoring the 1:1 ratio, this approach is not feasible for the fusion containing the PTX sensitive wild type $G_{i\alpha 1}$ sequence. To determine if agonist efficacy at the α_2A -C351X $G_{i\alpha 1}$ fusion protein is modulated by the physiochemical properties of residue 351 in the G-protein moiety, it was decided to create a range of α_2A -C351X $G_{i\alpha 1}$ fusion proteins each containing a different residue at position 351. The PTX insensitive nature of each of the G-proteins should allow the use of PTX to uncouple endogenous PTX sensitive G-protein coupling, thus ensuring a 1:1 ratio of receptor to G-proteins. Agonist potency would then be assessed in each of the α_2A -C351X $G_{i\alpha 1}$ fusions.

5.2 Results

5.2.1 Construction of α_2 A-C351X $G_{i\alpha 1}$ fusion proteins

Pertussis toxin insensitive mutants of the α_2 A adrenoceptor C351 $G_{i\alpha 1}$ fusion protein were constructed by substituting the last 707bp of the cDNA sequence corresponding to the C-terminus of the fusion protein with the equivalent cDNA sequence from PTX-insensitive C³⁵¹Xaa $G_{i\alpha 1}$ mutants

The α_2 A-C351 $G_{i\alpha 1}$ fusion protein cDNA in the pCDNA3 vector was digested with the restriction enzymes *Eco*R1 and *Sac*II to remove the last 707bp of the cDNA sequence as described in **Section 2.23**. The digestion products were resolved on a 1% (w/v) agarose gel and stained with ethidium bromide (**Figure 5.1**). The expected 7163 bp fragment corresponding to the pCDNA3- α_2 A-C351 $G_{i\alpha 1}$ fusion protein was excised from the gel and purified as in **Section 2.27**.

The PTX-insensitive C³⁵¹Xaa $G_{i\alpha 1}$ mutants were similarly digested with the restriction enzymes *Eco*R1 and *Sac* II to liberate a DNA fragment corresponding to the last 707bp of the $G_{i\alpha 1}$ cDNA sequence. The digestion products were resolved on a 1% (w/v) agarose gel and stained with ethidium bromide. The expected 707bp bands were excised, purified and ligated into the *Eco* RI/*Sac* II digested pCDNA3 vector containing the α_2 A- $G_{i\alpha 1}$ fusion protein cDNA. The ligation reactions were used to transform the DH5 α bacterial strain, which was subsequently grown on agar plates under ampicillin selection (**Section 2.18**).

Five clones were arbitrarily chosen from each of the transformations and cultured as in **Section 2.18**. Plasmid DNA was extracted from the cultures by small scale DNA preparation (**Section 2.22.1**) and digested with the restriction enzymes, *Eco*R1 and *Asp* 718 (**Section 2.23**). The restriction digests were resolved on a 1% (w/v) agarose gel and stained with ethidium bromide (**Figure 5.2**). The presence of a DNA fragment with an apparent molecular size of 2461 bp, corresponding to the expected size of the α_2 A-C351X $G_{i\alpha 1}$ fusion protein cDNA, indicated recombinant clones.

The 3' end of the α_2 A-C351X $G_{i\alpha 1}$ fusion protein cDNAs were sequenced using a primer annealing on the SP6 promoter priming site in the pCDNA3 vector. The

sequencing profiles were translated to confirm the identity of the residue at position 351 in the G-protein.

5.2.2 Transient expression of the α_2 A-C351X G_{iα1} fusion proteins

The HEK293T cell line was transiently transfected with either 10 µg of α_2 A-C351X G_{iα1} or α_2 A-C351 G_{iα1} cDNA in the pCDNA3 vector. The cells, with the exception of those transfected with α_2 A-C351 G_{iα1}, were treated with 50ng/ml of pertussis toxin 12 hours prior to harvesting (Section 2.7) in order to inactivate, by ADP-ribosylation, endogenously expressed PTX-sensitive G-proteins. At 72 hours after transfection, the cells were harvested and membranes were prepared as in Section 2.9.

5.2.3 The expression level of the α_2 A-C351I G_{iα1} fusion protein increases upon transfection with increasing amounts of cDNA

The α_2 A-C351I G_{iα1} fusion protein was arbitrarily chosen to see if a relationship existed between the amount of cDNA transfected into HEK293T cells and the amount of fusion protein detected by the α_2 A adrenergic receptor binding assay. This was in order to assess a convenient amount of cDNA required to obtain a reasonable expression level. HEK293T cells were transiently transfected with 2, 4, 6, 8 and 10 µg of α_2 A-C351I G_{iα1} cDNA in the pCDNA3 vector. The total amount of cDNA transfected (10 µg) was standardised in each experiment by using empty pCDNA3 vector. The expression level of the α_2 A-C351I G_{iα1} fusion protein was quantitated by the α_2 A adrenergic receptor binding assay. Analysis of the receptor binding data in Figure 5.3. shows that the expression level of the α_2 A-C351I G_{iα1} fusion protein increased upon transfection with up to 6 µg of cDNA, after which very little variation in expression was seen.

5.2.4 Quantitation of α_2 A-C351X G_{iα1} fusion protein expression in HEK293T cells

The relative expression levels of the α_2 A-C351X G_{iα1} fusion proteins were quantitated by a receptor binding assay directed against the receptor portion of the fusion protein. The α_2 A adrenergic receptor binding assay was carried out as described in Section 2.15.1. Analysis of the receptor binding data in Figure 5.4. shows that α_2 A-

C351X $G_{i\alpha 1}$ fusion expression fluctuated between 10 and 21 pmol/mg. It was also noted that no expression was detected in non-transfected HEK293T control cells.

5.2.5 A linear function exists between α_2A -C351I $G_{i\alpha 1}$ expression and [^{35}S]GTP γ S binding at a maximal agonist concentration

A correlation between α_2A -C351I $G_{i\alpha 1}$ expression and [^{35}S]GTP γ S binding at a non-limiting concentration of UK14304 (100 μ M) was determined in HEK 293T cells transfected with increasing amounts of α_2A -C351I $G_{i\alpha 1}$ cDNA (0, 2, 4, 6, 8 and 10 μ g). The cells were treated with 50ng/ml PTX to remove the endogenous G-protein signalling component 12 hours prior to harvesting and membrane preparation. The level of α_2A -C351I $G_{i\alpha 1}$ fusion protein expression was quantitated by an α_2A adrenergic receptor binding assay (Figure 5.3.) as described in Section 2.15.1. In parallel, a [^{35}S]GTP γ S binding assay was performed on the same membrane preparation. By stimulating the α_2A -C351I $G_{i\alpha 1}$ fusion protein with UK14304, an α_2A adrenoceptor agonist, it can be seen in Figure 5.5. that a linear function described [^{35}S]GTP γ S binding versus the level of fusion protein expression. The experimental points correlate with a correlation co-efficient close to 1 ($r=0.98$).

5.2.6 The relative efficacy of the α_2A -C351X $G_{i\alpha 1}$ fusion proteins to bind [^{35}S]GTP γ S in response to UK14304 stimulation

The capacity of each of the α_2A -C351X $G_{i\alpha 1}$ fusion proteins to bind the non-hydrolysable GTP analogue, [^{35}S]GTP γ S, in response to UK14304 stimulation of the α_2A adrenoceptor moiety in the fusion protein was measured as detailed in Section 2.15.2. In parallel, the expression level of each of the mutants was quantitated by an α_2A adrenoceptor binding assay as described in Section 2.15.1. The maximal [^{35}S]GTP γ S binding value of each of the fusion proteins was then normalised with regard to the level of fusion protein expression. The data (Figure 5.6.), now corrected for variation in G-protein expression, depicts the efficacy of the fusion proteins to bind [^{35}S]GTP γ S in response to activation by a non-limiting concentration of the α_2A adrenoceptor agonist, UK14304.

5.2.7 Agonist efficacy in the α_2 A-C351X G_{iα1} fusion proteins is modulated by the identity of residue 351 in the G-protein moiety

The α_2 A-C351X G_{iα1} fusion proteins containing the residues cysteine, isoleucine and glycine at position 351 in the G_{iα1} moiety were arbitrarily chosen to assess the identity of residue 351 and its effect on agonist efficacy. COS-7 cells were transfected to express α_2 A-C351X G_{iα1} fusion proteins containing cysteine, glycine and isoleucine at position 351 in the G-protein moiety. Cells transfected with α_2 A-C351I G_{iα1} or α_2 A-C351G G_{iα1} were treated with PTX to remove the endogenous G-protein signalling component 12 hours prior to harvesting. The efficacy of agonists on the fusion protein were measured by agonist stimulated [γ ³²P]GTP hydrolysis in membrane preparations from each of the transfections. The following agonists were assessed in this study – adrenaline, nor-adrenaline, α -methylnoradrenaline, UK14304, dexmedetomidine, BHT-933, xylazine, clonidine, guanobenz and oxymetazoline. The results were then plotted as a percentage of the activity stimulated by 100 μ M adrenaline (**Figure 5.7.**). It can be seen from **Figure 5.7.** that the order of intrinsic activity relative to adrenaline in each of the fusion proteins was as follows- Ile>Cys>Gly. This order was conserved throughout all of the partial agonists assayed.

