Chapter 1.
GPCR and G-protein engagement: signalling and exploitation.

"Facts are the air of scientists. Without them you can never fly."

Linus Pauling

1.1.   INTRODUCTION

Communication between cells and their environment is mediated by a variety of cell surface receptors with the largest of those families being the G-protein coupled receptors (GPCRs) (Howard *et al.*, 2001). GPCRs are encoded by the largest gene family in most animals genomes (Chalmers and Behan, 2002). GPCRs are activated by a huge spectrum of extracellular molecules including ions, amino acids, hormones, growth factors, light and odorant factors some of which are shown in **Table 1.1** which emphasises their physiological importance (Marinissen and Gutkind, 2001). The full repertoire of human GPCRs is thought to include approximately 747 receptors comprising about 350 olfactory receptors, 30 additional chemosensory receptors, and 367 other physiological receptors (Vassilatis *et al.*, 2003). Recently, 650 GPCR genes were identified and about 190 of these are known to be activated by one of the 70 identified ligands (categorised as known GPCRs) (Chalmers and Behan, 2002).

Acetylcholine	Glucagon	Opioids
Adenosine	Glutamate	Orexin
Adrenaline	Gonadotropin-releasing hormone	Oxytocin
Adrenocorticotropic hormone	Growth hormone-releasing factor	Parathyroid hormone
Angiotensin II	Growth-hormone secretagogue	Photons (light)
Bradykinin	Histamine	Platelet activating factor
Calcitonin	Luteinising hormone	Prolactin releasing peptide
Chemokines	Lymphotactin	Prostaglandins
Cholecystokinin	Lysophospholipids	Secretin
Corticotropin releasing factor	Melanocortin	Serotonin
Dopamine	Melanocyte-stimulating hormone	Somatostatin
Endorphins	Melatonin	Substances P, K
Endothelin	Neuromedin-K	Thrombin
Enkephalins	Neuromedin-U	Thromboxanes
Fatty acids	Neuropeptide-FF	Thyrotropin
Follitropin	Neuropeptide-Y	Thyrotropin releasing hormone
GABA	Neurotensin	Tyramine
Galanin	Noradrenaline	Urotensin
Gastric inhibitory peptide	Odorants	Vasoactive intestinal peptide
Gastrin		Vasopressin
Ghrelin		

Table 1.1:   A partial list of some of the known endogenous and exogenous GPCR ligands (McMurchie and Leifert, 2006).

Their physiological importance, together with their accessible cellular localisation and tissue specific expression, highlights GPCRs as ideal targets for pharmaceutical intervention (Nambi and Aiyar, 2003). Currently, these receptors are the target of more than 40-50% of marketed drugs (more than a quarter of the 100 top selling drugs) with annual sales of $US 47 billion in 2003 (Brink *et al.*, 2004) (see **Table 1.2**). Furthermore the availability of highly selective ligands makes this receptor superfamily very attractive pharmaceutical targets (Nambi and Aiyar, 2003). Meanwhile, the search for novel therapeutics is a constantly expanding activity in the pharmaceutical industry and the significance of GPCRs in this industry continues to be manifested by the number of research projects focused on GPCRs as primary targets (Stadel *et al.*, 1997).

Brand Name	Generic Name	G-protein coupled receptor(s)	Indication
Zyprexa	Olanzapine	Serotonin 5-HT_2 and Dopamine	Schizophrenia, Antipsychotic
Risperdal	Risperidone	Serotonin 5-HT_2	Schizophrenia
Claritin	Loratidine	Histamine H_1	Rhinitis, Allergies
Imigran	Sumatriptan	Serotonin 5-$HT_{1B/1D}$	Migraine
Cardura	Doxazosin	α-adrenoceptor	Prostate hypertrophy
Tenormin	Atenolol	β_1-adrenoceptor	Coronary heart disease
Serevent	Salmeterol	β_2-adrenoceptor	Asthma
Duragesic	Fentanyl	Opioid	Pain
Imodium	Loperamide	Opioid	Diarrhea
Cozaar	Losartan	Angiotensin II	Hypertension
Zantac	Ranitidine	Histamine H_2	Peptic ulcer
Cytotec	Misoprostol	Prostaglandin PGE_1	Ulcer
Zoladex	Goserelin	Gonadotrophin-releasing factor	Prostate cancer
Requip	Ropinirole	Dopamine	Parkinson's disease
Atrovent	Ipratropium	Muscarinic	Chronic obstructive pulmonary disease (COPD)

Table 1.2: **Some examples of prescription drugs which target GPCRs for the indicated disease state (McMurchie and Leifert, 2006).**

This literature review aims to explore some of the background pertaining to the structure and mechanism of action of GPCRs and their close association with the heterotrimeric G-proteins and the regulation of GPCR activity. In addition, aspects of

currently used drug discovery and ligand screening technologies (which are mainly cell-based) will be discussed and compared to cell-free approaches. The specific aims of this thesis and the significance of the project will also be addressed.

1.2. GPCRS AND PHARMACOLOGY

GPCRs represent the best characterised class of therapeutic targets. The seven transmembrane spanning domains, characteristic of this receptor class, give rise to several structural features and three specific subtype classifications. The often debated ligand activation mechanistic models will also be discussed briefly. To begin with, the most recent nomenclature recommendations concerning drug/ligand classification will be briefly defined as these terms are used throughout this thesis.

1.2.1. Classification of ligands

An **agonist** is a ligand that binds to a receptor and alters the receptor state resulting in a biological response. Conventional agonists increase receptor activity (i.e. the proportion of receptors in the active conformation) whereas this activity is reduced by inverse agonists (Neubig *et al.*, 2003). Inverse agonism is often observed when a receptor is constitutively activated. Agonistic drug actions are experimentally compared by expressing the EC_{50} which is the molar concentration of the half maximal possible effect of that agonist. Agonists that only partially increase receptor activity are referred to as partial agonists. An **antagonist** is a ligand that reduces the action of another ligand by inhibiting the orthosteric or primary binding site (both agonists and antagonists bind to this site). Antagonists are commonly divided into 2 categories; surmountable and insurmountable. Surmountable antagonists participate in competitive and reversible antagonism. In contrast, insurmountable antagonists either participate in irreversible competition at the binding site, non-competitive antagonism or indirect antagonism. These terms are defined by Neubig *et al.* (2003)

in more detail. Many small molecules have now been recognised to bind to allosteric sites at the GPCRs to increase or decrease the action of the agonist or antagonist (Neubig *et al.*, 2003).

1.2.2.   GPCR classification

It is generally accepted that GPCRs are heptahelical structures that share a common architecture. These receptors exhibit an extracellular N-terminus, a seven transmembrane spanning domain composed of helices connected by intracellular and extracellular loops (arranged in a barrel like structure with a tightly packed core) and an intracellular C-terminus (Gether and Kobilka, 1998; Gouldson *et al.*, 2001). The transmembrane regions support four extracellular segments that act as a cell surface receptor. The extracellular domain is composed of three loops and an N-terminal segment with conformation restricted by intra-strand disulfide bonds (Gether and Kobilka, 1998). In addition to the topographical organisation of these receptors, post translational modifications such as N-glycosylation (Lanctot *et al.*, 1999; Xu *et al.*, 2003) and phosphorylation (discussed later) increase the level of structural complexity.

GPCRs can be divided into three broad classes (see **Table 1.3**) based on the pharmacological nature of their ligand and sequence similarities: their members share >20% sequence identity in their transmembrane domains (Wess, 1998). These receptors are activated by a variety of extracellular signals that trigger an intracellular cascade. The putative ligand-binding domain on GPCRs is formed by the extracellular amino terminus and certain portions of the transmembrane domains. Ligands have been shown to bind to the internal transmembrane regions (biogenic amine receptors), the N-terminus and the extracellular loops (neurokinin, glucagon

receptors) and just the N-terminus (metabotropic glutamate receptors) (Gouldson *et al.*, 2001).

Class	Ligand (examples)	% of known GPCRs
I Rhodopsin-like	Biogenic amines, neuropeptides and chemokines	89
II Secretin like: Characterised by a large extracellular amino terminus with several cysteine	Calcitonin, glucagon	7
III Metabotropic-glutamate receptor like: Extremely large amino terminus which is thought to contain the messenger binding site.	Metabotropic glutamate, $GABA_B$	4

Table 1.3:   **GPCR classification taken from (Chalmers and Behan, 2002; Wess, 1998).**

There are four important factors identified by (Helmreich and Hofmann, 1996) that enable GPCRs to act as molecular signalling transducers allowing communication to occur in response to many diverse stimuli. These factors are:

1) Molecular recognition of the stimuli.

2) Initial signal amplification - further amplification occurs at the effector level.

3) Limited lifetime of the activated state - this ensures precise timing of signalling and will be discussed later.

4) Reversible covalent modification of signalling proteins - phosphorylation/ dephosphorylation (desensitisation).

The structural communication from receptor binding site to intracellular nucleotide binding site (G-protein engagement is discussed later), is relatively unresolved (Cherfils and Chabre, 2003). However, various mechanistic models have been used to describe the initial events occurring at the primary binding site which are likely to elucidate key events occurring in the signal transduction pathway.

1.2.3.   **Ligand activation of GPCRs**

The initial events following the molecular recognition of the stimuli, involve the formation of the agonist-receptor complex. Upon the formation of this complex, the signal is propagated downstream and incorporates the interaction with cognate G-proteins. Mechanistic models of ligand-receptor interactions such as the 3-state model of receptor activation as described by Scaramellini and Leff (2002) are used to simplify the GPCR activation occurring at receptor level. Their model assumes that the receptors exist in three conformations; an inactive state, and two active states, with the two active conformations interacting with the G-proteins. However, new evidence suggests limitations with this model (Scaramellini and Leff, 2002). Vauquelin and Van Liefde (2005) recently discussed a sequential binding and conformational selection model that includes intermediate receptor conformations. This model (**Figure 1.1**) assumes that after an initial interaction between receptor and the structural group of the agonist, each subsequent interaction will stabilize one or more transmembrane domains until the active state is finally reached. Thus a full agonist would need to bind to drive activation to completion, whereas a partial agonist would preferentially stabilise an intermediate or pre-activated state. In an intermediate or pre-activated state, the receptor has an increased chance of spontaneously isomerising into the active state (Vauquelin and Van Liefde, 2005). The sequential binding and conformational selection model also allows a receptor to adopt distinct inactive conformations (Vauquelin *et al.*, 2002). Furthermore, these mechanistic models describe the interactions at the orthosteric site on the receptor, i.e. the site which the endogenous agonist binds. However the complexity of these models will be increased by the addition of allosteric modulation and Christopoulos *et al.* (2004) describes this in more detail.
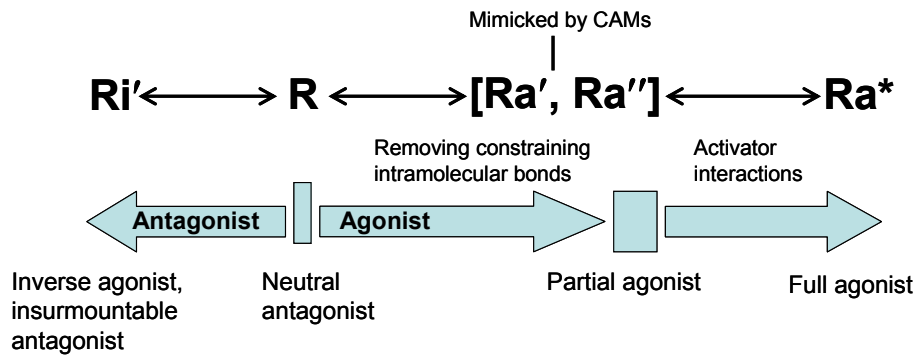
Figure 1.1: **Sequential binding and conformational selection model.**
Ra* is the fully active state; Ra′, Ra″ are the intermediate/pre-activated states; CAMs are constitutively active receptor mutants; R is the basal state and Ri′ is the inactive receptor formation (Vauquelin and Van Liefde, 2005).

G-proteins play an integral part in GPCR activation (as discussed below) and the formation of the ternary complex consists of the agonist, the receptor and the G-protein heterotrimer (Offermanns, 2003). Nevertheless, it remains to be shown how specificity of the receptor/G-protein interaction is achieved and how ligand-induced conformational change in the receptor molecule results in G-protein activation. (Offermanns, 2003).

1.3. G-PROTEIN ENGAGEMENT

Heterotrimeric G-proteins act as guanosine nucleotide dependent molecular switches in signalling pathways that connect transmembrane receptors with downstream effectors. The flow of information across the plasma membrane to initiate a particular response requires the transfer of the extracellular signal from receptor to G-protein to intracellular effector enzyme (Barritt, 1992). The G-protein complex is composed of 3-distinct polypeptide chains designated the α-(39-52 kDa), β-(35-36 kDa) and γ-subunit (7-8 kDa). There have been about 20 different α-subunits, 5 β-subunits and 13 γ-subunits identified in the mammalian system (Landry and Gies, 2002). The activation and deactivation of G-proteins are accomplished by a cascade of events called the GTPase cycle (see **Figure 1.2**).
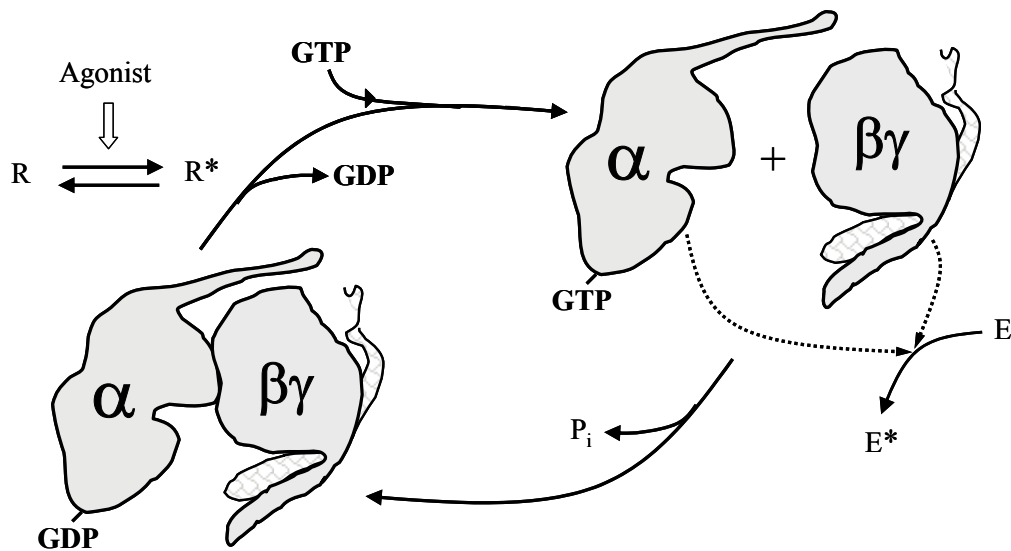
Figure 1.2: The GTPase Cycle: molecular switching.
The binding of the agonist to the unoccupied receptor (*R*) causes a change in conformation thus activating the receptor (*R**) which promotes the release of GDP from the heterotrimeric G-protein complex and rapid exchange with GTP into the nucleotide binding site on the Gα subunit. In its GTP-bound state, the G-protein heterotrimer dissociates into the Gα and Gβγ subunits exposing new surfaces allowing interaction with specific downstream effectors (*E*). The signal is terminated by hydrolysis of GTP to GDP (and P$_i$) by the intrinsic GTPase activity of the Gα subunit followed by return of the system to the basal unstimulated state. (*indicates activated state of receptor (R) or effector (E); P$_i$, inorganic phosphate, GDP, guanosine diphosphate; GTP, guanosine triphosphate) (McMurchie and Leifert, 2006).

When the G-protein complex is in the inactive state, guanosine diphosphate (GDP) is bound to the α-subunit while the βγ dimer stabilises this conformation by binding tightly to Gα-GDP enhancing the coupling of the inactive heterotrimer to a specific GPCR (Higashijima *et al.*, 1987). Thus this dimer acts as a guanine nucleotide (guanosine) dissociation inhibitor (GDI) by preventing GDP release. The rate of GDP dissociation is slow and this feature keeps the system in the inactive state. However, when the agonist binds to its receptor, the rate of GDP release is greatly accelerated, with guanosine triphosphate (GTP) replacing GDP. This leads to the dissociation of the GTP-liganded α (active state) and the free βγ dimer. Both G-protein-α and βγ complexes have the capacity to regulate effector systems such as adenylate cyclase (AC), phospholipase C and ion channels inhibiting or activating the production of a variety of second messengers such as cyclic adenosine

monophosphate (cAMP), diacylglyerol, and inositol trisphosphate (IP$_3$) (Marinissen and Gutkind, 2001). The intrinsic GTPase activity of the α-subunit as well as GTPase activating proteins allow the subunit to deactivate itself by cleaving the terminal phosphate group. Subsequently, the GDP-liganded α-subunit reassociates with the βγ dimer to form the heterotrimer of the resting state poised for re-activation by the next signal (Offermanns, 2003). In addition, several unconventional G-protein signalling pathways that diverge from this standard model have been identified (McCudden *et al.*, 2005).

1.3.1.   The Gα subunit

The Gα subunit is divided into two main domains separated by a cleft. The GTPase domain is involved in binding and hydrolysing GTP. Structurally identical to the superfamily of GTPases, rho, this helical domain is associated with GDP binding which occupies a binding site deep within the core of the subunit (Hamm, 1998). In the active conformation, the GTP bound α subunit has a lower affinity for the βγ subunit and this conformational change in the α subunit occurs around three flexible discontinuous regions, termed switch regions I, II, III (Hamm, 1998). As a result of GTP binding, the α subunit becomes better ordered particularly around the C-terminus and N-terminus which correspond to its dissociation from the GPCR and βγ respectively at those points of interaction (Hamm, 1998). The C-terminus of the third intracellular loop of the GPCR binds to the C-terminus of the α-subunit. The main properties of individual G-proteins appear to be primarily determined by the identity of the Gα subunit of the heterotrimeric G-protein (Offermanns, 2003). There are four main classes of Gα proteins:

1. Gα$_s$- stimulates/activates adenylate cyclase (includes α$_{olf}$-olfactory)

2. Gα$_{i1}$ - inhibits adenylate cyclase (includes α$_o$, α$_t$, α$_z$ and α$_{gust}$-gustducin)

3. $G\alpha_q$ - activates phospholipase C (α_q, α_{11}, α_{14}, α_{15}, α_{16})

4. $G\alpha_{12}$ & $G\alpha_{13}$ - unknown function (Hamm, 1998; Landry and Gies, 2002).

G-protein subunits such as $G\alpha_s$, $G\alpha_q$, $G\alpha_{11}$, $G\alpha_{12}$ and $G\alpha_{13}$ appear to be expressed more or less ubiquitously (Offermanns, 2003). In nature, somatic mutations that alter certain amino acids in the α subunit can lead to diseases (Landry and Gies, 2002). Loss and gain of function mutations (Rondard *et al.*, 2001) have been synthesised in the laboratory to study different aspects of G-protein signalling and receptor activation.

1.3.2. The $\beta\gamma$ dimer

The β subunit has a 7-membered β-propeller structure composed of seven blades (Hamm, 1998) whereas the γ subunit adopts an extended mainly α-helical conformation (Holler *et al.*, 1999). The γ subunit interacts with the β subunit through the N-terminal coil and then along the base of the β subunit making extensive contact. The $\beta\gamma$ subunit is a functional unit that is not dissociable except by denaturation and this subunit interacts with the residues in the switch II and N terminal regions of the α subunit (Hamm, 1998). In contrast to the pronounced changes that occur in the various conformations of the α-subunit, $\beta\gamma$ does not undergo any major structural changes (Holler *et al.*, 1999).

1.3.3. G-protein modification

G-proteins are subject to covalent modifications, which occur in both normal and pathological contexts. Adenosine diphosphate (ADP)-ribosylation of one of the amino groups on the $G\alpha$-subunit involves the donation of the ribose group from nicotinamide adenine dinucleotide (Chen and Manning, 2001). Two bacterial toxins specifically catalyse this modification thereby interfering with the activation-

inactivation cycle. They have been useful tools in studying G-protein mediated signalling (Barritt, 1992) (see **Table 1.4**).

	Pertussis Toxin	**Cholera Toxin**
Organism	Bordetella pertussis	Vibrio cholerae
Modifications	Ribosylation of a cysteine near carboxyl terminus of α-subunit	Ribosylation of an arginine residue
Action	Inhibition of signal	Prolongation of signal
Most probable classification of action	Interferes with the receptor mediated activation of G-protein	Inhibits the intrinsic GTPase activity thereby increases the lifetime of the active α-subunit
Effective against:	Gαβγ conformation	Gα-GTP conformation only
Subunit specificity	$G\alpha_i$, $G\alpha_o$, $G\alpha_t$	$G\alpha_s$, $G\alpha_t$

Table 1.4:   **A comparison of PTX/ cholera toxins (Barritt, 1992; Helmreich and Hofmann, 1996).**

Lipid modifications facilitate protein-protein and protein-membrane interaction of Gα-subunits at the N-terminus and the C-terminus. Isoprenoid modification of the γ subunit is responsible for the membrane association of the βγ complex (Wedegaertner *et al.*, 1995). These regions are relatively close together in the heterotrimer suggesting a site of membrane attachment. The acylation of the α subunit is thought to be responsible for its plasma membrane localisation (Hamm, 1998). Apart from membrane targeting, lipid modification also promotes subunit binding to effectors and inhibits association with protein regulators (described later) (Chen and Manning, 2001; Farazi *et al.*, 2001). Protein N-myristoylation is the covalent attachment of myristate, a 14 carbon saturated fatty acid, to the N terminal glycine of eukaryote and viral proteins (Farazi *et al.*, 2001). This amide linkage is irreversible (in most instances) and the reaction is catalysed by myristoyl CoA: protein N-myristoyl transferase (NMT), an enzyme that attaches the fatty acid to glycine (following cleavage of methionine) and sometimes to serine or threonine residues. $G\alpha_i$ subunits which contain a glycine/serine motif are substrates for NMT while $G\alpha_s$, $G\alpha_q$ and $G\alpha_{12}$ are not (Chen and Manning, 2001). Myristoylated proteins

interact weakly and reversibly with membranes and other proteins. N-myristoylation enables $G\alpha$ to interact with either $\beta\gamma$, the cell surface (due to moderate hydrophobicity) or to be targeted for palmitoylation (Chen and Manning, 2000)

Some $G\alpha$ subunits undergo post-translational covalent modification with one or more palmitoyl groups after N-myristoylation. This involves the attachment of palmitate (C16:0) to a cysteine residue near the N-terminus (of $G\alpha$ subunits) through a thioester bond. Palmitoylation is reversible and can be achieved enzymatically or non-enzymatically (Chen and Manning, 2000). $G\alpha_s$ and $G\alpha_{12}$ are palmitoylated at one site and $G\alpha_q$ and $G\alpha_{13}$ at potentially two sites. The reversibility of palmitoylation provides a mechanism for regulated interactions between N-myristoylated cellular membranes and /or other proteins.

1.4. REGULATION OF GPCR ACTIVATION

GPCRs not only associate with G-proteins but also a variety of other proteins (including other receptors) that control receptor localisation and/or trafficking and ligand binding properties of the receptor and hence modulate receptor signalling.

1.4.1. Receptor dimerisation

GPCRs have been reported to interact with other GPCRs to form receptor dimers and this interaction may influence receptor activation. Receptor dimerisation is being extensively researched and it is a phenomenon that may change the way and efficiency in which receptors recognize the agonist and the way they interact with the heterotrimeric G-protein complex (Bockaert and Pin, 1999). The function of receptor dimerisation has been addressed either through the use of peptides that block dimerisation or by investigating mutant receptors that do not dimerise (Gomes *et al.*, 2001). Stable heterodimerization between AT_1 and the bradykinin receptor (B_2)

caused an increase in activation of both $G\alpha_q$ and $G\alpha_{i1}$ proteins sharing the signal enhancement triggered by this interaction of receptors (Bond and Notides, 1987). Although several lines of evidence suggest that GPCRs can and do dimerise, it remains to be established whether this phenomenon is a general characteristic of all receptors in the superfamily. Therefore the importance of dimerisation in terms of receptor function is clearly an unresolved issue (Nature Reviews Drug Discovery GPCR Questionnaire Participants., 2004[1]).

1.4.2. Regulators of G-protein signalling (RGS)

The recently discovered family of RGS (Regulators of G-protein Signalling) proteins consists of more than 20 members. They function in part by acting as G-protein activating proteins (GAPs). The GAP activity reduces the lifetime of active GTP bound α subunits and increases the kinetics of signal termination. These effects are mediated by the RGS domain of approximately 120 amino acids that are highly conserved in the family (Hoffmann *et al.*, 2001). Various researchers have shown that most RGS proteins are selective in the Gα subunit. Hoffmann *et al.* (2001) showed rank order of effectiveness of different RGS proteins on α_2-adrenoreceptor implying selectivity. There is also evidence suggesting that RGS function to stabilise those G-proteins already in the transition state to enhance hydrolysis, in contrast to inducing a large conformation change as previously proposed (Lan *et al.*, 2000). There has been much recent interest into looking at the inhibition of RGS proteins as possible therapeutics targets (Neubig and Siderovski, 2002).

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[1]This reference was a whole feature in Nature Reviews and represents the contemporary view of 20 experts in the GPCR field. The participants were asked to discuss questions regarding emerging technologies for GPCR research, drug screening and where they anticipate the next wave of opportunity will fall.

1.4.3.   G-protein receptor kinases (GRKs)

Sustained stimulation of GPCRs has been shown to lead to decreased receptor responsiveness, a phenomena termed receptor desensitisation. This has been particularly well studied in the β-adrenergic receptor system (Helmreich and Hofmann, 1996) and families of regulatory molecules have been found to participate in desensitisation of GPCRs. These include second messenger-regulated kinases (eg protein kinase A and protein kinase C), G-protein receptor kinases (GRKs) and the family of arrestin/protein scaffolds (Lefkowitz, 1998). Desensitisation can be homologous, that is, involving only those receptors that have been activated by a given agonist, or the process can be more generalised (Helmreich and Hofmann, 1996). GRKs induce rapid, agonist-induced desensitisation of the receptor by C-terminal phosphorylation (Schnabel and Bohm, 1996). Once phosphorylated, the arrestins can bind to the GPCR preventing G-protein coupling (short-term desensitisation) and then target GPCRs to Clathrin-coated vesicles for endocytosis (GPCR internalisation) (Leurs *et al.*, 1998). Arrestins have been shown to associate with the receptor near the third intracellular loop (Hall and Lefkowitz, 2002).

The following section deals with measuring GPCR activity. In the past most of this activity has been explored using cell-based assays. It is important to recognise that this study is seeking to develop new technologies to facilitate the measurement of GPCR in a cell-free platform. The major challenge with this objective is to be able to incorporate the functional properties of these signalling complexes without having the biological complexities of a cell. Functionality of the cell-free signalling complex refers to the ability to induce receptor activated (upon cognate agonist addition) G-protein signalling. This signalling can be measured using radioactive techniques as

discussed later. The motivation to achieve such a cell-free functional platform is explored below.

1.5. MEASUREMENT OF GPCR ACTIVTY

Traditionally, the study of GPCR signalling has focused on classical second-messenger-generating systems, that is, by following downstream signalling events such as changes in intracellular Ca^{2+} or cAMP (cell-based assays). However, each GPCR subtype would be expected to stimulate not one but a large number of highly interconnected cytoplasmic signalling routes (Marinissen and Gutkind, 2001). Some cell-based approaches using heterologous expression systems[2] are discussed below. Although cellular assays are easily performed and can generate valuable information, they can be relatively expensive. In the section following, specific challenges and limitations of ligand screening approaches are examined and compared followed by a discussion on the development of functional cell-free approaches together with an assessment of the feasibility of such an approach.

1.5.1. Cell-based assays

GPCR activation in chromatophores from fish and frogs controls spectacular colour changes that can be detected using fluorescent and colorimetric techniques (Karlsson *et al.*, 2002; Lundstrom and Svensson, 1998). One important class of chromatophores is the melanin pigment containing specialised cells (melanophores). This pigment can disperse or aggregate depending on the stimulation and hence the degree of aggregation of the pigment can be monitored using simple optical techniques. Endogenously this downstream signal is linked to the adrenergic (α_2 adrenoreceptors) receptor subtype and thus activation detected by colorimetric changes in the melanophores has been used to detect noradrenaline changes

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[2] A heterologous expression system is one in which a protein (i.e. GPCR) is over expressed recombinantly that is normally not found in that system.

(Lundstrom and Svensson, 1998). The melanophores can also be functionally transfected with selected GPCRs such as human opioid receptors. This has lead to the development of a functional assay to detect opioids which could potentially be used to measure these compounds in body fluids such as blood, plasma or saliva (Karlsson *et al.*, 2002). Moreover, transfecting melanophores with constitutively active GPCRs may have specific applications for potentially screening compounds that may act as inverse agonists (Chalmers and Behan, 2002). Linking a down stream signal (in this case an optically detectable signal) to the activation of a specific GPCR forms the basis of these functional cell-based assays.

More sophisticated functional cell-based heterologous assays can be used to specifically classify GPCR ligand activity. Some agonists possess marked subtype selectivity in their efficacy and so assays are required for subtype selectivity discrimination (Jansson *et al.*, 1999). G-protein and receptor stoichiometry can easily be controlled in GPCR cell-based assays by using G-protein fusion proteins (Milligan, 2000) making these assays unique tools for exploring the basis of ligand efficacy. Studies have shown that the basic pharmacological features of full agonist function are preserved following construction of these fusion proteins (at least for the human adenosine A1 receptor and $G\alpha_{i1}$, $G\alpha_{i2}$ and $G\alpha_{i3}$). In addition, Watson and colleagues (2000) used a $G\alpha$-subunit enriched HEK cell system (also known as stimulus biased assays) to detect agonist-selective receptor active states. They observed different patterns of response to agonists in cells transfected with different G-protein subunits with some striking differences in relative potencies of the agonists. These stimulus-biased assay systems[3], have been used in whole cells to furnish unique information about agonists theoretically offering another level of

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[3] An assay whereby a GPCR is expressed recombinantly with an over expression of a certain G-protein subunit.

agonist selectivity (Watson *et al.*, 2000). Functional assays such as the methodologies discussed above are imperative for exploring different patterns of agonism involved in GPCR activation.

1.5.2.   Ligand screening assays

Determining the effect of a drug on a particular signal transduction pathway using a cell-based assay is complex as cellular regulatory mechanisms such as rapid receptor down regulation (Tang *et al.*, 2004) may hinder the feasibility and reproducibility of the process. In addition, signal transduction pathways are often transient in cells and some down stream signalling end points may be difficult to adapt to HTS (Waller *et al.*, 2003). In a single cellular assay, lack of efficacy for a certain biochemical event does not necessarily indicate lack of receptor activation (Gurwitz and Haring, 2003). Furthermore, partial agonism in such systems is often masked by spare receptors (Kenakin, 2002) and such cell-based screening is not suited to screening multiple receptors. Although cellular responses are insightful to toxicological effects (Johnston, 2002), the above limitations must be considered when exploring appropriate ligand screening platforms.