5.2.8 The efficacy of clonidine is modulated by the identity of residue 351 in the G-protein

The efficacy and potency of clonidine was assayed through its ability to competitively stimulate [γ ³²P]GTP hydrolysis in the α_2 A-C351X G_{iα1} fusion proteins in the presence of adrenaline. COS-7 cells were transiently transfected to express α_2 A-C351X G_{iα1} fusion proteins containing cysteine, glycine and isoleucine at position 351 in the G-protein. Cells transfected to express α_2 A-C351I G_{iα1} or α_2 A-C351G G_{iα1} were treated with PTX to remove the endogenous G-protein signalling component 12 hours prior to harvesting. Agonist stimulated [γ ³²P]GTP hydrolysis was assayed in membrane preparations from each of the transfections treated with varying concentrations of clonidine competing with a maximally effective concentration of adrenaline (100 μ M). The capacity of the agonists to stimulate [γ ³²P]GTP hydrolysis was recorded and presented as a percentage of the activity stimulated by 100 μ M adrenaline alone. It can be seen from **Figure 5.8.** that at a maximal concentration of clonidine, the efficacy of this drug to activate the fusion proteins was the same as estimated from direct

stimulation by a maximally effective concentration of clonidine (Figure 5.7.). The relative order of intrinsic activity for clonidine in the fusion proteins was as follows – Ile>Cys>Gly

5.2.9 The capacity of α_2 A adrenoceptor agonists and antagonists to compete with [3 H]RS-79948-197 binding to the α_2 A-C351 $G_{i\alpha 1}$ fusion protein

The capacity of α_2 A adrenoceptor antagonists and agonists to compete with [3 H]RS-79948-197 binding to the α_2 A-C351X $G_{i\alpha 1}$ fusion proteins was measured by a competition binding assay (Section 2.15.1). Membranes were prepared from COS-7 cells transiently transfected to express the α_2 A-C351 $G_{i\alpha 1}$ fusion protein. Samples of membrane preparation were used to assess the capacity of yohimbine, adrenaline, oxymetazoline and clonidine to compete with 1nM [3 H]RS-79948-197 for binding to the α_2 A adrenoceptor moiety of the fusion protein at a range of ligand concentrations. The competition binding data was plotted with the mathematical package Kaleidagraph (Fig. 5.9). It can be seen from Figure 5.9. that the antagonist and agonists displayed different affinities to compete with [3 H]RS-79948-197 binding for the α_2 A-C351 $G_{i\alpha 1}$ fusion protein. The order of affinity of the drugs at the α_2 A-C351 $G_{i\alpha 1}$ fusion protein is as follows- yohimbine> oxymetazoline> clonidine> adrenaline.

5.2.10 The affinity of yohimbine for the α_2 A-C351X $G_{i\alpha 1}$ fusion proteins is not affected by the identity of the residue at position 351 in the G-protein

The capacity of yohimbine, an α_2 A adrenoceptor antagonist, to compete with the radiolabelled antagonist, [3 H]RS-79948-197, for binding to α_2 A-C351X $G_{i\alpha 1}$ fusion proteins was measured by a competition binding assay. COS-7 cells were transiently transfected to express α_2 A-C351X $G_{i\alpha 1}$ fusion proteins containing either a cysteine, glycine or isoleucine residue at position 351 in the G-protein moiety. Competition binding experiments were performed between 1nM [3 H]RS-79948-197 and varying concentrations of yohimbine in membranes prepared from each of the transfections. The competition binding data was plotted with the mathematical package Kaleidagraph. It can be seen from Figure 5.10 that none of the fusion proteins displayed any difference in antagonist binding affinity. Therefore, the ligand binding site in the receptor moiety

is not affected by the covalent link with the G-protein mutant or by the mutations introduced in the G-protein moiety.

5.3 Discussion

Fusion proteins, composed of a receptor physically linked to its cognate G-protein, provide us with useful tools for determining pharmacological constants, such as agonist efficacy, with high precision (Bertin *et al.*, 1994; Burt *et al.*, 1998; Seifert *et al.*, 1988a and Wise *et al.*, 1997c). Measurements of agonist efficacy are highly susceptible to variations in the ratio of G-protein to receptor expression. Fusion proteins provide a means of predetermining a stoichiometric ratio of one G-protein for one receptor, and in addition ensure co-localisation of the receptor and its cognate G-protein in the same subcellular compartment, features which are extremely difficult to keep constant in simple transient transfection experiments. Unfortunately, the capacity of the receptor moiety of the fusion protein to also activate endogenously expressed G-proteins can modify the predicted 1:1 ratio (Burt *et al.*, 1998). To address this problem, as discussed in previous chapters, PTX-insensitive G-protein mutants have been employed as the G-protein moiety in the fusion construct (Wise *et al.*, 1997b, 1997c; Burt *et al.*, 1998). Following PTX treatment, endogenous PTX-sensitive G-protein coupling to the α_2A adrenoceptor is abolished by ADP-ribosylation, so restoring 1:1 G-protein to receptor stoichiometry. No such action needs to be taken to prevent endogenous $G_{s\alpha}$ G-protein coupling, as it has been demonstrated that the receptor moiety in the α_2A -C351G $G_{i\alpha 1}$ fusion construct has lost the capacity to activate this G-protein (Sautel and Milligan, 1998).

In spite of these advantages, there are some possible shortfalls in the use of receptor-G-protein fusions. It is possible that the covalent attachment of a G-protein to the extreme C-terminus of a receptor may alter or restrict the possible conformations adopted by the receptor moiety, so inhibiting its natural function. This is reflected in the findings of Burt *et al.* (1998) who have shown that a fusion construct between the α_2A adrenoceptor and the PTX resistant $G_{i\alpha 1}$ mutant (C351G $G_{i\alpha 1}$) lacks the capacity to activate the secondary effector adenylyl cyclase. There is also evidence to suggest that the covalent attachment of the G-protein moiety to the extreme C-terminus of a receptor may result in the constitutive activity in the absence of agonist (Seifert *et al.* 1998b). Whilst this is a feature which can be exploited to identify inverse agonists, such a property can have negative implications on the assessment on agonist efficacy.

It has been demonstrated that agonist efficacy at the α_2A -C351G $G_{i\alpha 1}$ fusion protein is lower than at a fusion constructed from the wild type $G_{i\alpha 1}$ G-protein (Carr *et al.*, 1998). The residue C351 in $G_{i\alpha 1}$ lies in the C-terminal tail of the G-protein α -subunit, a region which has been shown to undergo a dramatic conformational change during G-protein activation (Kisselev *et al.* 1998; Yang *et al.* 1999). Considering the important role the C-terminal tail of the G-protein α -subunit plays in receptor coupling and specificity, it would be of extreme interest to determine what effect the use of PTX-insensitive mutants may have on measurements of agonist efficacy using such fusion proteins.

Expression of the α_2A -C351X $G_{i\alpha 1}$ fusion constructs was confirmed by a receptor ligand binding assay using the α_2A adrenoceptor antagonist [3H]RS-79948-197. It can be seen from **Figure 5.4.** that while fusion protein expression was restricted to transfected cells, there was variation in expression between transfections. In order to accurately pharmacologically characterise a range of agonists at the fusion constructs, it was necessary to determine the window of expression in which agonist-stimulated [^{35}S]GTP γ S output could be accurately compared. The α_2A -C351I $G_{i\alpha 1}$ cDNA was arbitrarily chosen to determine the expression levels of fusion protein as a function of transfection with increasing amounts of plasmid cDNA. It can be seen from **Figure 5.3.** that α_2A -C351I $G_{i\alpha 1}$ expression increased upon transfection with up to 6 μ g of cDNA, after which expression was seen to plateau. Maximal agonist stimulated [^{35}S]GTP γ S binding was determined in membranes expressing known quantities of α_2A -C351I $G_{i\alpha 1}$ fusion construct. From the data in **Figure 5.5.** it can be seen that the α_2A -C351I $G_{i\alpha 1}$ fusion construct was insensitive to ADP-ribosylation by PTX. It was also noted that a linear function can be extrapolated from maximal agonist stimulated [^{35}S]GTP γ S binding in function of fusion protein expression. From these findings it was concluded that fusion protein expression levels determined in **Figure 5.4.** were suitable for assaying agonist efficacy at the fusion proteins using the [^{35}S]GTP γ S binding assay. Maximal (100 μ M) UK14304 stimulated [^{35}S]GTP γ S binding was then assessed for each of the fusion constructs. The [^{35}S]GTP γ S binding data was normalised with regard to fusion protein expression, to produce a rank order of coupling efficiency at each of the fusion proteins (**Figure 5.6.**). From the data in **Figure 5.6.** it can be seen that, with the exception of the wild type $G_{i\alpha 1}$ fusion protein, all of the fusion proteins were insensitive to ADP-ribosylation by PTX. It was also noted that the efficacy of the

agonist UK14304 varied between the fusion constructs. The agonist UK14304 was seen to be less efficacious at the α_2A -C351G $G_{i\alpha 1}$ fusion protein when compared to the equivalent construct composed of wild type $G_{i\alpha 1}$ sequence. Interestingly, many of the fusion constructs assayed performed better than the wild type fusion protein, with α_2A -C351I $G_{i\alpha 1}$ demonstrating the greatest efficiency of G-protein activation with UK14304. The rank order of efficiency of fusion protein activation of UK14304 correlates very closely with the order of coupling efficiency generated by co-transfectional studies involving freely interacting G-protein mutants with the α_2A adrenoceptor (**Figure 3.9 Chapter 3**). In fact, all of the fusion constructs demonstrated the same order of coupling efficiency upon maximal stimulation with UK14304 as the respective C351X $G_{i\alpha 1}$ mutants expressed independently from the receptor, with the exception of the α_2A -C351A $G_{i\alpha 1}$ fusion construct, which appeared to display greater efficiency of G-protein activation when compared to the separately expressed C351A $G_{i\alpha 1}$ subunit. However, the error associated with this particular measurement shows that this reversal of order is not significant. It has already been demonstrated in Chapter 3 that the coupling efficiency of C351X $G_{i\alpha 1}$ mutants to the α_2A adrenoceptor is closely related with the hydrophobicity of the residue at this position, with the general rule that the more hydrophobic the residue at this position, the greater the coupling efficiency of the mutant. Conversely, a charge at this position was seen to negatively affect G-protein coupling efficiency. Since the only difference between the fusion constructs lies in the identity of residue 351 in the $G_{i\alpha 1}$ moiety, it can be inferred that efficiency of agonist stimulated G-protein/receptor coupling is in part modulated by the physiochemical properties of residue 351.

The rank order of coupling efficiency of the fusion constructs was generated using a single agonist. It could be argued that the physiochemical properties of residue 351 in the $G_{i\alpha 1}$ moiety could affect the pharmacological profile of the receptor moiety. To determine whether this was the case, the intrinsic activity compared to adrenaline of a range of full and partial agonists was assayed on three selected fusion proteins. From the data in **Figure 5.7**, it can be seen that partial agonist relative intrinsic activity was consistently lower in the α_2A -C351G $G_{i\alpha 1}$ fusion construct than in the fusion protein constructed from wild type $G_{i\alpha 1}$ sequence for all of the partial agonists assayed. Similarly, agonist relative intrinsic activity was shown to be consistently greater at the α_2A -C351I $G_{i\alpha 1}$ fusion protein than for wild type regardless of the agonist assayed.

From these findings, it can be concluded that the order of coupling efficiency between the fusions cannot be related to the agonist, demonstrating that the pharmacological profile of the receptor moiety is not altered by the covalent addition of the C351X G_{1α1} mutants.