Another technology commonly used to screen ligands is target specific discovery platforms. These assays primarily address the non-functional structural dynamics of ligand binding (Moore and Rees, 2001). Evaluating molecular assemblies in a non-cellular manner may be more productive using new powerful, rapid and reliable nuclear magnetic resonance (NMR) spectroscopy based screening assay for the identification of inhibitors and for lead molecule identification (Dalvit *et al.*, 2003). This technology allows for the determination of the conformation of bound agonists to respective GPCRs and has the potential for high resolution structural determination. This approach would be an incremental advancement on the

conventional target specific discovery platforms that cannot distinguish between agonist and antagonist binding. For the most part both cell-based and target directed assays are conventionally utilised for the drug discovery campaign. However, the chemical diversity of screening compounds is rarely screened in both formats (Moore and Rees, 2001) which may lead to the dismissal of potential therapeutics. A suitable assay format is chosen based on the types of information required and this information is listed below (Moore and Rees, 2001):

1. Type of pharmacophoric/biological information sought.

2. Target type and cellular location (intracellular or membrane targets).

3. Preferred chemical sampling and screening strategy.

4. Stage of the project in the drug discovery process.

5. Technical/ logistical considerations

6. Financial burdens

Heterologous cell-based assays for drug screening have been designed for sensitivity, reproducibility and versatility (Nature Reviews Drug Discovery GPCR Questionnaire Participants., 2004). These assays have been shown to be well suited for screening targets within the cell membrane (cell adhesion inhibitors, ion channels and GPCRs). In contrast, isolated target approaches are preferred for intracellular targets (i.e. enzymes such as protein kinases and nuclear receptors). However, for membrane targets such as GPCRs, cell-based assay formats are simply preferred because the isolated target assay formats are somewhat limited in terms of functionality validation. In addition proximal events such as G-protein signalling are not measured or considered in either cell-based or isolated target assays (Nature Reviews Drug Discovery GPCR Questionnaire Participants., 2004). In fact, ligand efficacies and potencies compared at G-protein level and effector level in a given

signalling cascade were shown to be quite different (Seifert *et al.*, 1999). Membrane based functional assays offer the main benefits from both conventional assay formats and are increasingly becoming the format of choice for GPCR screening (Moore and Rees, 2001). Cell-free assays have the potential to extend the measurements of both cell-based and isolated target assays compared to other conventionally used methodologies as summarised in **Table 1.5**.

Isolated Target Assays	Cell-free functional screen	Cell-based functional screen
Early stage (lead discovery)	Both	Late stage (lead optimisation)
Kinetic mechanism of action	Both	Functional consequence of ligand activity
Displacement compounds only, allosteric modulators may be missed	Enable discernment between displacement compounds as well as allosteric modulators	Enable discernment between displacement compounds as well as allosteric modulators
Specific	Specificity can be controlled	Lack specificity due to complexities of cellular systems
HTS	HTS/minaturisable	HTS limited by cell size
G-protein signalling is not considered	Proximal endpoint –close to ligand binding events and G-protein signalling	Distal endpoint and monitoring earlier events is not conclusive.
Low system complexity	Moderate systems complexity	High system complexity

Table 1.5: **Comparison of current/potential methodologies used in the drug discovery process (Moore and Rees, 2001). Abbreviations: high throughput screening (HTS).**

The ligand screening platforms described above (including, heterologous cell-based assays) are unable to totally account for the cellular context of an endogenous system (Horrobin, 2003). There is a bewildering array of complicating factors such as GPCR homo- and hetero-dimerisation, interactive accessory proteins and regulatory protein networks. The importance of these factors on the physiological integrity of screening may influence the efficacy/behaviour of the drug *in vivo*. Furthermore, the proximity of a GPCR to a specific signalling molecule and the differences in cell machinery between cell systems and tissues may indicate that such complexities need to be considered (Nature Reviews Drug Discovery GPCR Questionnaire Participants.,

2004). Therefore, it is important to recognise that traditional cellular formats (as opposed to heterologous HTS cell-based assays) can never be replaced because they have application in secondary and tertiary screening prior to moving toward *ex vivo* and *in vivo* studies (Moore and Rees, 2001). Nevertheless, cell-free GPCR screening platforms may offer an alterative with specific advantages as discussed above to current HTS cell-based and isolated target assays.

1.5.3.   Functional cell–free assays

Measurement of cell-free GPCR signalling has been represented by Leifert *et al.* ((a) 2005) as five different levels whereby many different protocols/assays can be used to target the site of interest (see **Figure 1.3)**. Level 1 assays focus on the binding of ligands to the GPCR making them the perfect first port of call for discovery of novel ligands, however, these assays are non-functional. Conformational changes in the GPCR that can be measured upon agonist induction form the basis of the level 2 cell-free assays. There have been limited reports on this mode of assay (Ge and Selvin, 2004). The functional assays directed at the early signal transduction event of GTP binding was referred to as level 3 assays. These assays are discussed in more detail in Chapter 3. The next two levels downstream, further enhance the amount and type of information generated from functional GPCR assays thus, they may set up more possibilities for immobilisation and subsequent measurement in the development of HTS cell-free assays. Some of these levels will be discussed in further detail within the appropriate chapters in this thesis.
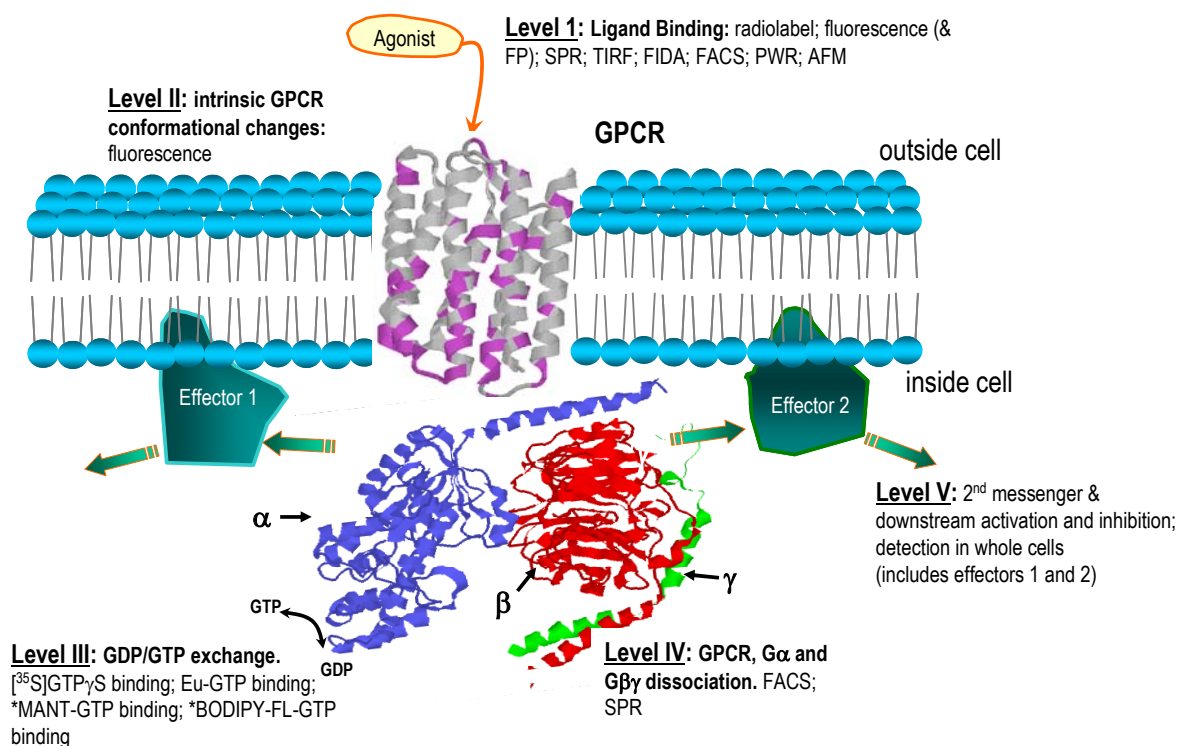
Figure 1.3: **Schematic representation of the GPCR signalling complex.**
The "levels" of signalling that may be exploited for detection in a cell-free assay are also shown (only levels I – IV are discussed in this review). A representative 7-transmembrane spanning GPCR (the rhodopsin receptor) is shown as a schematic, Gα is shown in dark blue, Gβ is shown in red and Gγ is shown in light green associated with the Gβ-subunit. The Gα and Gβγ subunits ribbon structures were created using "Protein Explorer". The size of the GPCR, G-proteins, effector proteins and lipid bilayer are not drawn to scale from Leifert *et al*. ((a) 2005). **Abbreviations:** AFM: atomic force microscopy, BODIPY-FL-GTP: 4,4-difluoro-4-bora-3a,4a-diaza-*s*-indacene labelled GTP, Eu-GTP: europium-labelled GTP, FACS: fluorescence activated cell sorting, FIDA: Fluorescence intensity distribution analysis, FP: fluorescence polarization, GDP: guanosine diphosphate, GPCR: G-protein coupled receptor, GTP: guanosine triphosphate, MANT-GTP: *N*-methyl-3'-*O*-anthranoyl labelled GTP, PWR: plasmon waveguide resonance, SPR: surface plasmon resonance, TIRF: total internal reflection fluorescence, *indicates that these fluorescent probes have been used for investigating G-protein interactions only.

Heterotrimeric G-proteins are key players in a network of interacting signalling pathways that operate in most mammalian cells. The complex G-protein mediated signalling system is subject to different regulatory processes. Many successful drugs today were identified 30-40 years ago using traditional pharmacological "gut bath" methodology in functional (endogenous) cell-based assay systems (Moore and Rees, 2001). Thus, this demonstrates the success of cell-based drug discovery. However, currently higher throughput target based assays together with heterologous cell-based assay have replaced these traditional approaches, and their limitations/advantages

were discussed. Novel technologies are required that enable high throughput lead compound validation without compromising on functionality. Using cell- free assays, receptor activation can be studied and interrogated under conditions that circumvent the complications that occur in a cellular system while maintaining functionality.

1.6. AIMS OF THIS PROJECT

1) To obtain a source of GPCRs and G-proteins that can be reconstituted as a functional signalling complex ("transductosome").

2) To validate the functionality of this artificial transductosome complex and to increase the throughput of this assay into a 96-well format.

To capture the activated transductosome complex on to a surface and validate functionality.

1.7. SIGNIFICANCE OF THE PROJECT

The study of disturbances in cell signalling processes is likely to have significant impact on the identification of novel biomarkers for disease, and in drug discovery. Therefore, developing new technologies for displaying integral membrane proteins, in a manner which will allow high throughput assays, would herald a significant development in diagnosis. GPCRs were chosen as the class of cell membrane signalling system because of their ubiquity, close correlation to many different diseases, and their interaction with G-proteins which will help in detection purposes. In the future it will be important to characterise the interactions between GPCRs and their cognate G-proteins in more detail as well as exploit these interactions for developing cell-free ligand screening assays. This will be pivotal in deriving more information on disease processes and for preventative health strategies. The novelty of this thesis lies in the application (s) of the developed approach to cell-free GPCR ligand screening.

Chapter 2.
GPCR and G-protein preparations

"Experience is what you get when you don't get what you want."

Baroness Professor Susan Greenfield

2.1. INTRODUCTION

This study focuses specifically on two different GPCR systems, the adrenergic receptor system and the angiotensin II receptor system.

2.1.1. Adrenergic receptors

The β-adrenergic receptor system has become a premier model system to study the nature and regulation of receptors. The ubiquity and diversity of the physiological responses that this system mediates makes it an ideal candidate to study GPCR signal transduction (Lefkowitz *et al.*, 1983). This transduction system is one of the major pathways mediating cardiac contraction (Yoshida *et al.*, 2001). The two major subtypes of this receptor are the β_1 and β_2 adrenergic receptors and both major subtypes in the heart activate adenylate cyclase via stimulatory $G\alpha_s$ to trigger the formation of cyclic AMP (Chakraborti *et al.*, 2000; Xiang and Kobilka, 2003). These two adrenergic receptor subtypes have been cloned along with β_3 and α_{1A}, α_{1B}, α_{1D}, α_{2A}, α_{2B} and α_{2C} adrenergic receptors and each is encoded by a distinct gene. Physiological agonists to the adrenergic receptors include the endogenous catecholamines adrenaline and noradrenaline (Small *et al.*, 2002). Synthetic agonists to the β_1AR include isoproterenol and iodocyanopindolol (ICYP) and antagonists include Propranolol and alprenolol (Shorr *et al.*, 1981).

The α_{2A}AR is widely expressed in the human body particularly being present in the cardiovascular system (Gao *et al.*, 2003) and, most commonly, in the central nervous system (Schwartz, 1997) and sections of the brain (Gonzalez-Maeso *et al.*, 2000). Through coupling with the $G\alpha_{i/o}$ family (Small *et al.*, 2002), α_{2A}AR activation results in physiological effects such as a decrease in blood pressure and mediation of voltage sensitive calcium channels in the sympathetic nervous system (Gao *et al.*, 2003;

Schwartz, 1997). Synthetic agonists to α_{2A}AR include UK-14304, iodoclonidine (partial agonist (Schwartz, 1997), and antagonists, including Yohimbine and Rauwolscine (Wade *et al.*, 2001).

2.1.2. Angiotensin receptors

Angiotensin II (Ang II) receptors have as their cognate ligand Ang II, which is a multi-functional 8 amino acid peptide product formed following the processing of the 10 amino acid precursor Ang I by Ang converting enzyme (ACE) (Berk, 1998). The peptide sequence of Ang II is Asp-Arg-Val-Try-Ile-His-Pro-Phe in the human, horse and pig (de Gasparo *et al.*, 2000). Ang II exerts diverse physiological effects including critical roles in hypertension (as a vasoconstrictor), inflammation, atherosclerosis and congestive heart failure (Berk, 1998). At least two high affinity receptors, designated as the Ang II type 1 receptor and the Ang II type 2 receptor, mediate the effects of this octapeptide (Guo *et al.*, 2001). The receptor is conventionally abbreviated to AT followed by a numerical subscript (de Gasparo *et al.*, 2000). AT_2 is highly expressed in fetal tissue (Capponi, 1996) whereas the tissue from adult vasculature, the kidneys, the adrenal gland, the heart, the liver and the brain have a large numbers of AT_1 receptors (Berk, 1998; Crane *et al.*, 1982).

The structure of both receptor subtypes (AT_1 and AT_2) indicates that they are both members of the GPCR family, and subtype selective antagonists have been used to distinguish between them (Guo *et al.*, 2001). The non-peptide biphenylimidazoles Losartan (Tamura *et al.*, 1997), candesartan and irbesartan (Dinh *et al.*, 2001) are selective AT_1 antagonists. Peptide agonists for this receptor include Ang II and its various peptide analogues such as saralasin (Hunyady *et al.*, 1996). Based on the two state model of receptor interaction, two types of antagonism; surmountable and insurmountable, have been identified in this receptor system (Verheijen *et al.*, 2003).

The first successful cloning of the AT_1 receptor in the early 1990s in rat vascular smooth muscle cells (Murphy *et al.*, 1991) and the bovine adrenal gland (Sasaki *et al.*, 1991) led to the identification of 2 distinct subtypes of rodent AT_1 (Guo *et al.*, 2001) which may explain the diverse actions of Ang II in different tissues (Iwai and Inagami, 1992). Although there is no noticeable difference in terms of ligand binding, the gene for the more predominant AT_{1A} is found on chromosome 17 and with AT_{1B} on chromosome 2 (Iwai and Inagami, 1992).

Activation of the AT_1 receptor has been shown to be triggered by a multistep process (Vauquelin and Van Liefde, 2005), mediated most often by $G\alpha_{q/11}$ signal transduction activating intracellular Ca^{2+} and the protein kinase C pathway (Guo *et al.*, 2001). AT_1 coupled to $G\alpha_{i/o}$ proteins can inhibit adenylate cyclase in several target tissues (Dinh *et al.*, 2001).

2.1.3. Muscarinic receptors (M_2)

The M_2 receptor was used in experiments described in Chapter 3 however it seems appropriate to describe them here. Briefly, muscarinic acetylcholine receptors are widely expressed in the human body being present in both the central nervous system and the periphery and are stimulated by acetylcholine release (Krejci *et al.*, 2004). There are 5 Muscarinic subtypes and the M_1, M_3 and M_5 subtypes preferentially couple to the $G\alpha_{q/11}$ pathway. Subtypes such as M_2 signal through the pertussis sensitive G-protein ($G\alpha_{i/o}$) pathway and their activation results in decrease in cAMP (Krejci *et al.*, 2004). Agonists to M_2 include acetylcholine and carbachol (Krejci *et al.*, 2004).

2.1.4.   Overview of radioligand binding assays

Ligand binding studies still remain a very popular technique to analyse receptor ligand interactions. Equilibrium experiments are used conventionally to calculate receptor number (B_{max}) and affinity (K_d) using one of two commonly performed experiments; saturation binding experiments or homologous displacement experiments (Rovati, 1998), both of which contain exactly the same type of information from a mathematical point of view. Heterologous displacement or competition binding experiments involves using a different chemical species to the tracer and these assays can be used to characterise the receptor in terms of rank order potency and agonist affinity (Rovati, 1998). Association and dissociation time courses are mainly used in preliminary phases of the characterisation of a receptor system to optimise some of the conditions for subsequent use in equilibrium experiments and to demonstrate the reversibility of the ligand –receptor interaction (Rovati, 1998). In the simplest case, only 3 conditions are necessary to demonstrate specific binding in a membrane (or whole cell) preparation. First, the total amount of radioligand binding on the filter, total binding, needs to be defined. Next, non-specific binding is defined, that is, the binding of the radioligand to other lower affinity sites that are not the receptor. Specific binding is the difference between total and non-specific binding. Finally, the total number of counts added to the assays must be determined and total binding should be less than 10% of the total number of counts added to the reaction abiding to the law of mass action[4] (Windh and Manning, 2002).

To study β-adrenoreceptors many different radio ligands have been characterised and utilised, each having a unique pharmacological profile. Iodinated iodocyanopindolol

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[4] This is a kinetic consideration that ensures that the concentration of free radioligand is approximately equal to the concentration added.

(ICYP) is a lipophilic antagonist that has been used to characterise the β_1AR receptors including turkey β_1AR (Shorr *et al.*, 1982). The hydrophilic βAR radioligand (-)-[^{3}H]CGP-12177 (Staehelin and Hertel, 1983) has been shown to be a partial agonist in various native heart tissues including rat (Joseph *et al.*, 2004) and in recombinant β_1AR (Pak and Fishman, 1996).

Several antagonist and agonist radioligands have been used to characterise the AT_1 receptor system. In whole cell assays, an antagonist ligand is preferred (Caballero-George *et al.*, 2003) as AT_1 receptors undergo rapid internalisation and desensitisation upon agonist stimulation (Guo *et al.*, 2001). Alternatively if receptor internalisation is prevented by lowering the temperature, an agonist may be used. However for membrane preparations either [^{125}I]Ang II or [^{125}I]Ang II (saralasin) have been used (de Gasparo *et al.*, 2000).

2.1.5.   Natural sources of GPCRs

The β-adrenergic receptor has been well characterised in a number of animals including turkey, frog and rat (Lefkowitz *et al.*, 2000). Interestingly, the plasma membranes of nucleated turkey erythrocytes (which contain β_1-adrenergic receptors coupled to adenylate cyclase) can be easily prepared and have been well characterised pharmacologically (Shorr *et al.*, 1982). Hence, they are a convenient source of the receptors and were initially chosen as a GPCR system to use in this study.

Although tissue density of AT_1 receptors varies between species, receptors are found in target tissues involved in its cardiovascular actions i.e. adrenal cortex, medulla, brain, kidney, vascular smooth muscle and heart (Capponi, 1996) and its

gluconeogenic and glycogenic actions in the liver (Sernia *et al.*, 1985). Many of the properties of the AT_1 receptors were first identified in studies in the adrenal gland and liver, both of which are abundant sources of receptors (de Gasparo *et al.*, 2000). Whereas two molecularly distinct types of AT receptors have been identified in mammals, only a single type has been identified in avians (Murphy *et al.*, 1993).

2.1.6. Recombinantly expressed GPCRs and G-proteins

Cloning technology enables the generation of receptor molecules and associated signal transducing and modulator proteins in sufficient quantities to permit study in isolation. Before the introduction of molecular techniques, GPCRs (as described above) and G-proteins (Dingus *et al.*, 2002) were only predominately isolated from natural sources. There are a few different types of recombinant expression systems available that have been utilised for the production of GPCRs and G-proteins and they have been reviewed in (Massotte, 2003; McIntire *et al.*, 2002). Some of the advantages and disadvantages of each system are recorded in the following (**Table 2.1**).

2.1.7. The baculovirus expression system

The baculovirus expression system permits high level expression of recombinant GPCRs and G-proteins with characteristics almost identical to native counterparts. Cells derived from the fall army worm *Spodoptera frugiperda* (*Sf*9), as the host cells, manufacture the required protein after infection with a recombinant baculovirus encoding the gene(s) to be expressed. These recombinant viruses are constructed by the introduction of a gene of interest by homologous recombination into the genome of the baculovirus. The powerful polyhedrin gene promoter (formerly from the *Autographica californica* nuclear polyhedrosis virus) is used to drive the expression

and post infection can usurp over 60% of the infected cells protein producing machinery (McIntire *et al.*, 2002).

Expression System	Advantage	Disadvantage
Bacteria e.g., *Eschericia coli*	• Many host species to choose from • Many DNA expression vectors available • Relatively cheap • Fast process and easy to scale-up • Yield can be very high	• Prokaryotic, not eukaryotic • Truncated proteins can be produced • The expressed proteins often do not fold properly and so are biologically inactive • Insufficient post-translational modifications made e.g., GPCR glycosylation, G-protein palmitoylation • Overexpression can be toxic to the host cells • Functional yield can be very low
Yeast e.g., *Saccharomyces cerevisiae*	• Eukaryotic • Fast process and relatively easy to scale-up • Yield can be very high • Relatively cheap • Performs many of the post-translational modifications made to human proteins	• Cell wall may hinder recovery of expressed proteins • Presence of active proteases that degrade foreign (expressed) proteins, therefore may reduce yield • Constitutive activity
Insect e.g., *Spodoptera frugiperda Sf9*, Hi-5	• High levels of expression • Correct folding • Post-translational modifications similar to those in mammalian cells • Biosafety issues minimal	• Expensive to up-scale • Slow generation time • Difficult to work with
Mammalian e.g., CHO, HEK, COS	• Good levels of expression • Correct folding and post-translational modifications	• Relatively low yields • Very expensive to up-scale • Slow generation time • Difficult to work with • Health and safety implications involved

Table 2.1: **Comparison of the advantages and disadvantages of various commonly used expression systems to obtain GPCRs and/or G-proteins (adapted from McMurchie and Leifert 2006).**

The baculovirus expression system is commonly used to produce GPCRs and G-proteins and successful over expression of these proteins has been achieved (Massotte, 2003). The amounts of GPCR generated from this expression system are several hundred fold higher than that which is typically seen in tissue systems where

natural expression is occurring. The artificial expression levels can range from 5-30 pmol/mg protein (which corresponds to 1-2 x10^6 receptor sites per cell) as reviewed in (Grisshammer and Tate, 1995). These receptor yields are constantly being improved and have, in some cases, well exceeded 30 pmol/mg protein (Massotte, 2003). Eukaryotic insect cells are able to perform post translational modifications identical to those of mammalian cells (i.e. phosphorylation, fatty acid acylation, glycosylation) and recombinant proteins exhibit characteristics very similar to their native mammalian counterparts (Massotte, 2003). Protein misfolding is a limitation of the prokaryotic expression systems, even though these systems can generate very large quantities of proteins.

2.1.8.   Urea treatment of membranes

The process of viral infection reduces the expression of native insect cell proteins including endogenous G-proteins (McIntire *et al.*, 2002). However, Gα_s and Gα_q have been unambiguously identified in *Sf*9 cells and inconclusive evidence shows the presence of members from the G$\alpha_{i/o}$ family (Massotte, 2003). Consequently, receptors in membranes prepared from these insect cells are able to couple with the native G-proteins (Massotte, 2003). This is not desirable in experiments aimed at studying GPCR interaction with specific exogenously supplied G-proteins (Lim and Neubig, 2001), and specifically not desirable for a commercial biosensor whereby a certain "effect" must be directly accountable to the constituents/composition of the assay. Several methods have been used to inactivate G-proteins including alkaline pH, pertussis toxin, CHAPS and the chaotropic agent urea (Lim and Neubig, 2001). Chaotropic agents denature proteins by disrupting hydrophobic interactions that normally stabilize native conformation. The authors demonstrated that urea extraction mainly denatures G-proteins *in situ* and that 5 M urea is sufficient to inactivate Gα subunits, while 7M is required to functionally inactivate $\beta\gamma$. They

showed that for multiple GPCRs (including the α_{2A}AR) in different cell lines (including insect cells), 7 M urea uncouples receptor from endogenous G-proteins (Lim and Neubig, 2001).

2.1.9.   High affinity binding assays

The affinity shift assay allows the measurement of the ability of the agonist to promote receptor-G-protein coupling which is evident experimentally as a shift in the receptor to a higher affinity state than is observed in the uncoupled receptor (Cabrera-Vera *et al.*, 2002). The high affinity state of a GPCR most likely represents the ternary complex of the receptor and heterotrimeric G-protein (minus guanine nucleotides) (Cabrera-Vera *et al.*, 2002). These assays have been used to determine the uncoupling effects of introduced mutations or unknown therapeutic compounds (Cabrera-Vera *et al.*, 2002). Furthermore, affinity shift assays have been used with GPCRs to show receptor coupling to G-proteins (McIntire *et al.*, 2002). This technique is very effective in assays which utilise receptors which are over-expressed in membranes from insect cells because over expression in these membranes often yields an excess of receptors over the available G-proteins. Urea treatment also has been shown to increase the fraction of cells in the low affinity uncoupled state (Lim and Neubig, 2001). This technique allows for interrogation for the best combination of subunits that restores high affinity agonist binding and enables investigation into studying the relationship between the subunits, receptor and ligand (McIntire *et al.*, 2002).

This chapter deals with various techniques used to isolate receptors and G-proteins in preparation for functional reconstitution. Specifically the chapter deals with;

1) Isolation/characterisation of β_1AR and AT$_1$ from natural sources.

2) Isolation/characterisation of β_1AR, α_{2A}AR and AT$_{1A}$ from expressed sources.

3) Purification of a repertoire of G-proteins from cloned sources to be used in functional reconstitution.

4) Restoration of high affinity binding in the AT_{1A} receptor system.

2.2.   MATERIALS AND METHODS

All general chemicals and reagents were of the highest grade available and purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2.1.   Membrane preparations from natural sources

2.2.1.1.   Membranes containing β_1AR from turkey erythrocyte

The plasma membrane enriched in β-adrenergic receptors were isolated from turkey erythrocytes using a method modified from that of (Shorr *et al.*, 1982). Whole turkey blood (2 L) was obtained from live turkeys (Aldinga turkeys, Aldinga SA). The blood was placed into 1L plastic containers with 50 mL of 100 mM EDTA and delivered on ice. The isolation was performed on the day that the blood was collected. Blood was poured through 4 layers of wide-weave cheese cloth and spun at 500 g in a Beckman JA-14 rotor (Beckman J2-21 centrifuge) at 4ºC for 10 min. The supernatant and fluffy coat was discarded by vacuum aspiration. The pellet was washed twice with buffered saline (140 mM NaCl, 5 mM $MgCl_2$, 10 mM Tris-HCl pH 7.4) and protease inhibitors at a final concentration of 100 μg/mL bacitracin, 200 μM benzamidine and 30 μM phenylmethylulfonyl fluoride (PMSF). The centrifugation was repeated and the pellet was resuspended in high Tris (HT) buffer (75 mM Tris, 24 mM $MgCl_2$, 1mM EDTA, pH 7.4) containing protease inhibitors. Erythrocytes (200 mL) were homogenised using short bursts (5-10 sec) on a large polytron tissue homogeniser (Kinematica, GmbH, Switzerland) on maximum setting for a total time of 1 min. Membranes were obtained by centrifuging these homogenised cells at 40,000 g (using a JA 20 rotor in a Beckman ultracentrifuge) for 15 min. The supernatant was discarded and the pellet resuspended in HT buffer and protease inhibitors and homogenised with 10 passes of a large dounce glass homogeniser. The membranes were re-centrifuged as above, washed and

homogenised 3-4 times until the pellet was a pink/white colour. Then, the pellet was resuspended in a small volume of HT buffer and protease inhibitors (10 mL), aliquoted and snap frozen in liquid N_2. Membranes were stored at -80°C until required. Protein concentration was determined by Lowry *et al.* (1951) in the range of 8-10 mg/mL per preparation.

2.2.1.2. Membranes containing AT_1 from rat liver

Plasma membranes enriched in AT_1 receptors were isolated from rat liver (using a method by Prpic *et al.*, (1984)). The liver (1.4 g) from a male Sprague Dawley rat was removed and placed in ice cold isolation media (250 mM sucrose, 5 mM potassium HEPES, 1 mM EDTA, pH 7.4) containing protease inhibitors at a final concentration of 100 μg/mL bacitracin, 200 μM benzamidine and 30 μM PMSF. The tissue was minced with scissors and homogenised with 13 passes with a loose fitting Dounce homogeniser (Wheaton) followed by 3 passes with a tight fitting homogeniser. The homogenate was diluted to 6% (w/v) with isolation buffer and centrifuged at 1400 *g* for 10 min at 4°C using a Beckman JA 20 rotor in a Beckman J2-21 centrifuge. The pellet was resuspended to 6% (w/v) in isolation media and with 4 passes of the tight fitting homogeniser. Density-gradient medium, Percoll (Amersham Biosciences, Melbourne, VIC) was added at 13% (v/v) of liver homogenate and this mixture was centrifuged at 34,000 *g* for 30 min at 4°C using 50.2Ti rotor in the Beckman ultracentrifuge. Two distinct layers close to the top of the tube were revealed and the top fluffy layer was recovered and diluted 1:5 in incubation media (250 mM sucrose, 50 mM Tris pH 8.0) containing protease inhibitors (same as previous). This layer was centrifuged at 34,000 *g* for 30 min at 4°C and the resulting pellet was resuspended in approximately 2 mL of incubation media. After 3-5 passes in the glass homogeniser the membranes were aliquoted to approx 10 mg/mL protein and snap frozen in liquid N_2. The membranes were stored

at -80°C until required. Protein concentration was determined by the method of Lowry *et al.*, (1951).

2.2.1.3. Membranes containing AT$_1$ from turkey liver

Large amounts of enriched plasma membranes (containing AT$_1$ receptors) were isolated from turkey liver. Fresh turkey liver (approx 17 g) were obtained from Aldinga Turkey (Aldinga, SA) and the liver was couriered on ice in isolation media without protease inhibitors. The membranes were prepared exactly as previously described for rat liver membranes (see above).