It is possible that the receptor moieties in the fusion proteins may display different affinities for the same agonist and therefore behave as different receptors. To determine the rank order of agonist potency at the fusion proteins, the capacity of clonidine to compete with adrenaline for stimulation of high affinity GTPase activity was compared in the same three fusions used to assess the relative intrinsic activity of a range of agonists. It can be seen from the data in **Figure 5.8.** that clonidine was able to compete with 100 μM adrenaline at each of the three fusion constructs with IC₅₀ values of (α₂A-C351G G_{1α1}) 4.8±0.5x10⁻⁶M, (α₂A-C351 G_{1α1}) 2.5±1.3x10⁻⁵M and (α₂A-C351I G_{1α1}) 4.1±0.4x10⁻⁵M. While there was greater divergence of efficiency of G-protein activation between the fusion constructs towards maximally effective concentrations of clonidine, the rank order of efficiency of G-protein activation in the fusion proteins remained unchanged throughout the agonist concentrations. It could be argued that the divergence in efficiency of G-protein activation in the constructs at maximally effective concentrations of clonidine could be due to the inability of clonidine to fully compete with adrenaline for binding at the receptor moiety in the fusion construct. To determine if this was true, the capacity of clonidine and adrenaline to compete with [³H]RS-79948-197 binding was determined in the wild type fusion construct. It can be seen from **Figure 5.9.** although both clonidine and adrenaline could completely displace binding of [³H]RS-79948-197 from the receptor moiety of the fusion construct, clonidine had a higher affinity. Therefore, at maximally effective concentrations of clonidine (**Figure 5.8.**) the plateau of stimulated GTPase provides a direct measurement of the relative intrinsic activity of clonidine versus adrenaline. These values mirrored those produced by direct addition of clonidine alone (**Figure 5.7.**).

It could be argued that the covalent bond linking the α₂A adrenoceptor to the G_{1α1} mutant might alter ligand binding affinity at the receptor moiety. To determine whether the ligand binding site was affected by the presence of the covalently linked G-protein, the capacity of the antagonist yohimbine to compete with the radiolabelled antagonist [³H]RS-79948-197 for binding at the receptor moiety was determined for each of the three selected fusion proteins. It can be seen from **Figure 5.10.** that all of the

fusion proteins displayed a similar affinity for yohimbine ($K_i=3.1-4.0 \times 10^{-9} \text{M}$). This observation is in good accord with the findings of Bertin *et al.* (1994), Seifert *et al.* (1988a) and Burt *et al.* (1998), who have shown that agonist and inverse agonist affinity at the receptor moiety in the fusion protein is that same as that at the freely expressed receptor. It can therefore be concluded that differences in the relative agonist efficacy at the fusion proteins is not related to a modification in the ligand binding affinity at the receptor moiety, but more probably to the capacity of the receptor to activate its covalently linked G-protein.

The data generated herein has demonstrated that agonist efficacy at the $\alpha_2\text{A-C351X G}_{\text{tail}}$ fusion proteins can be modulated by the physiochemical properties of the residue 351 in the G_{tail} moiety. This modulation of agonist efficacy cannot be attributed to a modification in the agonist affinity or pharmacological profile of the receptor moiety in the fusion construct. Therefore, agonist efficacy at the fusion constructs must be related to the coupling efficiency of the G-protein mutant to the receptor, which is in turn defined by the hydrophobicity of the residue at position 351 in the G_{tail} moiety. These findings are in good accord with the observations of Kisselev *et al.* (1998) and Yang *et al.* (1999), who have presented evidence that, in the GDP bound inactive form, the hydrophobic residues in the C-terminal tail of G_{tail} adopt an ordered conformation the hydrophobic nature of which is stabilised by receptor/G-protein interactions. Receptor catalysed guanine nucleotide exchange in the α -subunit causes the transmission of a conformational change to the C-terminal tail resulting in stabilisation of the hydrophobic residues by alternate means and so loss of receptor association. Therefore, as the data generated herein has shown, increasing the hydrophobic nature of the C-terminal tail should strengthen G-protein/receptor coupling. In conclusion, the fusion proteins generated herein provide valuable tools to measure agonist efficacy and efficiency of G-protein/receptor coupling. In order to use fusion proteins to measure the efficacy of agonists at other G_i coupled receptors, it would be of interest to determine if PTX-insensitive mutant coupling to other G_i linked receptors is similarly modulated by the hydrophobic nature of residue 351. Future work could involve constructing PTX-insensitive G_{tail} -protein/ G_i linked receptor fusions and then characterising the efficiency of G-protein activation in combination with specificity of endogenous G-protein coupling and effector activation.

Figure 5.1 Analysis of the restriction digestion products of the α_2 A-C351 G_{1 α 1} fusion protein cDNA in the pCDNA3 vector

α_2 A-C351 G_{1 α 1} fusion protein cDNA in the pCDNA3 vector was digested with the restriction endonucleases *EcoRI* and *SacII* (Section 2.23) and resolved on a 1% agarose gel. Cleavage of the vector containing the construct yielded two fragments of 7163 and 707 bp (lane 2). A 1Kb ladder provided size markers (lane 1).

Figure 5.1

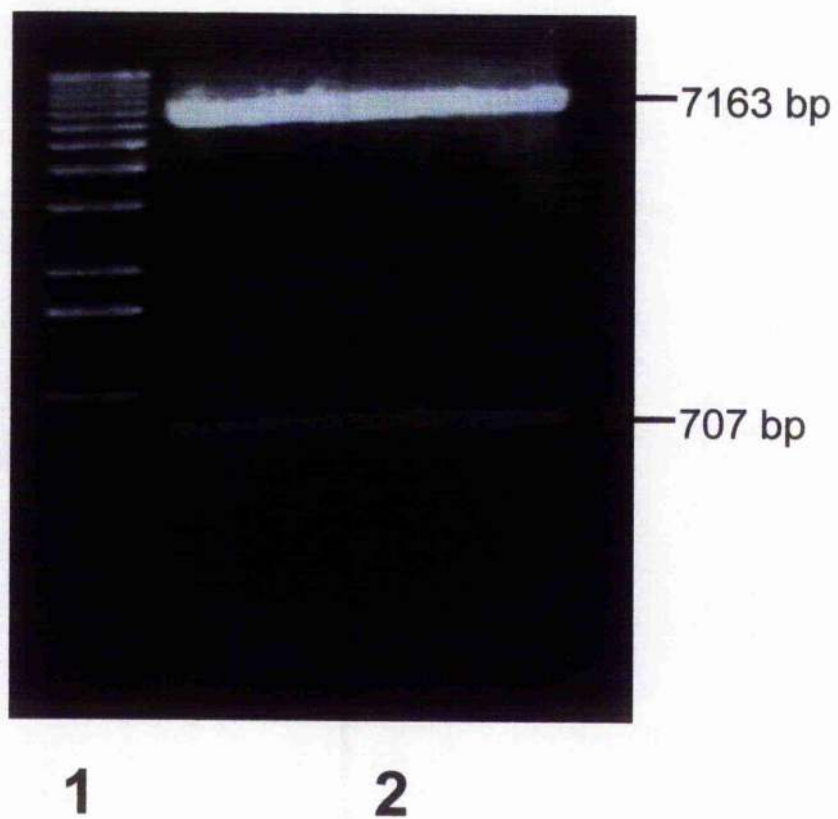


Figure 5.2. Analysis of clones transformed with α_2 A-C351X G_{1 α 1} fusion protein cDNA in the pCDNA3 vector

DNA was isolated from clones thought to be transformed with an α_2 A-C351X G_{1 α 1} fusion protein cDNA in the pCDNA3 vector. Samples (1 μ g) were restriction digested with the enzymes *EcoRI* and *Asp718* (Section 2.23) and resolved on a 1% agarose gel. Cleavage of the vector containing the insert yielded fragments of 5456 and 2461 bp (lanes 1, 4 and 5).

Figure 5.2

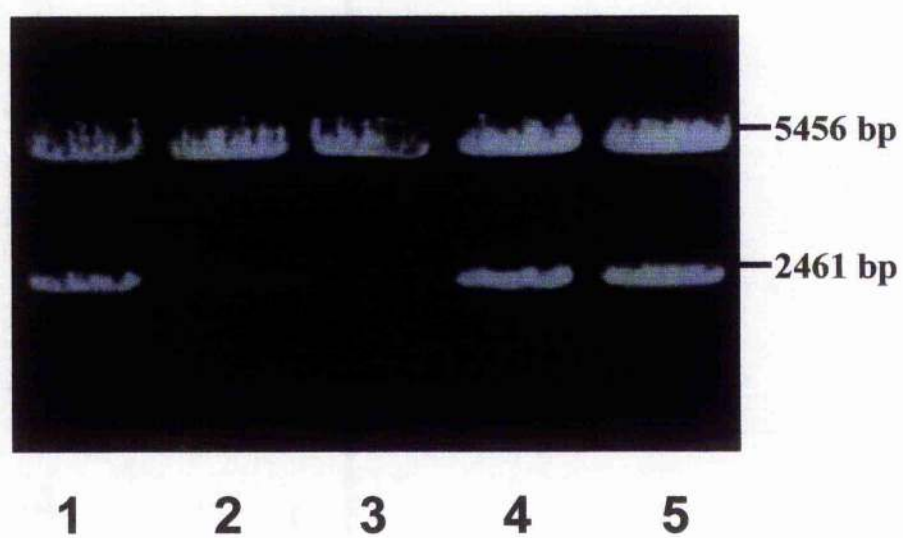


Figure 5.3. Transient transfection of increasing amounts of α_2A -C351I $G_{i\alpha 1}$ cDNA into HEK293T cells results in higher levels of α_2A -C351I $G_{i\alpha 1}$ expression

Membranes were prepared from HEK293T cells transfected with increasing quantities of α_2A -C351I $G_{i\alpha 1}$ cDNA (2, 4, 6, 8 and 10 μ g). The amount of DNA transfected was standardised to 10 μ g with empty pCDNA3 vector. All cells were treated with 50ng/ml PTX 12 hours prior to harvesting and membrane preparation. The level of α_2A -C351I $G_{i\alpha 1}$ expression was measured in each of the membrane samples by the α_2A adrenoceptor binding assay. The results of the receptor binding assay were plotted as a function of the amount of α_2A -C351I $G_{i\alpha 1}$ cDNA transfected. Each data point represents the average of triplicate measurements \pm S.D. of a single experiment. The data represents a typical experiment.

Figure 5.3

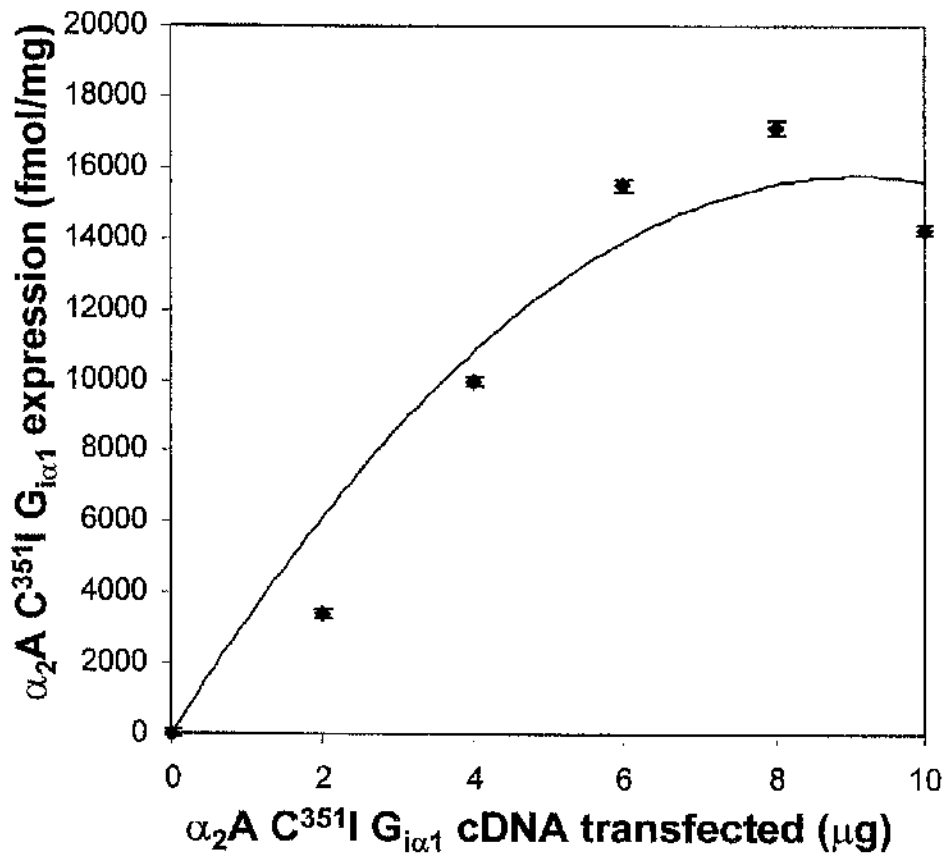


Figure 5.4. The expression of α_2 A-C351X G_{i α 1} fusion proteins transfected into HEK293T cells

Membranes were prepared from HEK293T cells transfected to express α_2 A-C351X G_{i α 1} fusion proteins. With the exception of untransfected cells or the α_2 A-C351 G_{i α 1} transfection, cells were treated with 50ng/ml PTX prior to harvesting. The level of fusion protein expression was assayed in each of the membrane samples by the α_2 A adrenergic receptor binding assay (Section 2.15.1). The identity of the residue 351 in the G-protein portion of the fusion protein is denoted by the conventional three letter code. Membranes prepared from untransfected cells (C) were used to assay endogenous α_2 A adrenoceptor expression. The results shown are the means \pm S.D. of triplicate measurements from two separate experiments (1 and 2)

Figure 5.4

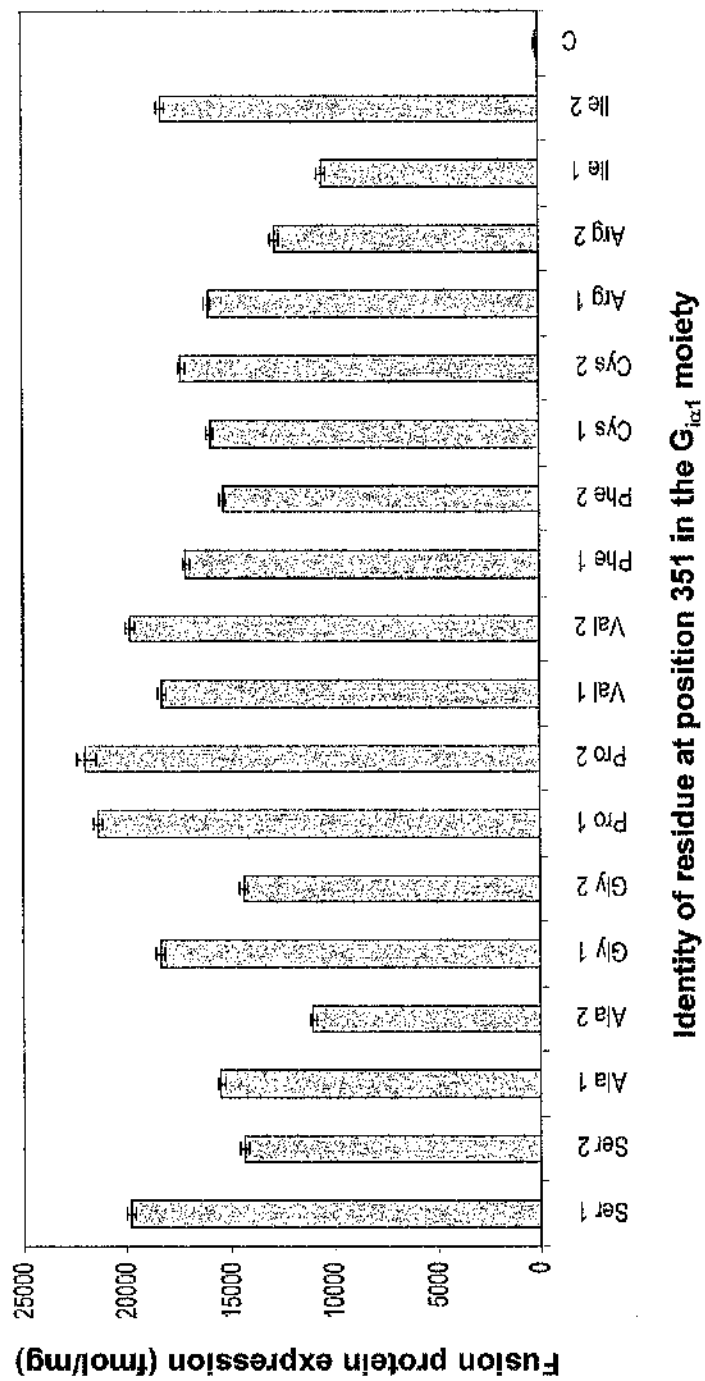


Figure 5.5 A linear relationship exists between α_2 A-C351I $G_{i\alpha 1}$ expression and [35 S]GTP γ S binding at a maximal agonist concentration

Membranes were isolated from HEK293T cells transiently transfected with increasing amounts of α_2 A-C351I $G_{i\alpha 1}$ cDNA (2, 4, 6, 8 and 10 μ g). Cells were treated with 50ng/ml PTX 12 hours prior to harvesting. UK14304 stimulated (100 μ M) [35 S]GTP γ S binding was assayed in each of the membrane preparations. The levels of α_2 A-C351I $G_{i\alpha 1}$ expression were quantitated in the same membrane samples by the α_2 A adrenergic receptor binding assay. The results from the [35 S]GTP γ S assay were plotted as a function of α_2 A-C351I $G_{i\alpha 1}$ expression in each of the membrane samples. The relationship between fusion protein expression and agonist stimulated [35 S]GTP γ S binding can be described by a linear equation whose experimental points correlate with a co-efficient of $r=0.98$. The data shown represents a typical experiment.

Figure 5.5

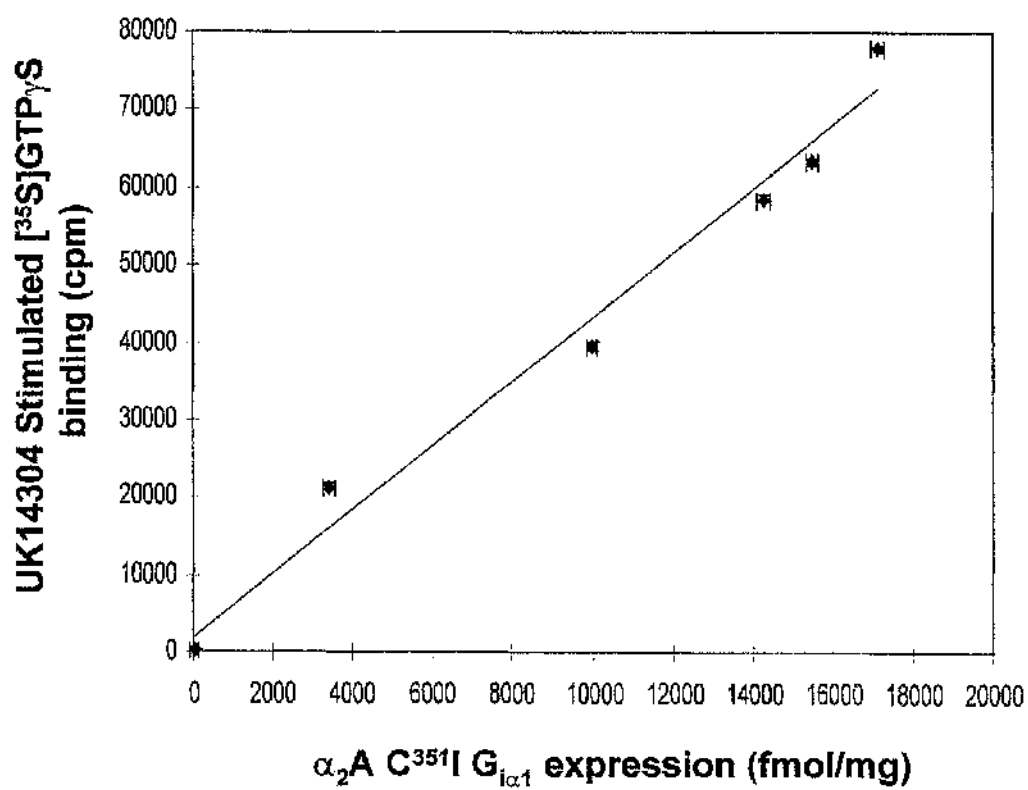


Figure 5.6. A comparison of the efficiency of α_2A -C351X $G_{i\alpha 1}$ fusion proteins to bind [^{35}S]GTP γ S at maximal UK14304 concentrations

The capacity of maximal concentrations of UK14304 (100 μ M) to stimulate [^{35}S]GTP γ S binding was assayed in membrane preparations prepared from HEK293T cells transfected to express α_2A -C351X $G_{i\alpha 1}$ fusion proteins. All transfected cells, with the exception of those transfected with the wild type fusion cDNA, were treated with 50ng/ml PTX 12 hours prior to harvesting and membrane preparation. The level of α_2A -C351X $G_{i\alpha 1}$ fusion protein expression was quantitated in the same membrane preparation by the α_2A adrenergic receptor binding assay. The [^{35}S]GTP γ S binding capacity index for each of the mutants was calculated by dividing agonist-stimulated [^{35}S]GTP γ S binding by the level of fusion protein expression. The identity of the residue at position 351 in the G-protein is denoted by the conventional three letter code. The results shown are the means \pm S.E.M. of triplicate measurements from four separate experiments.