2.2.2. Baculovirus expression system

2.2.2.1. Insect cell culture

Spodoptera frugiperda (*Sf*9) insect cells (Invitrogen, Mt Waverley, VIC) which were adapted to serum free media were chosen overcoming the requirement for expensive and time consuming serum media which facilitated scale up (Massotte, 2003). The semi-adherent insect cells were grown in suspension *Sf*900II (serum-free media) (Invitrogen, Mt Waverly, VIC) using Schott bottles. Cells were cultured at 28°C (non-humidified) with agitation at 140 oscillations/min in an orbital shaker. A more consistent atmospheric exposure for the cells was obtained by maximising cell culture volume to one quarter that of the total bottle volume. For example. for a 2 L bottle, a 500 mL culture was used, and loosening the cap to allow for aeration. Cells were counted using a haemocytometer by standard methods and cell viability was determined by trypan blue staining. Cells were subcultured back to a density of 0.5 x 10^6 cells/ml when the cell density exceeded 2 x10^6 cells/ml (the cells are growing in the logarithmic stage). Each subculture was referred to as a passage. As a general rule, cells were only used to passage 60. Stocks of *Sf*9 cells (2 x10^6 cells/mL with a cell viability greater than 90%) were preserved in sterile preservation media (7.5%

DMSO (v/v) in SFM-900) and stocks were slowly frozen in liquid nitrogen according to instruction by GIBCO[5].

2.2.2.2.   Baculovirus amplification and infection

The baculovirus clones used were generous gifts from a number of international research institutes (see **Table 6.1** in Appendix page 235). Virus titre was assumed at 5×10^7 plaque forming units (PFM) /mL unless otherwise stated. A multiplicity of infection (MOI) of 2 is required for viral infection and 0.1 for viral amplification. The following formula was used to determine the volume of inoculum required for infection or virus amplification:

$$\text{Inoculum required (ml)} = \frac{\text{desired MOI x total no. cells}}{\text{viral titre (PFM)}}$$

For long term storage, baculovirus samples were kept in 2-4% (v/v) fetal bovine serum (Invitrogen, Mt Waverley, VIC) and maintained at 4°C in the dark to protect degradation of baculovirus DNA. For expression of G-proteins or receptors, *Sf*9 cells in log phase (i.e. 1.5 to 2 $\times 10^6$ cells/mL) were infected with the appropriate combinations of baculovirus with a multiplicity of infection (MOI) ratio of 1-2. Cells/membranes were harvested 72 hours post infection, when, ideally, the protein expression was maximal but large scale cell death had not occurred. Large scale cell death must be avoided as it leads to the release of a cocktail of proteases which will degrade the expressed protein (Massotte, 2003). Cell viability following 72 h infection was approximately 40-70% as determined by trypan blue staining.

2.2.2.3.   Membrane preparation from insect cells

Infected *Sf*9 cells (up to 2 L) were collected and centrifuged at 1000 *g* for 10 min and the pellet was washed carefully with ice cold phosphate buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4) and then

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[5] Invitrogen product manuals online: http://www.invitrogen.com/content/sfs/manuals/3408.pdf

centrifuged again at 1000 g for 10 min. The cell pellet was gently suspended in 200 mL ice-cold lysis buffer (50 mM HEPES, 0.1 mM EDTA, 3 mM MgCl$_2$, 10 mM β-mercaptoethanol, pH 8.0) with protease inhibitors; 0.02 mg/mL PMSF, 0.03 mg/mL benzamidine, 0.025 mg/mL bacitracin, 0.03 mg/mL Lima bean trypsin inhibitor (Sigma-Aldrich, St. Louis, MO, USA). All subsequent steps were performed at 4°C. Cells were subjected to N$_2$ cavitation at 500 psi for 15 min (using a custom made nitrogen cavitation chamber) followed by sedimentation of nuclei and unbroken cells (750 g, 10 min). Membranes were obtained by pelleting of the post-nuclear supernatant at 100,000 g for 30 min. The membrane pellets (from cells expressing receptors) were resuspended in incubation buffer (250 mM sucrose, 10 mM Tris, 3 mM MgCl$_2$, pH 8.0) or wash buffer (50 mM HEPES, 3 mM MgCl$_2$, 10 mM β-mercaptoethanol, 50 mM NaCl, 10 μM Guanosine Diphosphate (GDP), pH 8.0) containing fresh protease inhibitors, from cells expressing G-proteins. These resuspended pellets were homogenised by 10 strokes of a glass homogeniser, aliquoted and snap frozen in liquid nitrogen and stored at -80°C. Membranes from cells expressing receptors were urea treated as described below prior to snap freezing. Membranes from cells expressing G-proteins were stored at -80°C prior to purification (see section on page 63). Total protein concentration was determined using the Bradford protein assay (Bradford, 1976)[6].

2.2.2.4. Urea extraction

A modification of the methods of (Lim and Neubig, 2001; McIntire *et al.*, 2001) were used to remove endogenous G-proteins from *Sf*9 membranes expressing the β$_1$AR and α$_{2A}$AR. The 100,000 g membrane pellet was resuspended in (50 mL per 1 L of the original *Sf*9 cell culture) in incubation buffer (250 mM sucrose, 10 mM Tris,

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[6] From this point forward the Bradford method of protein determination was used in place of the Lowry method because it was more time efficient and was higher throughput.

3 mM MgCl$_2$, pH 8.0) containing 7 M urea (Sigma-Aldrich, St. Louis, MO, USA) and protease inhibitors. Urea stock solutions were prepared fresh by dissolution of urea crystals in wash buffer at room temperature. After 30 min incubation on ice with slow stirring, membranes were diluted to 4 M urea with incubation buffer and protease inhibitors and centrifuged at 100,000 g at 4ºC for 30 min. The urea-treated membrane pellet was resuspended to approximately 1-3 mg/mL protein and rapidly frozen in liquid N$_2$ and stored at -80ºC until use. Total protein concentration was determined using the Bradford protein assay (Bradford, 1976).

2.2.3. Mammalian expression system

2.2.3.1. Chinese Hamster cell culture

Chinese hamster ovary cells (CHO-K1) stably transfected with Clone 117 expressing rat AT$_{1A}$ receptor were a gift from Dr Walter Thomas (Baker Institute, Melbourne, VIC). The cells were obtained at passage 21. Adherent CHO-K1 cells with epithelial morphology were maintained in complete α-MEM medium. Incomplete media was maintained at 4ºC up to 2 weeks in the dark and prepared following the directions set out by Gibco BRL (Invitrogen, Mt Waverley, VIC). The media was completed by the addition of foetal bovine serum (FBS) (10% v/v), penicillin G sodium (100 μg/mL), streptomycin sulphate (100 μg/mL), amphotericin B (0.25 μg/mL) and geneticin (0.2 mg/mL) (purchased from Gibco BRL, Invitrogen, Mt Waverley, VIC). Subculturing (2-3 times per week) was performed when cells were sparse (W.Thomas personal communication) to optimise better monolayer coverage. Cultures were maintained in a humidified incubator with 5% CO$_2$ and kept at 37ºC. When cells were ready for subculturing, the monolayer was washed twice with sterile PBS (Surgical and Medical, Adelaide, SA) to remove the FBS which inhibits trypsin activity. For dissociation of the monolayer, a small volume (enough for monolayer coverage) of

Trypsin/0.53 mM EDTA (Invitrogen, Mt Waverley, VIC) was added, followed by a 5 min incubation at 37°C. Complete media containing FBS was added and the cells were either re-seeded or harvested for membrane preparation. Cells were counted using a haemocytometer and trypan blue staining was used to analyse cell viability. Cell stocks were maintained in liquid nitrogen. Cells were frozen at concentrations between 5-15 x 10^6 cells/mL in 1:2 ratio with freezing solution (FBS and 20% (v/v) DMSO). The final cell stock was frozen in 10% (v/v) DMSO.

2.2.3.2.   Membrane preparation containing rat AT$_{1A}$

CHO cell membranes were prepared using methods adapted from (Auger-Messier *et al.*, 2003; Inada *et al.*, 2002). Confluent monolayers were washed twice with PBS and then trypsinised (as above). Cells were resuspended in a small volume of complete media to inhibit trypsin activity and then centrifuged at 500 *g*. PBS was used to resuspend the cells and the centrifugation was repeated. Cells resuspended in PBS were either frozen in liquid nitrogen or used fresh. All subsequent steps were performed at 4°C. Cells were washed using an isotonic HEPES buffer (20 mM HEPES, 2.1 mM MgCl$_2$, 137 mM NaCl, 2.7 mM KCl, pH 7.4). A hypotonic buffer (10 mM Tris, pH 7.4) was used in some preliminary experiments to compare buffer composition, however, the isotonic buffer was determined to be preferable. Nitrogen cavitation (using a custom designed Parr bomb) was used to homogenise the cells. Another method of mechanical homogenisation using a Potter elvehjem homogeniser (15 passes using a Teflon drill piece) was investigated; however, this method was only used where indicated in the results section. Following homogenisation, unbroken cells were pelleted by centrifugation at 500 *g* for 10 min. The supernatant was then spun at 100,000 *g* for 40 min and this centrifugation step was repeated when the hypotonic buffer was used. Membranes were resuspended in HEPES buffer, aliquoted and snap frozen in liquid N$_2$, or alternatively, urea treated using the

following protocol. Total protein concentration was determined by using the Bradford assay {689}

2.2.3.3. Urea treatment

The membranes from CHO cells were urea treated exactly as described previously for insect cells. The urea-treated membrane pellet was resuspended to approximately 0.5-1.5 mg/mL protein (as concentrated as possible) and rapidly frozen in liquid N_2 and stored at -80 $^{\circ}$C until use.

2.2.3.4. Up scaling of stable CHO cell line

After the initial establishment of the CHO cell lines expressing the AT_{1A} was verified by radio ligand binding, it was necessary to investigate other means of obtaining large amounts of membrane protein as facilities were not available to upscale manufacture on site. Subsequently, production was up-scaled using roller bottles, performed by George Lovrecz at CSIRO, Parkville, VIC. Harvested cells were washed with PBS (as per section 2.2.3.2), snap frozen in liquid nitrogen and then transported to this laboratory (CSIRO, Adelaide, SA). Cell pellets were kept at -80°C until prepared into cell membranes using the same procedure as above.

2.2.4. Binding assays

2.2.4.1. (-)-[^{125}I]iodocyanopindolol and (-)-[^{3}H]CGP-12177 binding assay

Two receptor binding assays for detection of βAR receptors were optimised using the ligands [^{125}I](-)- Iodocyanopindolol (ICYP) and [^{3}H]CGP-12177. The approach was based on methods in (McMurchie *et al.*, 1987). These assays were used to verify and compare β_1AR activity in turkey erythrocyte membranes, rat cardiac membrane fractions and *Sf*9 cell membranes over expressing the β_1AR. Time course assays,

saturation binding isotherms and competition assays were performed and the assay conditions were optimised. $[^{125}I]$ICYP was purchased from Perkin Elmer Life Sciences (Boston, MA, USA) and had a specific activity of 2200 Ci/mmol. $[^{3}H]$CGP-12177 was purchased from NEN life science products (Boston, MA, USA) and the specific activity was 36 Ci/mmol. Fresh or once only thawed membrane protein (2.5-20 μg per assay) was incubated with either $[^{125}I]$ICYP (25- 700 pM) or $[^{3}H]$CGP-12177 (50-700 pM) and TEAM buffer (20 mM Tris, 1 mM EDTA, 1mM ascorbic acid, 10 mM $MgCl_2$, pH 7.4). Non-specific binding was determined by the addition of 10 μM (-) Propranolol (Sigma-Aldrich, St. Louis, MO, USA). Propanolol (specific β_1AR antagonist) and the specific α-adrenergic agonist (-) arterenol (Sigma-Aldrich, St. Louis, MO, USA) were used at the indicated concentrations to displace the radio ligand in competition binding experiments. Assays were incubated in 5 ml polypropylene tubes (in a total volume of 100 μL unless otherwise stated) and incubated for 60 min at 30ºC in a water bath. The addition of the radio ligand started the reaction and the reaction was terminated by rapid filtration over a glass fibre filters (GF/C; Whatman, Kent, UK) using a manifold apparatus (Millipore Australia, North Ryde, NSW). Filters were washed (3 x 4 mL) with TMN buffer (100 mM Tris pH 7.4, 125 mM $MgCl_2$, 500 mM NaCl). and air dried. For $[^{125}I]$ICYP assays, filters were counted in a LKB gamma counter. For $[^{3}H]$ detection, 4 mL of Ultima Gold^{TM} scintillation cocktail (Perkin Elmer Life Sciences, Boston, MA, USA) was added. The radioactivity was counted using a Wallac 1410 Liquid Scintillation counter (Pharmacia, Turku, Finland) with a counting efficiency of 57%.

2.2.4.2. [^{125}I] Angiotensin II binding assay for liver plasma membranes

Radio ligand binding assays using either [^{125}I] [Sar^1, Val^5, Ala^8]Ang II (saralasin) (Amersham Biosciences, Melbourne VIC; 2200 Ci/mmol) or [^{125}I]Ang II (Auspep, Parkville VIC; 2139 Ci/mmol) were performed to verify AT$_1$ receptor activity in the membrane preparations. Binding assays were performed according to a procedure by (Glossmann *et al.*, 1985). Membranes were thawed at room temperature and diluted in TN buffer (20 mM Tris, 100 NaCl, 0.2% BSA (w/v) pH 7.4) with fresh protease inhibitors added (30 μM PMSF, 0.03 mg/mL benzamidine, 100 μg/mL bacitracin) to a final concentration of 50-100 μg of protein/assay tube. For binding isotherms, displacement assays and time course assays, membranes were incubated with 50-700 pM. Non radioactive Ang II (human) (Auspep, Parkville, VIC) and Ang II (saralasin) (Auspep, Parkville, VIC) were used to determine non-specific binding at 10 μM and stocks of these protein were prepared in TN buffer. Losartan (gift from Merck Sharp and Dohme) was also diluted in TN buffer. Assays, initiated by the addition of radioligand were incubated in 5 ml polypropylene tubes (in a total volume of 100 μL unless otherwise stated) and incubated for 60 min at 27 °C in a shaking water bath. The reaction was terminated by pouring the entire contents of the tube over a GF/C filter (pre-soaked in TMN stop buffer with 0.1% BSA (w/v) for 2 hour) on a manifold apparatus. The filters were washed 3 times with TMN buffer (100 mM Tris, 125 mM MgCl$_2$, 500 mM NaCl, pH 7.4) containing 0.1% BSA (w/v). The filters were carefully placed into clean 5ml polypropylene tubes and the tubes were counted for [^{125}I] using a LKB gamma counter.

2.2.4.3.   [^{125}I] Angiotensin II binding assay for CHO cell membranes

To verify that the CHO membranes expressed the AT_1 receptor, a radioligand binding assay utilising the agonist [^{125}I]Ang II was performed following previously published methods by (Auger-Messier *et al.*, 2003; Inada *et al.*, 2002), similar to that described above. The radioligand [^{125}I]Ang II was purchased from Protech (Melbourne, VIC) with specific activity of 2200 Ci/mmol. CHO cell membranes were diluted in binding buffer (50 mM Tris, 6.5 mM $MgCl_2$, 1 mM EDTA, 125 mM NaCl, 0.1% (v/w) BSA, pH 7.4) with fresh protease inhibitors added (20 mg/mL PMSF, 30 mg/mL benzamidine, 25 mg/mL bacitracin, 30 mg/mL soybean trypsin inhibitor). The stock of [^{125}I]Ang II was diluted to a working stock of 10 nM with binding buffer (without protease inhibitors) and kept at -20°C. Non radioactive Ang II (human) (Auspep, Parkville, VIC) at a concentration of 10 μM was used for the determination of non-specific binding. Non-specific binding was also determined using the Ang II receptor antagonist Losartan (gift from Merck Sharp and Dohme) at 10 μM diluted in binding buffer. Assays were initiated by the addition of the radioligand and incubated in 5 ml polypropylene tubes (in a total volume of 100 μL unless otherwise stated) and incubated for 60 min at 27 $^{\circ}$C in a water bath. The reaction was terminated and filtered as per previous section.