Figure 5.6

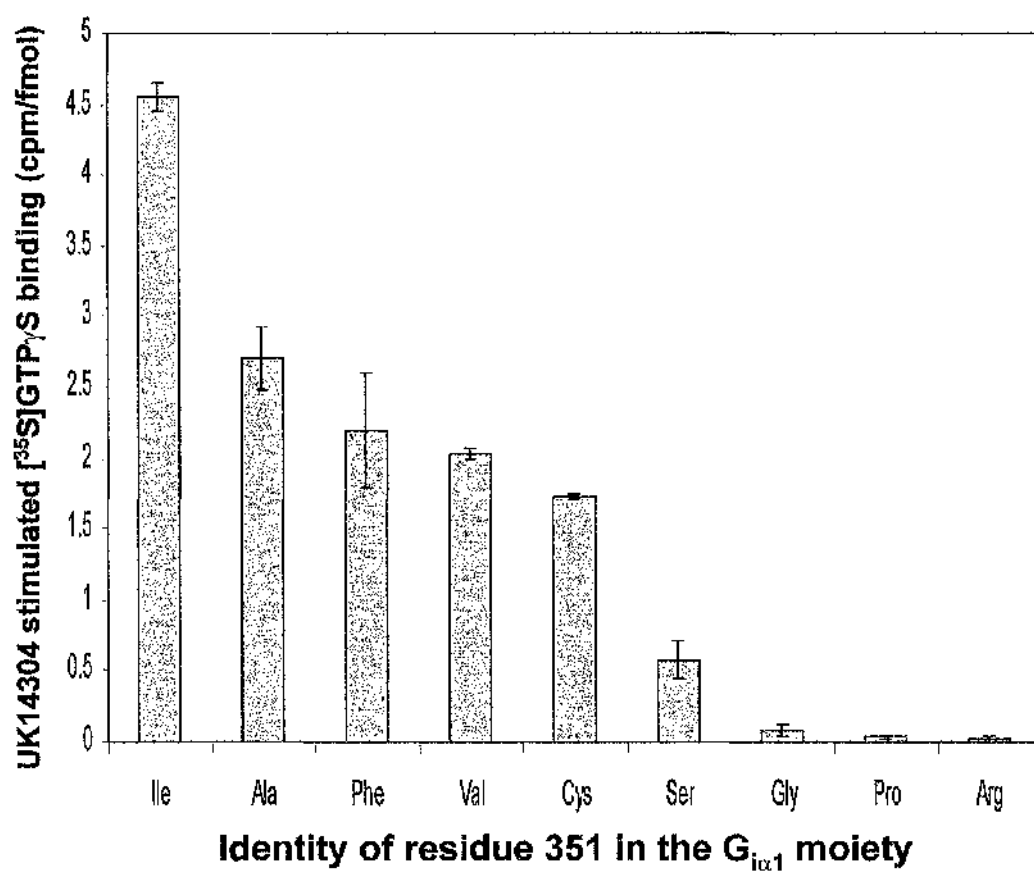


Figure 5.7. The relative intrinsic activity of agonists on the α_2 A-C351X G_{I α 1} fusion proteins

Membranes were prepared from COS-7 cells transfected to express one of three α_2 A-C351X G_{I α 1} fusion proteins containing either Ile (open bars), Gly (filled bars) or Cys (hatched bars) at position 351 in the G-protein. The capacity of maximally effective concentrations of adrenaline (1), noradrenaline (2), α -methylnoradrenaline (3), UK14304 (4), dexmedetomidine (5), BHT-933 (6), xylazine (7), clonidine (8), guanobenz (9) and oxymetazoline (10) to stimulate [γ ³²P]GTP hydrolysis was measured in membrane preparations from each of the transfections. The results shown are represented as a percentage of 100 μ M adrenaline stimulation, which was considered as a full agonist. The data shown represents the means of three independent experiments; errors in all cases were less than 5%.

Figure 5.7

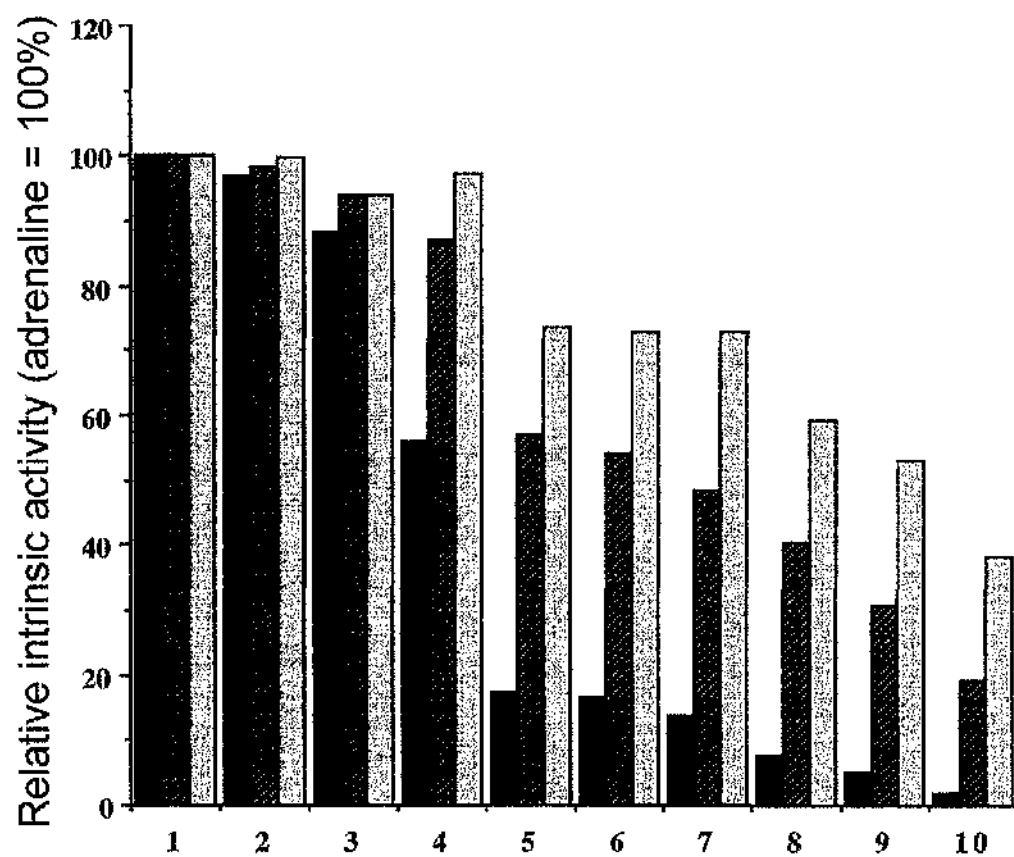


Figure 5.8. The relative intrinsic activity of clonidine is dependent upon the identity of the G-protein residue 351 in the α_2 A-C351X G_{i α 1} fusion protein

Membranes were isolated from COS-7 cells transfected to express either α_2 A-C351I G_{i α 1} (squares), α_2 A-C351G G_{i α 1} (circles) or α_2 A-C351 G_{i α 1} (triangles). Cells transfected with α_2 A-C351I G_{i α 1} or α_2 A-C351G G_{i α 1} were treated with 50ng/ml PTX 12 hours prior to harvesting. Adrenaline stimulated (100 μ M) high affinity GTPase activity was measured in each of the membrane samples in the presence of varying concentrations of clonidine. In the experiment shown, clonidine was seen to inhibit the effects of adrenaline with IC₅₀ values of (α_2 A-C351G G_{i α 1}) $4.8 \pm 0.5 \times 10^{-6}$ M, (α_2 A-C351 G_{i α 1}) $2.5 \pm 1.3 \times 10^{-5}$ M and (α_2 A-C351I G_{i α 1}) $4.1 \pm 0.4 \times 10^{-5}$ M. The results shown are represented as a percentage of stimulation generated by 100 μ M adrenaline, which was treated as a full agonist. The data shown represents the means \pm S.D. of triplicate measurements of a single experiment. The results represent a typical experiment.

Figure 5.8

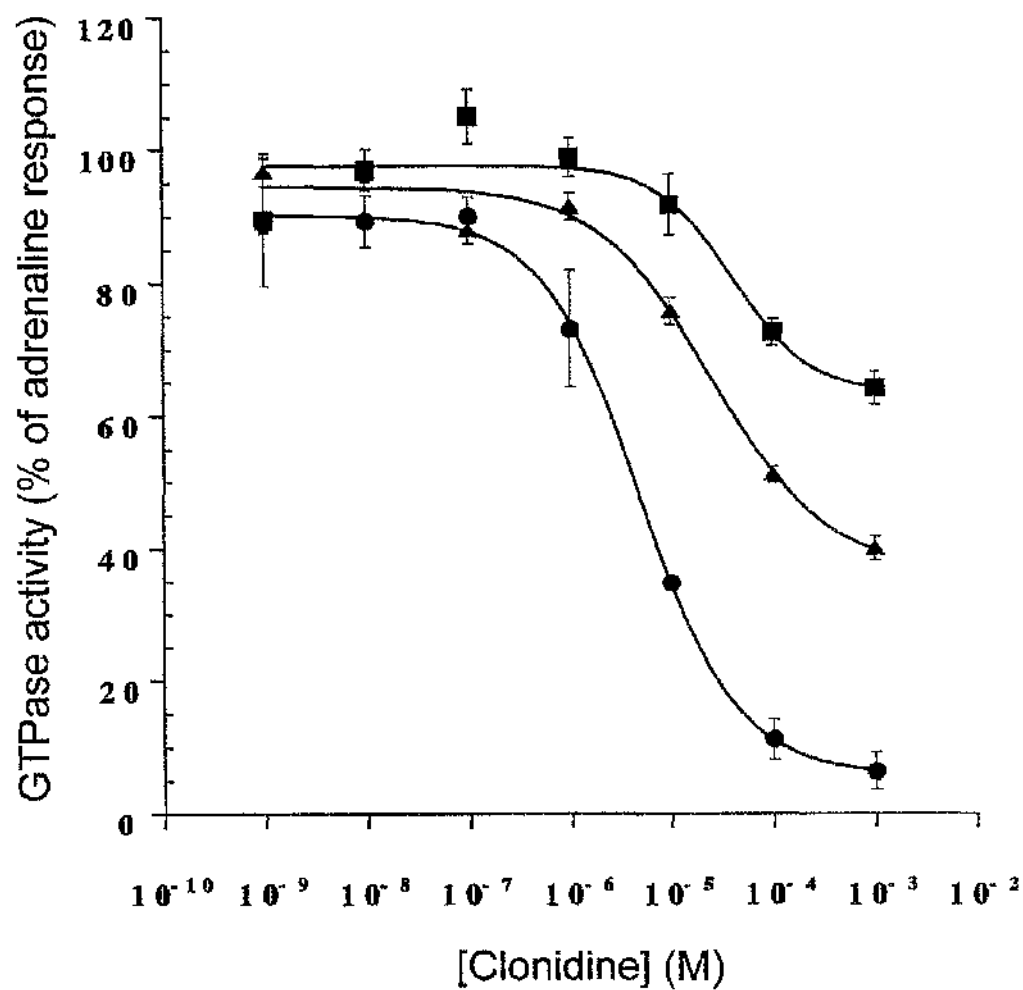


Figure 5.9 The capacity of α_2A adrenoceptor agonists and antagonists to compete with [3H]RS-79948-197 for binding to the α_2A -C351 $G_{i\alpha 1}$ fusion protein

Membranes were prepared from COS-7 cells transfected to express the α_2A -C351 $G_{i\alpha 1}$ fusion protein. Membrane samples were used to assess the capacity of yohimbine (filled circles), oxymetazoline (filled triangles), clonidine (open triangles) and adrenaline (open circles) to compete with (1 nM) [3H]RS-79948-197 binding for the α_2A -C351 $G_{i\alpha 1}$ fusion protein. The results shown are represented as a percentage of [3H]RS-79948-197 binding in the absence of competing agonist or antagonist. The data shown represents the means \pm S.D. of triplicate measurements of a single experiment. The results represent a typical experiment.

Figure 5.9

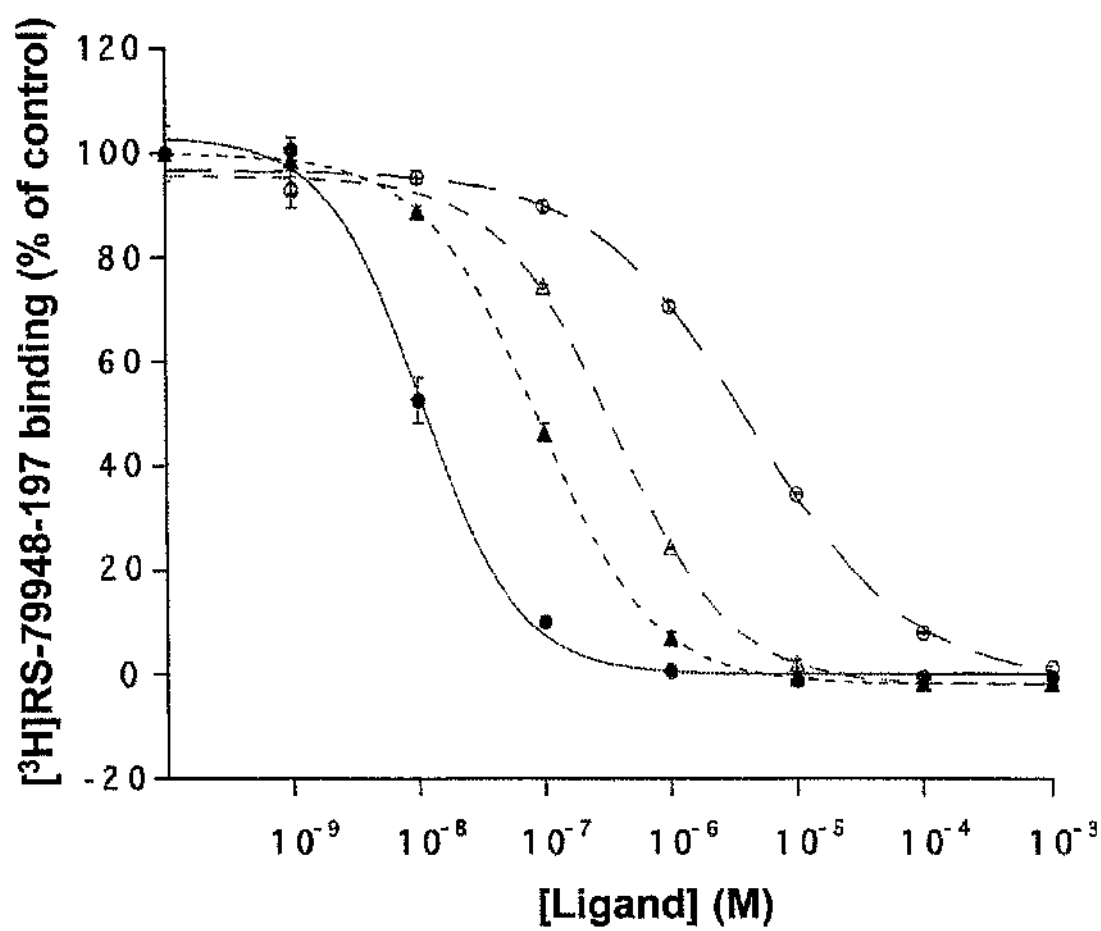
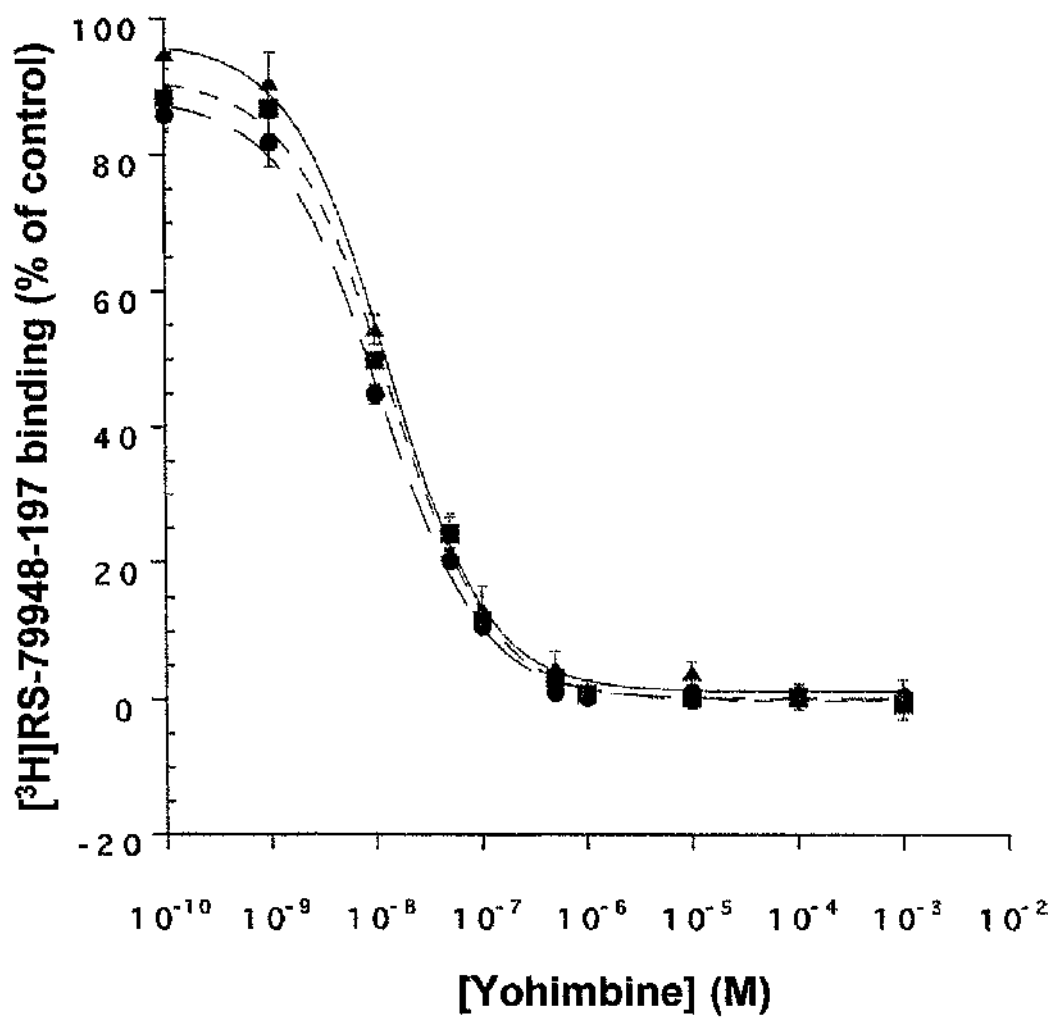


Figure 5.10. The ability of yohimbine to compete with [³H]RS-79948-197 binding to the α_2 A-C351X G₁₂₁ fusion proteins is not affected by the identity of the residue at position 351 in the G-protein

Membranes were prepared from COS-7 cells transiently transfected to express α_2 A-C351X G₁₂₁ fusion proteins with either Gly (circles), Cys (triangles) or Ile (squares) at residue 351 in the G-protein. The capacity of yohimbine to compete with (1 nM) [³H]RS-79948-197 binding for the α_2 A-C351X G₁₂₁ fusion proteins was assessed in membrane preparations from each of the transfections. In the experiment shown, each of the fusions displayed a similar affinity for yohimbine to compete for specific binding of [³H]RS-79948-197 ($K_i=3.1-4.0 \times 10^{-9}$ M). The results shown are presented as a percentage of [³H]RS-79948-197 binding in the absence of yohimbine. The data shown represents the means \pm S.D. of triplicate measurements of a single representative experiment.