2.2.4.4.   [^{3}H]MK-912 binding

[^{3}H]MK-912 (Perkin Elmer Life Sciences, Boston, MA, USA) is a selective potent antagonist to the $\alpha_{2A}AR$ receptor and was used to verify that the *Sf*9 insect cell membranes contained appropriate receptor activity. Membranes were thawed at room temperature and diluted in TMN buffer (50 mM Tris, 100mM NaCl, 10 mM $MgCl_2$) plus 1 mM dithiothreitol (DTT) (added fresh each day), pH 7.6) to a final concentration of 20-90 μg of protein per assay tube. For binding isotherms,

membranes were incubated with 50-5000 pM [^{3}H]MK-912 in the presence of the potent α_{2A}AR antagonist 10 μM Yohimbine (Sigma-Aldrich, St. Louis, MO, USA) for 90 min at 27°C. Yohimbine was diluted from a 4 mM stock prepared in 50% (v/v) ethanol. Saturation binding isotherms were performed on each new membrane preparation. Binding was terminated by vacuum filtration over GF/C filters using a Millipore manifold apparatus. The filters were rinsed three times with 4 mL of ice cold TMN buffer, dried and placed in Picopro vials. Bound radioactivity was determined by liquid scintillation counting using 4 mL Ultima Gold^{TM} scintillation fluid per filter and a Wallac 1410 Liquid Scintillation counter with 57% counting efficiency for the [^{3}H] nucleotide.

2.2.5. G-protein purification

A modification of the method by (Kozasa and Gilman, 1995) was used for the G-protein purification. All steps were performed at 4°C unless otherwise stated. Frozen membranes (at $\geq$ 5 mg/mL protein) containing combinations of G-proteins (Gα and $\beta\gamma$ subunits) were thawed and diluted to 5 mg/mL protein with wash buffer containing fresh protease inhibitors and 1% (w/v) cholate (final concentration diluted from 20% stock). Membranes were stirred on ice for 1 hour to allow detergent extraction of proteins. The sample was then centrifuged at 100,000 g for 40 min and the supernatant was collected and diluted 5 fold with buffer A (20 mM HEPES, 100mM NaCl, 1mM MgCl$_2$, 10 mM β-mercaptoethanol, 0.5% (w/v) polyoxyethylene-10-lauryl ether (C$_{12}$E$_{10}$) and 10 μM GDP pH 8.0). The heterotrimer was purified from the membrane extract by immobilised metal affinity chromatography (IMAC) using the hexa-histidine tag (on either the Gα or γ subunits). The sample was loaded onto a 1 mL nickel-nitrilotriacetic acid (Ni-NTA) column (Qiagen Pty Ltd, Clifton Hill, VIC) and the void volume was allowed to pass

through (under gravity). Then, the sample was collected and re-applied to the column. The column containing the histidine tagged subunit(s) was washed with 50 mL of buffer A containing 5 mM imidazole and 300 mM NaCl to remove non-specifically bound proteins. The column was warmed to room temperature ($22^{o}C$) and non-histidine tagged subunits were eluted from the column with 1 mL (void volume) fractions of buffer E (20 mM HEPES, 50 mM NaCl, 10 mM β-mercaptoethanol, 10 μM GDP, 1 % (w/v) cholate, 50 mM $MgCl_2$, 5 mM imidazole, pH 8.0) also containing 10 mM NaF and 30 μM $AlCl_3$ (AlF_4^-). The remaining histidine tagged subunits were eluted from the column with buffer E containing 150 mM imidazole. Confirmation of protein expression was verified by SDS polyacrylamide gel electrophoresis. Fractions containing protein were pooled and injected into either a 7 kDa (Gα) or 3.5kDa (βγ) "Slide-A-Lyzer®" dialysis cassette (Pierce Chemical Company., Rockford, IL, USA). Dialysis was carried out according to manufacturer's instructions, against several changes of buffer F (20 mM HEPES, 3 mM $MgCl_2$, 10 mM NaCl, 10mM β-mercaptoethanol, 1 μM GDP[7] and 0.1% (w/v) sodium cholate, pH 8.0). Dialyisis was performed to remove imidazole and/ AlF_4^-[8]. Protein concentration was determined by SDS-PAGE followed by laser densitometry which compared G-protein subunits against a bovine serum albumin (BSA) standard. Protein aliquots were snap frozen in liquid nitrogen and subsequently stored at −$80^{o}C$.

2.2.5.1.   Gel electrophoresis

Protein samples (20 μl) were combined with an equal amount of 2x sample buffer (BIO-RAD Laboratories, CA, USA) then heat denatured for 3 min at $100^{o}C$. After samples were cooled, 30 μl of sample was run on the precaste 15% gel (BIO-RAD

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[7] For the purification of $G\alpha_q$, Buffer F contained 5 μM GDP.
[8] Centricon was also investigated however this method was more tedious and for large preparations of G_proteins dialysis was the preferred method.

Laboratories, CA, USA) and run at 150V for 20 min and 200 V until dye front reached end of separating gel. Protein was detected by either coomassie blue (BIO-RAD Laboratories, CA, USA) or silver staining.

2.2.6. Data analysis

Data was analysed using Prism^{TM} (GraphPad Software Inc., San Diego CA, USA). Data are presented as mean and +/- SEM where (n) is greater than or equal to three. Where error bars are not visible they are hidden within the data point symbol. When n = 2, error bars where visible represent the range of duplicates. K_d and B_{max} were calculated in Prism^{TM} using non-linear regression analysis for one site binding. All data showing radioactivity bound, refer to specific binding of the ligand (i.e. total binding of radio ligand minus non-specific binding of the radio ligand). For radioligand displacement assays, the effective concentration at 50% (EC_{50}) or the inhibitory concentration at 50% (IC_{50}) was calculated in Prism using sigmoidal dose response. Statistical analysis (Students unpaired t-test) was performed using Prism^{TM}.

2.3.   RESULTS

The first part of this study focused on the β-adrenergic receptor system and the AT_1 receptor system, their native ligands being a catecholamine and a peptide respectively. Initially, both receptors were isolated from non-recombinant sources and receptor ligand binding activity was verified by radioligand binding using $[^{125}I](-)$- Iodocyanopindolol (ICYP) and $(-)-[^3H]CGP$-12177 for the $β_1AR$ and $[^{125}I]$Angiotensin II (human) and $[^{125}I]$Angiotensin II (saralasin) for the AT_1 receptor. In the second phase of the study, recombinant protein production techniques were introduced. The baculovirus expression system using *Sf*9 insect cells was investigated for over expression of GPCRs and G-proteins. The $β_1AR$ was expressed and this expression was verified and compared to the natural receptor using $(-)-[^3H]CGP$-12177 binding. G-protein subunits were also expressed and purified with the aim of reconstituting the receptor and G-proteins together in a functional "transductosome"(an *in vitro* reconstituted, signalling complex). Due to the inability to express and purify $Gα_s$ (which couples to $β_1AR$), the $α_{2A}AR$, which signals through the $Gα_{i1}$ pathway, was expressed and this expression was verified by $[^3H]$MK-912 antagonist binding. Some preliminary investigations into receptor purification were performed, however, urea-treatment was the only procedure routinely utilised.

Recombinant AT_1 could not be expressed using the baculovirus system, thus mammalian over expression was explored. CHO-K1 cells stably expressing AT_{1A} were used to obtain a recombinant source of the receptor. Reconstitution with both $Gα_{i1}$ and $Gα_q$ was evaluated with the aim of shifting the affinity of the agonist radioligand (Cabrera-Vera *et al.*, 2002). These results are explained in detail in the sections that follow.

2.3.1.   The β_1AR receptor from turkey erythrocytes

Membranes from turkey erythrocytes were prepared using differential centrifugation techniques in the presence of protease inhibitors (Shorr *et al.*, 1982). Freshly thawed membranes were examined for β_1AR binding using the radioligand [^{125}I]ICYP. Specific and total [^{125}I]ICYP binding increased over time as can be seen in **Figure 2.1**. Non-specific binding was minimal and less than 5% of total binding suggesting that 10 μM Propranolol was adequate to compete with the radioligand. Rat heart membranes were also prepared using a method by (McMurchie *et al.*, 1987) (data shown in the Appendix on page 235). However, non specific binding was between 40-60% and as a result this source was not considered further. Maximum [^{125}I]ICYP binding occurred after 15 min, which correlated well with published data (Shorr *et al.*, 1981).

Saturation binding was performed to quantify the β_1AR population capable of binding the antagonist radioligand in the turkey erythrocyte membranes prepared. [^{125}I]ICYP concentration was increased from 10 pM to 700 pM and binding plateued at about 500 pM. A one site binding curve was fitted to this data using Prism and shown in **Figure 2.1**. The K_d was calculated as 467 pM and the B_{max} was 1037 fmol/mg protein. Analysis of the data suggested a B_{max} closer to 600-650 fmol/mg protein which correlates to the data of (Shorr *et al.*, 1982). These workers reported B_{max} values between 600-800 fol/mg protein using a lipophilic radioligand [^{3}H] dihydroalprenolol.
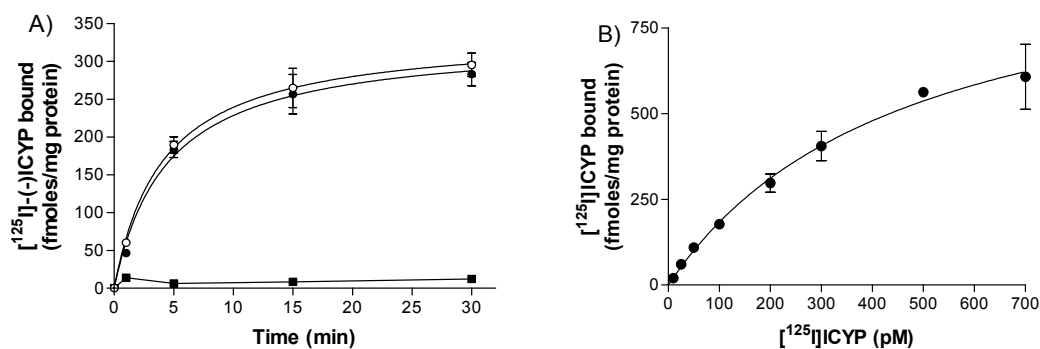
Figure 2.1: [^{125}I]ICYP binding to turkey erythrocyte membranes.
Assays were incubated as described in the materials and methods section ensuring the following: **A**) Membranes from turkey erythrocytes (0.5 mg/mL) were incubated with 300 pM [^{125}I]ICYP over time. **B**) Membranes from turkey erythrocytes (0.1 mg/mL) were incubated with [^{125}I]ICYP (concentrations as indicated) for 15 min. The dissociation constant (K_d) was calculated to be 467 pM and the receptor number (B_{max}) was determined to be 1037 fmoles/mg protein. Non-specific binding (■) was determined in the presence of 10 µM Propranolol (only shown in A. Total binding is represented by (o) and specific binding is represented by (●). Specific binding is defined as total binding minus non-specific binding. Each data point represents n = 3 samples, mean ± SEM. Where error bars are not visible they are contained within the data point.

To further characterise the turkey membrane preparations for β_1AR activity, competition binding curves were performed with Propranolol, a selective βAR antagonist, and arterenol, an α_1AR agonist (Cedazo-Minguez *et al.*, 2001) as depicted in **Figure 2.2**. A sigmoidal dose response curve was obtained with Propranolol displaying an inhibitory concentration at 50% (IC_{50}) of 13.2 nM. This value closely approximates with the literature for turkey erythrocyte membranes preparations whereby (De Lean *et al.*, 1982) found the IC_{50} for Propranolol to be 18 nM. In contrast, Propranolol (10 µM) did not compete with specific [^{125}I]ICYP binding added after radioligand binding had already occurred (i.e. after a 10 min incubation) (data not shown). This inability of the potent βAR antagonist to dissociate the radioligand may likely suggests that upon [^{125}I]ICYP binding the receptor is internalised (i.e. inside out vesicles) and Propranolol can not pass through the membrane to displace this radioligand or that [^{125}I]ICYP is not readily displaced. As expected, arterenol was not as effective in displacing the radio ligand being an α_1AR agonist and a much higher IC_{50} of 1.49 µM was obtained. Catechol (containing

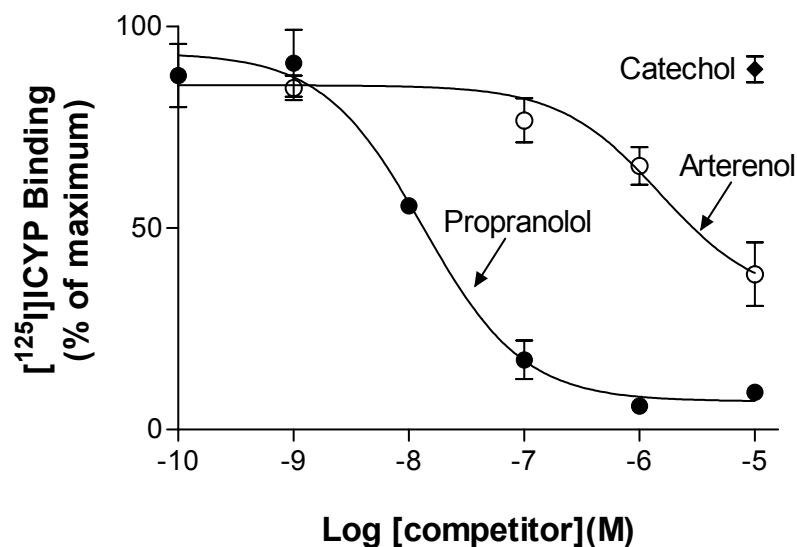the catecholamine ring structure that is evident in all catecholamines) added at 10 µM did not compete with binding.

Figure 2.2: **[^{125}I]ICYP competition binding curves for Propranolol and arterenol to turkey erythrocyte membranes.**
Assays were incubated as described in the materials and methods section ensuring the following: membranes from turkey erythrocytes (0.1 mg/mL) were incubated with [^{125}I]ICYP (300 pM) for 15 min. Competition of radioligand binding by Propranolol is shown by ($\bullet$) and the IC$_{50}$ was calculated as 13.2 nM. Competition of radio ligand binding by arterenol is represented by (o) and the IC$_{50}$ was calculated as greater than 1.49 µM. A high concentration of 10 µM catechol was also added which is shown by ($\blacklozenge$).Each data point represents n = 3 samples, mean ± SEM. Where error bars are not visible they are contained within the data point.

2.3.1.1. (-)-[^{3}H]CGP-12177 binding to β$_1$AR

Binding assays were repeated with the selective hydrophilic radioligand [^{3}H]CGP-12177 (which selectively binds to high affinity binding site of β$_1$AR (Riva and Creese, 1989)) in order to establish an alternative assay and for further verification purposes. In addition, there are advantages in using a titrated labelled radioisotope over an iodinated isotope such that the radioligand is chemically unaltered and thus biologically indistinguishable from the unlabelled compound and that it has a longer half life (Bylund and Toews, 1993). Freshly thawed turkey erythrocyte membranes were diluted 0.1 mg/mL protein and incubated with 300 pM [^{3}H]CGP-12177 for varying times. Specific and total binding increased over time as is illustrated in **Figure 2.3**. The antagonist Propranolol, was added to compete with the radioligand

binding and this was achieved. After 5 minutes, non-specific binding was less than 7% of total binding. Maximum binding occurred after 5 min using this radioligand compared to 15 min using [^{125}I]ICYP (see **Figure 2.1**). An incubation time of 10 min was chosen for further experimentation using this radioligand.