Figure 5.10



Final Discussion

Final discussion

The characterisation of the G-protein coupling specificity of receptors is often confounded by the interaction of multiple G-protein subtypes with the same receptor. A simple solution to this problem has been to use bacterial toxins to functionally uncouple selected G-proteins, so that the true extent of G-protein/receptor coupling can be analysed. One frequently used bacterial toxin is pertussis toxin (PTX) from the bacterium *Bordetella pertussis*. This toxin has been shown to act with a high degree of specificity at Gi family members which contain a cysteine residue four amino acids from the C-terminus. In such G-proteins, pertussis toxin catalyses the transfer of an ADP-ribose group from NAD⁺ to this cysteine residue, resulting in the loss of G-protein receptor coupling. This loss of receptor coupling is assumed to be due to steric hindrance of the ADP-ribose moiety. The G_{zα} subunit, which contains an isoleucine residue at this position, has been shown not to be substrate for this toxin. Based on this observation, Senogles (1994) and Hunt *et al.* (1994) have shown that replacement of this cysteine residue in both G_{iα1} and G_{iα3} can also confer resistance to post-translational modification by PTX. This would suggest that the substitution of this cysteine residue might provide a general strategy for the production of PTX insensitive Gi protein mutants.

While the substitution of such residues has been shown to confer resistance to pertussis toxin, one major flaw in this strategy has been that these mutants tend to couple less efficiently than the pertussis toxin sensitive wild type G-protein (Wise *et al.*, 1997d). As such, without prior investigation into the effects that these substitutions may have on the efficiency of G-protein/receptor coupling, these mutants are of little or no use in characterising agonist efficacy at a receptor. In spite of this limitation, little has been done to quantify the physiochemical properties of the substituted residue and its affect on receptor/G-protein coupling. It should also be noted that there has never been comprehensive study in which the cysteine residue was substituted to all other amino acids.

In the first results chapter, I have focussed on these limitations in the current understanding of PTX resistant mutants. Senogles (1994) and Hunt *et al.* (1994) have shown that the substitution of the conserved cysteine residue (C351) in G_{iα1} or G_{iα3} confers resistance to ADP-ribosylation catalysed by pertussis toxin. From these findings it has been postulated that the substitution of this cysteine residue may provide a general

strategy for the production of pertussis toxin resistant G-protein mutants. In order to assess if this was a feasible strategy, the conserved cysteine residue (C351) in $G_{i\alpha 1}$ was systematically substituted to all other amino acids to provide a comprehensive set of G-protein mutants. The coupling efficiency of each of these mutants was then assessed following agonist stimulation of the G_i linked α_2A adrenoceptor. As expected, the substitution of this cysteine residue did confer resistance to pertussis toxin. Indeed, these finding confirm that pertussis toxin is specific in its substrate requirements as, with the exception of the wild type $G_{i\alpha 1}$, none of the mutants generated in this study were seen to be substrates for ADP-ribosylation.

Substitution of this conserved cysteine residue in G_i family members has only ever been observed to negatively affect G-protein receptor coupling. Wise *et al.* (1997d) have shown that a glycine substitution at this position in $G_{i\alpha 1}$ reduces coupling efficiency at the α_2A adrenoceptor by an order of magnitude when compared to the wild type α -subunit. Similarly, Osawa and Weiss (1995) have demonstrated that the substitution of the equivalent residue for alanine in $G_{i\alpha}$ (C347A) also results in a reduced affinity of the G-protein for its cognate receptor. In a more extreme example, the substitution of a tyrosine residue at this position in $G_{i\alpha}$ has been shown to completely ablate coupling of this G-protein to its cognate receptor (Owasa and Weiss, 1995). These finding would lead us to believe that the cysteine residue is an essential components for optimal G-protein receptor coupling and therefore, the substitution of any other amino acid at this position would be detrimental to the capacity of the G-protein to couple to its cognate receptor. The findings of this study provide the first direct evidence challenging this preconception. While, as expected from the findings of Wise *et al.* (1997d), certain substitutions such as glycine did indeed reduce the capacity of the mutants to couple to the α_2A adrenoceptor, other substitutions such as isoleucine were shown to enhance G-protein/receptor coupling relative to the wild type G-protein. To summarise, the mutants assayed in this study displayed a spectrum of responses when assessed for coupling efficiency at the α_2A adrenoceptor. Since this was the first experiment of its kind to comprehensively substitute this residue for all other amino acids, the data generated herein was used to analyse possible relationships between the physiochemical properties of the residue at position 351 and the coupling efficiency of the G-protein. Following analysis of various amino acid descriptors such as hydrophobicity, hydrophilicity, and volume to surface area, a strong relationship was

finally determined between the octanol/H₂O partition co-efficient of the substituted residue and the capacity of the mutant to couple to the α_2 A adrenoceptor. This relationship indicated that the more hydrophobic the amino acid at this position, the greater the coupling efficiency at the α_2 A adrenoceptor. This same descriptor also demonstrated the best correlation with the predicted EC₅₀ value of the agonist UK14304 to stimulate the mutant. Analysis of the physiochemical properties of the residues also revealed some other interesting relationships, one of which was that the size of the residue did not appear to be a negative factor in G-protein receptor coupling. Evidence for this came from the capacity of G-protein mutants containing bulky amino acids such as tyrosine to couple to the α_2 A adrenoceptor with greater efficiency than the C351G G_{iα1} mutant. This is contrary to what might be expected according to the current model of attenuation of receptor coupling following ADP-ribosylation at this position. In contrast to this, charge was shown to be a negative factor in G-protein/receptor coupling. The substitution of charged residues such as arginine or lysine at this position was seen to result in a total loss of G-protein/receptor coupling. From these findings, it would seem as if the mechanism underlying the loss of G-protein/receptor coupling following ADP-ribosylation may be related more to the charge associated with this group than, as previously thought, size.

Amino acids which failed to conform well to the proposed hydrophobicity rule include proline and methionine. Possible explanations why these residues failed to conform to the general trend are as follows. For the methionine residue, it is possible that the amino acid was present as methionine sulfoxide. If this was the case, this would drastically alter the hydrophobic character of the residue and so its octanol/H₂O partition co-efficient value. As for the proline residue, this amino acid is known to introduce a kink into the peptide backbone which, in turn, can result in a loss of flexibility. The substitution of this residue at position 351 in G_{iα1} may have resulted in a loss of flexibility in the extreme C-terminus of the α -subunit and so a loss of G-protein/receptor coupling. Evidence supporting this hypothesis comes from the findings of Osawa and Weiss (1995) who have shown the substitution of a proline residue in G_{iα} at a position equivalent to residue 352 in G_{iα1} to result in loss of receptor coupling at this G-protein.

The findings of Osawa and Weiss (1995) have also identified another side effect of substituting the conserved cysteine residue in Gi G-proteins, the loss of nucleotide

binding. It could have been assumed that the differential coupling efficiencies observed at the C351x G_{iα1} mutants were due to a modification in the capacity of the G-protein mutant to bind or exchange nucleotide. Contrary to what would be expected from the study of Osawa and Weiss (1995), none of the mutants assayed in this study demonstrated any detectable change in either the capacity or the rate of guanine nucleotide exchange relative to the wild type G_{iα1} subunit. From these findings it can be concluded that the difference in capacity of C351x mutants of G_{iα1} to couple to the α₂A adrenoceptor are not due to a modification in nucleotide binding or exchange.

To summarise, this study shows that the capacity of PTX resistant G_{iα1} mutants to couple to the α₂A adrenoceptor can be influenced by the physiochemical properties of the residue present at position 351 in the G_{iα1} moiety.

In the second results chapter I examined the role the C-terminal tail of the G-protein plays in defining the specificity of G-protein/receptor coupling. Various groups including Komatsuzaki *et al.* (1997), Voyno-Yasenetskaya *et al.* (1994a) and Conklin *et al.* (1993) have shown that the substitution of as little as three amino acids in the extreme C-terminus of a G-protein is sufficient to switch the receptor selectivity of the G-protein while preserving the specificity of effector coupling. These studies have concentrated on the use of pertussis toxin insensitive G-proteins and have measured the efficiency of chimera coupling through secondary messenger based assays. A limitation of this approach lies in the use of effector activation to assay receptor/chimera selectivity. Measurements of effector activation can frequently be modulated, either synergistically or in opposition, by free βγ subunits dissociated either from the chimera or from endogenous G-protein cognate to the receptor being assayed. It is therefore laborious to prove that the chimera under investigation is solely modulating effector activation. A more accurate way of measuring receptor/chimera interactions would be to assay chimera activation at the level of the G-protein through guanine nucleotide binding or exchange based assays. However, the low guanine nucleotide exchange rate associated with PTX insensitive G-proteins does not make this a viable option. It would therefore be useful to determine if G-proteins of the Gi family, which possess a higher intrinsic guanine nucleotide exchange rate, could be feasibly used as the functional partner of the G-protein chimera, and if the substitution of the extreme C-terminus in these subunits could switch receptor selectivity in a manner similar to that seen in PTX insensitive G-proteins.

To determine if this was the case, the last 6 amino acids of $G_{i\alpha 1}$ were substituted for those of $G_{s\alpha}$, $G_{q\alpha}$ or $G_{16\alpha}$. The length of this substitution was determined from the findings of Conklin *et al.* (1993) who have shown optimal coupling of a $G_{q\alpha}/G_{i\alpha 2}$ chimera at the A1 adenosine receptor following the substitution of between 4 and 9 amino acids, substitutions beyond this point were shown to negatively affect the coupling efficiency of this chimera. Each of the chimeras generated in this study was then assayed for functional coupling at a bank of non-Gi linked receptors. The findings of this study show that the substitution of 6 amino acids in the C-terminus of $G_{i\alpha 1}$ is not sufficient to confer coupling of the G-protein to all non-Gi linked receptors. Of the chimeras assayed in this study, the $G_{i1\alpha}G_{q\alpha}$ chimera was only seen to functionally couple to the P_2Y_4 , TRH and oxytocin receptors. Similarly, the $G_{i1\alpha}G_{s\alpha}$ chimera was only seen to functionally couple with the V_2 vasopressin receptor and the β_2 adrenoceptor. Interestingly, none of the receptors assayed in this study were shown to functionally couple to the $G_{i1\alpha}G_{16\alpha}$ chimera. This is in direct opposition to what would be expected from the findings of Offermans *et al.* (1995) who have demonstrated $G_{16\alpha}$ coupling at quite a diverse array of receptors. Further immunological characterisation of the chimeras showed that the lack of functional coupling in the $G_{i1\alpha}G_{16\alpha}$ chimera could not be attributed to either a variation in chimera expression or the expression of an incomplete protein product. Nor, as determined by tryptic protection assays, could it be attributed to a loss or diminished ability of the chimera to exchange guanine nucleotides.

Wise *et al.* (1997a) have shown that variations in receptor expression can influence the output from guanine nucleotide binding assays. Therefore, it could be argued that the lack of functional coupling at many of the receptors assayed in this study could be due to variations in their expression levels. In light of this, it would have been prudent to assay receptor expression to determine if this was indeed adversely affecting the observation of receptor/chimera coupling. Unfortunately, the lack of suitable radiolabelled ligands restricted the use of this readily available simple control. However, in a few select cases, receptor expression was indirectly demonstrated through activation of the wild type $G_{i\alpha 1}$ subunit in a selective manner. As immunological characterisation showed the levels of chimera expression to be remarkably consistent between transfections, it was assumed that receptor expression would be equally consistent. Therefore, it can be concluded that in such cases, the

lack of chimera activation was solely due to incompatibility of the receptor for the chimera.

Osawa *et al.* (1990) have previously demonstrated that the loss of bacterial toxin ADP-ribosylation sites in G-protein chimeras can confer insensitivity to bacterial toxin treatment. To determine if this was the case in the $G_{i1\alpha}G_{s\alpha}$ chimera, which is missing ADP-ribosylation sites for both pertussis and cholera toxin, functional coupling of this chimera was assayed at the IP prostanoid receptor following treatment with either a combination of PTX and CTX or vehicle. As expected from the findings of Osawa *et al.* (1990), the data generated in this study shows that the elimination of bacterial toxin ADP-ribosylation sites in the $G_{i1\alpha}G_{s\alpha}$ chimera confers resistance to both PTX and CTX. The data herein also indicate that the substitution of the C-terminus of $G_{i\alpha1}$ for $G_{s\alpha}$ is sufficient to switch receptor specificity of $G_{i\alpha1}$ while retaining the G-proteins high guanine nucleotide exchange rate. Further analysis of this data showed a loss of total functional coupling in the assay following treatment with bacterial toxins. This loss of functional coupling was attributed to coupling to endogenous bacterial toxin sensitive G-proteins. These findings therefore indicate that coupling of the chimera to the receptor is not saturating, and that while the substitution of the last 6 amino acids of $G_{i\alpha1}$ for those of $G_{s\alpha}$ is sufficient to switch the coupling specificity of the G-protein, it is not sufficient to provide optimal coupling at non-Gi linked receptors.

To summarise, the findings of this study corroborate those of Voynoy-Yasenetskaya *et al.* (1994a), Komatsuzaki *et al.* (1997) and Conklin *et al.* (1993) in demonstrating that the substitution of the extreme C-terminus of a G-protein is sufficient to switch the specificity of receptor coupling. However, the data generated herein also address issues not covered in previous studies and show that while the C-terminus of the α -subunit is unarguably an important determinant of receptor specificity, it is not the only determinant involved in G-protein/receptor coupling.

Finally, in the third results chapter, I investigated the effects of substituting the residue C351 in $G_{i\alpha1}$ on the measurement of agonist relative intrinsic activity at the α_2A adrenoceptor. Measurements of agonist intrinsic activity are extremely susceptible to variations in the ratio of signal transduction component expression. This can be particularly problematic when assessing agonist intrinsic activity in systems constructed from transiently transfected components. While transient

transfection provides a convenient means of overexpressing signal transduction components, quantitation and consistent reproduction of transfection efficiencies is often problematic. This is especially true with regard to the transient expression of G-proteins, for which there is no convenient way of measuring expression levels. Reconstituted systems containing known quantities of purified signal transduction components provide a more reproducible system for measuring agonist intrinsic activity. However, due to the difficulties involved in purifying sufficient quantities of functional receptors and G-proteins, this is often not a viable option. In contrast to these two systems, a third approach, first proposed by Bertin *et al.* (1994), of covalently fusing the receptor to the G-protein provides the convenience of transient transfection with the reproducibility of a reconstituted system. The fusion approach also ensures a 1:1 stoichiometric ratio of receptor to G-protein expression, cellular co-localisation of the signal transduction components as well as an accurate means of quantifying G-protein expression through receptor binding assays.

Recently, the fusion strategy, as proposed by Bertin *et al.* (1993), has been shown to be flawed. While Sautel and Milligan (1998) have shown that a fusion construct of the α_2A adrenoceptor and $G_{i\alpha 1}$ has lost the ability to couple to endogenous $G_{s\alpha}$ subunits, Burt *et al.* (1998) have demonstrated that this fusion retains the capacity to interact with endogenous $G_{i\alpha 1}$. This endogenous G-protein coupling can be expected to upset the predicted 1:1 stoichiometric ratio of receptor:G-protein expression, so confounding agonist potency measurements. While the 1:1 ratio of G-protein to receptor can be restored by using a PTX resistant G-protein in the fusion construct in combination with PTX treatment to abolish endogenous G-protein coupling, the use of this PTX insensitive mutant itself has been shown to affect measurements of intrinsic activity. Carr *et al.* (1998) have shown the intrinsic activity of adrenaline at a PTX insensitive α_2A -C351G $G_{i\alpha 1}$ fusion construct to be significantly lower than at an equivalent construct containing the wild type G-protein. Residue 351 in $G_{i\alpha 1}$ is located four amino acids from the extreme C-terminus of the α -subunit, in a region which is known to undergo a conformational change during G-protein activation (Kisselev *et al.* 1998; Yang *et al.* 1999). Considering the predicted role of this region in defining the efficiency and specificity of receptor coupling, it would be of interest to determine what effects a substitution at this position may have on the measurements or agonist relative intrinsic activity.

To determine if intrinsic activity at α_2A -C351x $G_{i\alpha 1}$ fusion proteins can be modulated by the physiochemical properties of residue 351 in the $G_{i\alpha 1}$ moiety, agonist relative intrinsic activity was assayed in a series of α_2A -C351x $G_{i\alpha 1}$ fusion constructs each containing a different residue at position 351. The fusion constructs assayed in this study showed a spectrum of responses to the agonist UK14304. In accordance with the findings of Carr *et al.* (1998), the fusion construct containing the G-protein mutants C351G $G_{i\alpha 1}$ displayed lower intrinsic activity for UK14304 compared to adrenaline than an equivalent construct containing the wild type sequence. However, in contrast to this, relative to the wild type $G_{i\alpha 1}$ fusion, UK14304 displayed greater relative intrinsic activity at the fusion construct containing an isoleucine residue at position 351 in the $G_{i\alpha 1}$ moiety. Further analysis of these fusion constructs showed that the order of intrinsic activity of UK14304 closely mirrored the order of coupling efficiency previously determined using the separately expressed components.

To determine if the pharmacological profile of the receptor moiety of the fusion construct was altered by the covalent addition of the G-protein, the study was repeated using a diverse array of agonists at 3 selected fusion constructs containing either isoleucine, glycine or cysteine at position 351 in the $G_{i\alpha 1}$ moiety. Analysis of these fusion constructs showed that the order of agonist intrinsic activity relative to adrenaline was conserved regardless of the agonist being assayed. This observation is consistent with the findings of Wise *et al.* (1997c) and Burt *et al.* (1998), who have not been able to detect a change in the pharmacological profile of the α_2A adrenoreceptor following its fusion with $G_{i\alpha 1}$. The measurements of relative intrinsic activity could also be affected by a change in the agonist affinity at the receptor moiety. To investigate this property, agonist and antagonist affinity was assessed in the same three fusion constructs used to determine the pharmacological profile of the receptor moiety. None of the fusions assayed in this study were shown to differ in affinity for the antagonist yohimbine. Also, the affinity of the antagonist yohimbine at the fusion constructs was seen to be comparable to that determined in the separately expressed receptor. This observation is in good accord with the findings of Bertin *et al.* (1994), Seifert *et al.* (1998a) and Burt *et al.* (1998), all of whom have shown agonist and antagonist affinity at a fusion construct to be comparable to that displayed by the freely expressed receptor.

To summarise, the findings of this study have expanded on those of Carr *et al.* (1998) and have shown that the intrinsic activity of partial agonist at the α_2A adrenoceptor, relative to adrenaline, can be modulated by the physiochemical properties of residue 351 in the $G_{i\alpha 1}$ moiety. This discrepancy of relative intrinsic activity at the fusion proteins can not be attributed to a change in the pharmacological profile of the receptor moiety or to a modification of its affinity for agonist. In conclusion, the data generated throughout the three results chapters serves to reinforce the importance of the C-terminus of the G-protein α -subunit in defining the efficiency and specificity of G-protein/receptor coupling.

List of publications

List of publications

This thesis includes data from the following publications.

Vicky N. Jackson, Daljit S. Bahia, and Graeme Milligan (1999) "Modulation of Relative Intrinsic Activity of Agonists at the *Alpha*-2A Adrenoceptor by Mutation of Residue 351 of G Protein G_{11α}." *Mol Pharmacol* **55**, 195-201.

Chee Wai Fong, Daljit S. Bahia, Stephen Rees, and Graeme Milligan (1998) "Selective Activation of a Chimeric G₁₁/G_s G Protein Subunit by the Human IP Prostanoid Receptor: Analysis Using Agonist Stimulation of High Affinity GTPase Activity and [³⁵S]Guanosine-5'-O-(3-thio)triphosphate Binding." *Mol Pharmacol* **54**, 249-257

Daljit S. Bahia, Alan Wise, Francesca Fanelli, Melanie Lee, Stephen Rees, and Graeme Milligan (1998) "Hydrophobicity of Residue³⁵¹ of the G Protein G_{11α} Determines the Extent of Activation by the α₂A-Adrenoceptor." *Biochemistry* **37**, 11555-11562

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