Non-specific binding increases with increasing receptor concentration and in order to minimise non-specific binding, membrane protein concentration and [^{3}H]CGP-12177 binding was investigated. Using freshly thawed turkey red blood cell membranes total protein concentration in the assay was increased from 10- 50 μg (see **Figure 2.3**) and β$_1$AR binding was assessed. Specific and total [^{3}H]CGP-12177 binding increased linearly with an increase in total membrane protein concentration as expected and non-specific binding was less than 6% of total binding.

In order to directly compare the usefulness of both radioligands in terms of determining β$_1$AR binding, a saturation binding isotherm was performed with [^{3}H]CGP-12177. Freshly thawed turkey erythrocyte membranes (0.25 mg/mL protein) were incubated with increasing concentrations of the radioligand. Non-specific binding, determined by adding Propranolol (at a final concentration of 10 μM) was kept to approximately 5.7% of total binding. A one site binding curve was fitted to this data using Prism as shown in **Figure 2.3**. A K_d of 365 pM was determined which was close to the K_d using [^{125}I]ICYP (**Figure 2.1**). The total number of receptors (B_{max}) was calculated to be 621 fmol/mg protein which was less than the 1037 fmol/mg protein calculated from the binding saturation isotherm with [^{125}I]ICYP. This correlates with the reported findings of others (Riva and Creese, 1989; Staehelin and Hertel, 1983) that [^{3}H]CGP-12177 is specific for cell surface receptors only whereas the more lipophilic radioligand ([^{125}I]ICYP) can bind to

receptors that have been internalised (i.e. inside out membrane ghosts in the case of this study). The determination of receptor binding activity is important as the aim is to have a preparation of receptors that will be used to reconstitute a functional preassembled signalling complex (transductosome) to be attached to a surface. In this case it might be more advantageous to use the hydrophilic radioligand to determine the total receptor number of exposed receptors and not all receptors.
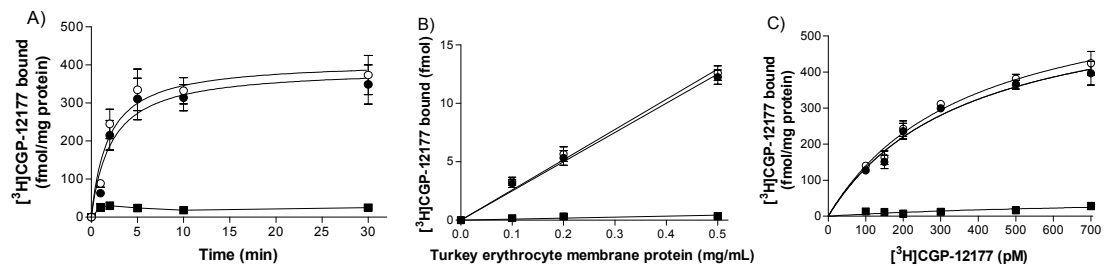

Figure 2.3: [³H]CGP-12177 binding to turkey erythrocyte membranes.
Assays were incubated as described in the materials and methods section ensuring the following: **A)** Membranes from turkey erythrocytes (0.1 mg/mL) were incubated with 300 pM [³H]CGP-12177 at 37°C for the specified time. **B)** Membranes (concentrations as indicated) from turkey erythrocytes were incubated with 300 pM [³H]CGP-12177 for 10 min. **C)** Membranes from turkey erythrocytes (0.25 mg/mL) were incubated with [³H]CGP-12177 (concentrations as indicated). The dissociation constant (K_d) was calculated to be 365 pM and the receptor number (B_{max}) was determined to be 621 fmoles/mg protein. Non-specific binding (■) was determined in the presence of 10 μM Propranolol. Total binding is represented by (o) and specific binding is represented by (●). Specific binding is defined as total binding minus non-specific binding. Each data point represents n = 3 samples, mean ± SEM. Where error bars are not visible they are contained within the data point.

2.3.2. The AT$_1$ receptor from natural sources

2.3.2.1. Liver membrane preparation

Enrichment of liver plasma membranes (containing receptors such as AT$_1$) has been achieved by using a self-forming Percoll gradient (as described previously by (Prpic *et al.*, 1984). A relatively quick and simple method, there were 2 distinct layers that appeared close to the top of the tube while all other material remained at the bottom. The second layer was shown to be enriched with plasma membrane markers (Prpic *et al.*, 1984) and thus this layer was kept. Self forming gradient Percoll consists of silica particles (15-30 nm diameter) coated with non-dialysable polyvinylpyrrolidone (a

non-toxic, inert chemical that doesn't adhere to membranes). Percoll gradients can be formed within the density range of 1-1.3 g/mL (technical notes[9]).

To verify that these plasma membrane enriched rat liver preparations had AT_1 binding activity, the agonist [^{125}I]Ang II (saralasin) radioligand was used. Initially, time dependent incubation of plasma membranes with [^{125}I]Ang II (saralasin) was investigated (see **Figure 2.4**). Specific and total [^{125}I] Ang (saralasin) binding increased over time and maximum agonist binding was completed in 30 min. Non-specific binding was determined in the presence of 10 μM Ang II (saralasin). Heat-denatured membrane protein did not bind [^{125}I]Ang II (saralasin) (data not shown) suggesting that the binding is specific to a non-denatured binding site. These data verify that the membranes prepared contained specific Ang II binding sites.

The specific [^{125}I]Ang (saralasin) binding to rat liver membranes was further characterised by competition binding analysis with Ang II (human), Ang II (saralasin) and Losartan. Freshly thawed membranes at a concentration of 1 mg/mL were incubated with 150 pM [^{125}I]Ang II (saralasin) and with increasing concentrations of the competing ligands as shown in **Figure 2.4**. The rank order potency obtained was Ang II (human) ≡ Ang II (saralasin)>Losartan which compares well with previous reports (Inada *et al.*, 2002). Both analogues of Ang II displayed similar affinities for the receptor with IC_{50} values calculated as 1.83 nM (Ang II human) and 2.2 nM (Ang II saralasin) and these values are in good agreement with recent reports (Inada *et al.*, 2002). Losartan, with an IC_{50} of 28 nM, was less effective in displacing the radioligand used in this study, and 7 fold less effective than previously reported efficacies for rat liver membrane (Inada *et al.*, 2002).

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[9] http://www5.amershambiosciences.com/aptrix/upp00919.nsf/Content/WD:Percoll,+Method

Nevertheless, these data further characterise the specific AT_1 binding activity of the rat liver membrane preparations.
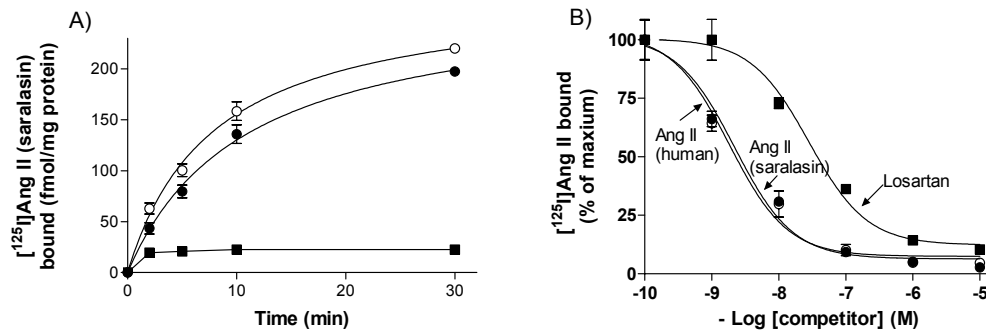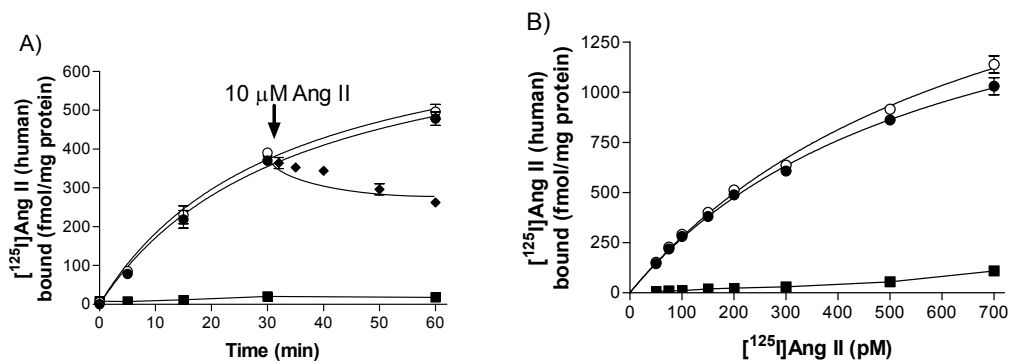

Figure 2.4: **[^{125}I]Ang II(saralasin) binding to rat liver membranes.**
Assays were incubated as described in the materials and methods section ensuring the following: **A)** membranes from rat liver (0.5 mg/mL) were incubated with 500 pM [^{125}I]Ang II(saralasin) at 27°C for the specified time. Non-specific binding (■) was determined in the presence of 10 μM Ang II (saralasin). Total binding is represented by (o) and specific binding is represented by (●). **B)** Membranes from rat liver (1 mg/mL) were incubated with 150 pM [^{125}I]Ang II(saralasin) at 27°C for 30 min. Radioligand binding was competed with Ang II (human) (●), Ang II (saralasin) (O) and Losartan (■). The IC_{50} were 1.83 nM, 2.2 nM and 28 nM for Ang II (human), Ang II (saralasin) and Losartan respectively. Each data point represents n = 3 samples, mean ± SEM. Where error bars are not visible they are contained within the data point.

Using the self-forming Percoll gradient procedure, turkey liver plasma membranes were prepared and then AT_1 binding activity was determined using the [^{125}I]Ang II binding assay[10]. Freshly thawed turkey liver membranes were assessed for time dependent radioligand binding (see **Figure 2.5**). As expected, specific and total [^{125}I]Ang II binding increased in a time dependent manner. Non-specific binding (binding in the presence of 10 μM Ang II) was less than 5% of total binding at all time points except at 5 minutes (where it was 8%), reflecting the lower amount of total membrane protein added in this assay (i.e. 10 fold less than the amount added in **Figure 2.4**). Displacement of the radioligand was monitored by the addition of unlabelled Ang II after a 30 minute incubation of turkey liver plasma membranes with [^{125}I]Ang II. Unlabelled Ang II displaced the labelled agonist by 48% and similar effects were evident using rat liver plasma membranes and in turkey erythrocytes (data not shown). Incomplete displacement is possibly due to receptor

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[10]The radioligand [^{125}I] Ang II (human) was purchased from Auspep and was considerably cheaper than [^{125}I] Ang II (saralasin) which was used in the previous binding assays.

internalisation upon exposure to an agonist or that the "off" binding is significantly slower than the "on" rate. Nevertheless, this data shows that liver plasma membranes specifically bind the agonist [^{125}I]Ang II over time indicating active AT_1 binding sites in this preparation.

Saturation binding isotherms are useful tools to quantify receptor numbers and determine some of the binding kinetics. Freshly thawed turkey liver membranes were incubated with increasing ranges of the agonist radioligand [^{125}I] Ang (50-700 pM) shown in **Figure 2.5**. Specific and total binding increased as the concentration of radioligand increased. However a plateau was not conclusively established. The Prism computer program was able to predict a B_{max} of 1896 fmol/mg protein and a K_d of 599 pM. This may reflect a biphasic binding pattern with a saturable high affinity binding site and a low affinity binding site. However, further characterisation of the turkey AT_1 would need to be performed as it was not an aim of this thesis.

Figure 2.5: [^{125}I]Ang II(human) binding to turkey liver membranes
Assays were incubated as described in the materials and methods section ensuring the following: **A)** Membranes from turkey liver (0.05 mg/mL) were incubated with 150 pM [^{125}I]Ang II (human) at 27°C for the specified time. At time 30 min, 10 μM Ang II was added and dissociation of [^{125}I]Ang II is shown by (♦). **B)** Membranes from turkey liver (0.05 mg/mL) were incubated with [^{125}I]Ang II(human) (concentration as indicated). The B_{max} was calculated as 1896 fmol/mg protein and K_d was 599 pM. Non-specific binding (■) was determined in the presence of 10 μM Ang II (human). Total binding is represented by (o) and specific binding is represented by (●). Each data point represents n = 3 samples, mean ± SEM. Where error bars are not visible they are contained within the data point.

To further characterise the prepared turkey liver membranes, competition binding curves were performed with Ang II (human), Ang II (saralasin) the non peptide antagonist Losartan shown in **Figure 2.6**. Both Losartan and Ang II(saralasin) did

not competitively displace [^{125}I]Ang II (human) at all the concentrations tested (0.1 nM - 10µM) which suggests that perhaps this membrane preparation did not contain the AT$_1$ receptor. Previous studies (Ji *et al.*, 1994; Murphy *et al.*, 1993) have suggested that avian (and amphibian) Ang II binding receptors may be pharmacologically more homologous to AT$_2$ receptors than mammalian AT$_1$ albeit showing structural homology to AT$_1$ receptors. Murphy *et al.* (1993) found that turkey adrenal membranes showed affinity (EC$_{50}$ reported at 17 nM) for both peptide analogues of Ang II, but not for Losartan. The data in **Figure 2.6** agrees with the findings reported by Murphy *et al.* (1993) for the Ang II (human) analog. However, a separate AT$_1$ receptor subtype that does not show affinity for Ang II (saralasin) may exist in turkey liver. Though interesting, this phenomenon was not investigated further as it was not considered a primary aim of this thesis.
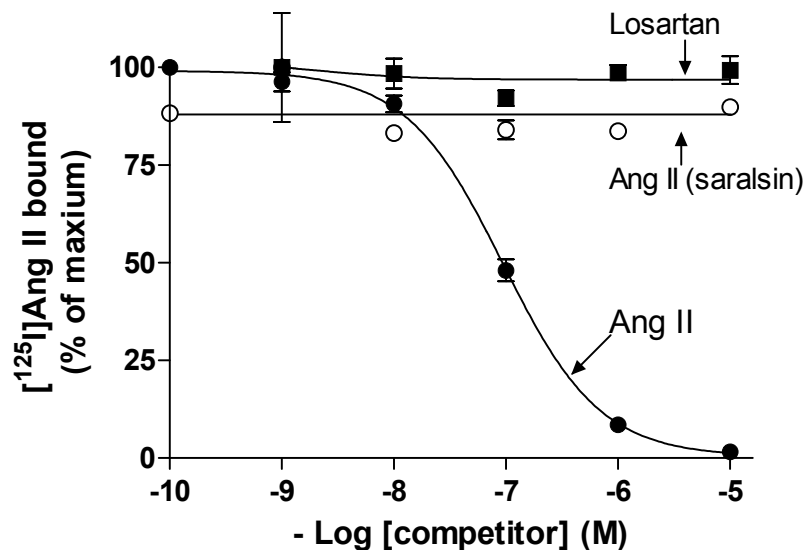
Figure 2.6: [^{125}I]Ang II(human) competition binding curves for Ang II (human), Ang II (saralasin) and Losartan to turkey liver membranes.
Assays were incubated as described in the materials and methods section ensuring the following: membranes from turkey liver (0.05 mg/mL) were incubated with 150 pM [^{125}I]Ang II(human). The assays were incubated at 27°C for 30 min. Radioligand binding was competed with using Ang II (human) (●), Ang II (saralasin) (o) and Losartan (■). The IC$_{50}$ was calculated as 94 nM for Ang II (human). Each data point represents n = 3 samples, mean ± SEM. Where error bars are not visible they are contained within the data point.

2.3.3.   Receptors from cloned sources: insect cell culture

The baculovirus/*Spodoptera frugiperda* (*Sf*9) insect cell expression system was chosen as an alternative to natural sources to express the GPCRs. Although this system has been well characterised for sourcing certain GPCRs and G-proteins previously (Massotte, 2003), it was necessary to establish and understand how this expression system worked, as these techniques (cell culture and expression techniques) were new to the laboratory. Culture techniques are included in the Appendix (page 238).

2.3.4. β_1 adrenergic receptor system: urea treatment

β_1AR receptor binding activity for membranes prepared from *Sf*9 cells were examined using the radioligand [^{3}H]CGP-12177. The membranes were treated with the chaotropic agent urea to remove endogenous G-proteins so that a pre determined complement of G-proteins in a specific stoichiometry could be used to reconstitute this receptor (Lim and Neubig, 2001). To rule out the possibility that antagonist binding is affected by the urea treatment of membranes, [^{3}H]CGP-12177 binding to urea treated and non-urea treated *Sf*9 cell membranes expressing the β_1AR was compared (**Figure 2.7**) No significant difference in the specific radio ligand binding (above concentrations of 100 pM [^{3}H]CGP-12177) between the two membrane preparations was found verifying that this treatment does not affect [^{3}H]CGP-12177 binding. Interestingly, the binding was substantially elevated for each concentration of radio label for the urea treated membrane set compared to the untreated membranes (and this increase was significant at 50 pM ($p<0.001$)). Thus, a higher B_{max} (4297 pmol/mg protein) and K_d (413 pM) was calculated in the urea-treated set compared with a B_{max} of 3853 pmol/mg protein and K_d of 483 pM for non-urea treated membranes. Heat denatured membranes did not show [^{3}H]CGP-12177

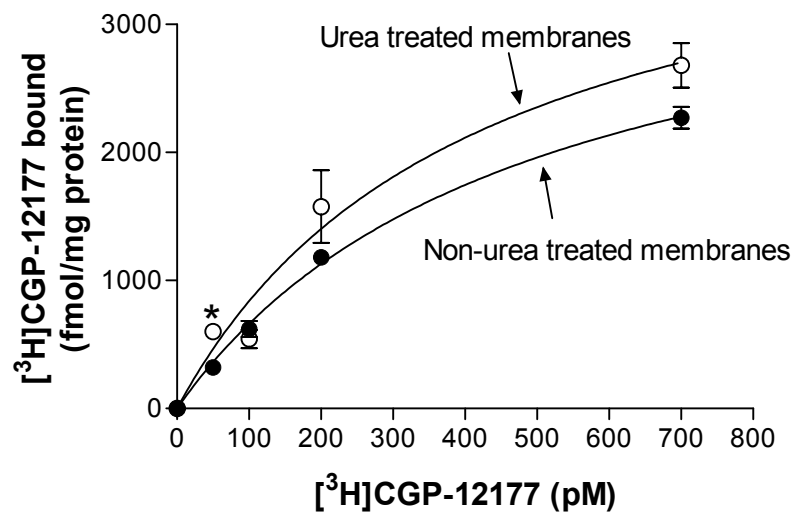binding (data not shown) suggesting that the binding observed is specific to the active protein.

Figure 2.7: **The effect of 7M urea on the specific binding of [³H]CGP-12177 to *Sf*9 membranes expressing β_1AR.**
Assays were incubated as described in the materials and methods section ensuring the following: *Sf*9 cell membranes over expressing β_1AR were incubated with [³H]CGP-12177 at various concentrations for 60 min at 30°C. Non-specific binding was determined by adding 10 μM Propranolol to the incubation mix. Specific binding is defined as total binding minus non-specific binding and is shown. Membranes that were treated with 7M urea are represented by open circles (o) and non-urea treated membranes are represented by closed circles (●).Each data point represents n = 3 samples, mean ± SEM. Where error bars are not visible they are contained within the data point. $p < 0.001$.

2.3.5. G-protein expression and purification.

Specific adsorption and elution from a Ni(NTA) column yields a highly enriched product (Kozasa and Gilman, 1995). SDS PAGE electrophoresis was used to confirm G-protein expression and purification for every purification performed. A representative coomassie stained gel is shown in **Figure 2.8**. G$\alpha_{i1\ (6xHIS)}$ (lane 2 **Figure 2.8**) is approximately 41 kDa which corresponds with the actual size of the subunit[11]. AlF$_4^-$ mimics GTP at the high affinity site allowing subunit dissociation (Higashijima *et al.*, 1987). The β_1 G-protein in lane 3 is approximately 37 kDa as expected from the amino acid sequence.

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[11] The website www.signalling-gateway.org/molecule provides up-to-date data regarding molecular sizes and other properties of G-proteins among other proteins.
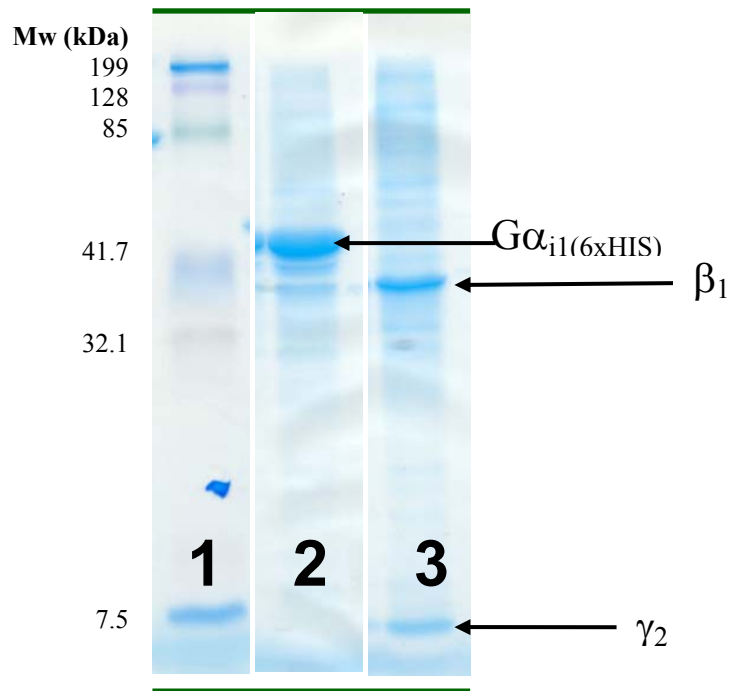
Figure 2.8: **A representative SDS PAGE gel electrophoresis photograph.**
G-Protein purification, G-proteins were resolved on a 10% acrylamide SDS-PAGE gel and coomassie blue stained. Lane 1 contains the molecular weight standard. Lane 2 contains $G\alpha_{i1HIS}$ (approximately 41 kDa). Lane 3 contains β_1 (approximately 37 kDa). The γ_2 subunit is approximately 8 kDa and runs with the tracking dye. The heterotrimer is dissociated by the addition of AlF_4^-, hence the $\beta_1\gamma_2$ dimer is eluted from the column and run on the gel whilst the $G\alpha_{i1(6xHIS)}$ protein remains associated with the column until it is eluted. Imidazole (150mM) can be used to remove the histidine tagged subunit $(G\alpha_{i1(6xHIS)})$. Dialysis was used to remove the AlF_4^- and imidazole.

2.3.5.1. The mystery of $G\alpha_s$

In order to reconstitute the β_1AR receptor to produce a functional transductosome which would mimic the initial stages of signal transduction, the $G\alpha_s$ subunit was required. Attempts to express and purify this subunit were unsuccessful. Three different recombinant baculovirus constructs were utilised in an attempt to generate this subunit including a version of the short $G\alpha_s$ gene. Similarly, Dr Michelle Glass and colleagues at the University of Auckland (personal communication) along with others (Kozasa and Gilman, 1995) found it difficult to express this protein using the baculoviral expression system due to low yields. Her group commonly used bacterial expression system to produce G-protein subunits. However, this system was not possible for use with $G\alpha_s$ as palmitoylation is not possible in *E.coli* (M. glass suggested cloning palmitoyl transferase). There was in fact no problem in obtaining

the $G\alpha_{i1}$ subunit and so it was decided to investigate functional reconstitution utilising a receptor system that signalled through this $G\alpha$ class.

2.3.6. α_{2A}-adrenergic receptor system

The α_{2A}AR signals through the $G\alpha_{i1}$ pathway and so the baculovirus expressing this receptor was obtained from Professor Richard Neubig (University of Michigan). This receptor system was previously characterised by other members of the laboratory and data pertaining to that aspect is not documented here. The radioactive antagonist [^{3}H]MK-912 was used to verify that the partially purified membranes contained the α_{2A}AR (shown in **Figure 2.9**). A saturation binding isotherm was performed on urea-treated membranes prepared from insect cells infected with the α_{2A}AR baculovirus. Non-specific binding was determined using the α_{2A}AR antagonist Yohimbine and greater than 95% specificity of [^{3}H]MK-912 was observed. The B_{max} was calculated to be 1.2 pmol/mg protein and the K_d was approximately 500 pM. Typical values of B_{max} were between 5 and 25 pmol/mg, and for the K_d values between 500 and 1000 pM were routinely achieved in this laboratory. These compare well with previously reported results (Uhlen *et al.*, 1994)

These membranes were then urea treated to partially purify the membranes and denature or remove endogenous G-proteins that may be associated with the receptor (Lim and Neubig, 2001). Urea treatment of insect cell membranes expressing the α_{2A}AR did not interfere with [^{3}H]MK-912 binding (see Appendix page 240).
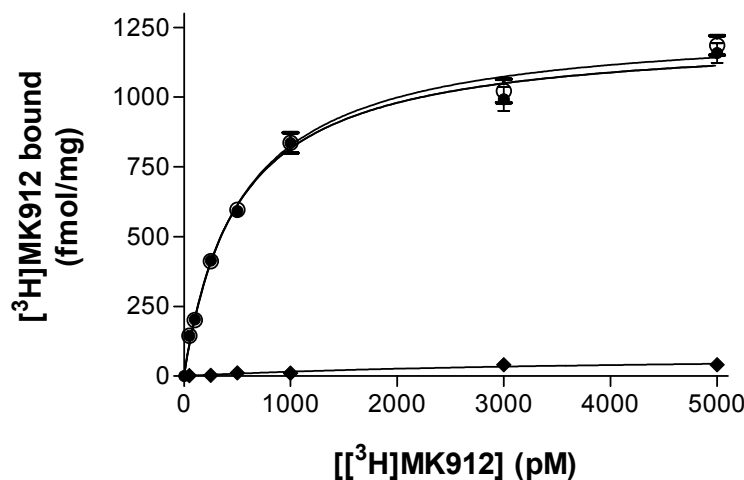
Figure 2.9: **Specific binding of [^{3}H]MK-912 to urea treated *Sf*9 membranes expressing α$_{2A}$AR: saturation binding isotherm.**

Assays were incubated as described in the materials and methods section ensuring the following: urea treated membranes expressing α$_{2A}$AR (0.2 mg/mL) were combined with increasing concentration of the α$_{2A}$AR antagonist [^{3}H]MK-912 and incubated for 90 min at 30°C. Non-specific binding was determined in the presence of 100 μM Yohimbine and is represented by filled diamonds (♦). Total binding is represented by open circles (o). Specific binding is shown as closed circles (●). The K$_d$ and B$_{max}$ were determined to be 499.8 pM and 1225 fmol/mg protein respectively. Each data point represents n = 3 samples, mean ± SEM. Where error bars are not visible they are contained within the data point.

2.3.7.   CHO cell membranes expressing the AT$_1$ receptor.

Membranes stably expressing the rat AT$_{1A}$ receptor from CHO cells, donated by Dr Walter Thomas (Baker institute, Melbourne) were prepared. Mammalian cell culture was established in this laboratory and preliminary observations are detailed in the appendix (see page 240). Radioligand binding using the agonist [^{125}I]Ang II had been demonstrated in whole cells with the expression levels calculated at between 500-600 fmol/mg protein prior to exportation to Adelaide at passage 19-20 (W.Thomas personal communication). AT$_{1A}$ expression levels in cell membranes from this clone had previously not been established by the Thomas laboratory thus it was necessary to characterise these membranes using the [^{125}I]Ang II binding assay.

Radioligand binding was employed to verify that the method of cell membrane preparation limited the damage to the AT$_{1A}$ receptor. Several different ways to prepare the membranes were investigated. Firstly, agonist binding was compared in

membranes prepared from freshly harvested cells to cells which had been once-thawed. The latter cells were harvested and then snap frozen in liquid N_2 and stored at -80°C until use. This enabled larger quantities of membrane to be prepared in batch mode. There was no statistical difference between [^{125}I]Ang binding as shown in **Figure 2.10** as a result of these differing procedures. The procedure for the preparation of cell membranes from a variety of cultured mammalian cells over expressing the AT_1 receptor has used either an isotonic isolation buffer (Auger-Messier *et al.*, 2003; Boucard *et al.*, 2003), or a hypotonic buffer (Lim and Neubig, 2001). Thus, the choice of isolation buffer was examined and the preliminary data showed that only an isotonic buffer was effective at preserving [^{125}I]Ang II binding properties in the prepared membranes (see **Figure 2.10**). Furthermore, various researchers have utilised many different methods for cell destruction and homogenisation (Auger-Messier *et al.*, 2003; Boucard *et al.*, 2003; Caballero-George *et al.*, 2003; Inada *et al.*, 2002). Potter elvehjem homogenisation and nitrogen cavitation were compared as methods of cell disruption/homogenisation with these CHO cell membrane preparations. Preliminary experiments showed that Potter elvehjem homogenisation was not visibly breaking up cells as effectively as nitrogen cavitation. Thus, the latter cell disruption method was utilised for future preparations.

A protein dose curve was performed (**Figure 2.11**) in order to establish the linearity of [^{125}I]Ang II receptor binding as a function of membrane protein concentration. As the amount of membrane protein added increased in the reaction, the amount bound increased linearly between the range of 0.5µg protein/assay and 10 µg protein/assay which corresponds to concentrations of 0.005 mg/mL and 0.1 mg/mL. Non-specific binding was determined using 10 µM Angiotensin II and was less than 8% of the total binding for protein concentrations above 0.02 mg/mL. Minimising the use of

AT$_{1A}$ containing membranes because of the expense and time investment associated with mammalian cell culture, was desirable. It should be noted that in a similar study (Inada *et al.*, 2002) between 10-18 µg protein per assay was used.
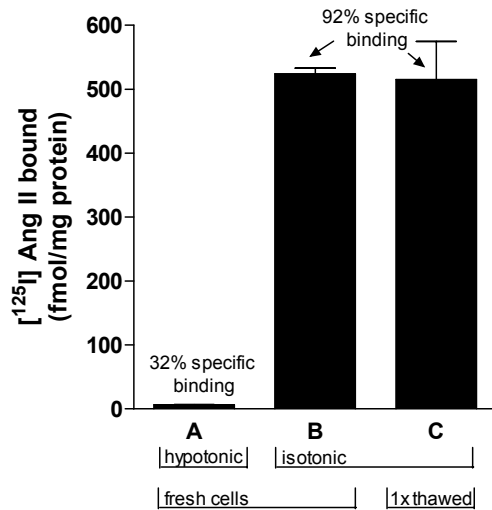

Figure 2.10: **Specific [^{125}I]Ang II binding to CHO cell membranes expressing the Angiotensin II receptor (AT$_1$) prepared in different ways.**
CHO cell membranes stably expressing AT$_{1A}$ were incubated in binding buffer (plus protease inhibitors and 0.1% (w/v) BSA) with 2 nM [^{125}I]Ang II for 60 min at 26°C. Non-specific binding was determined using 10 µM Ang II. All membranes were prepared using N$_2$ cavitation as method of cell disruption and the following differences; **A**) Hypotonic buffer was used with fresh CHO cells; **B**) Isotonic buffer with fresh CHO cells and **C**) Isotonic buffer with once thawed harvested cells. The reaction was terminated by filtering the total assay volume (150 µl) using 0.1% (w/v) BSA pre-soaked GF/C filters. Specific [^{125}I]Ang II binding is shown and was calculated as 32% for **A** and 92% for **B** and **C**. (n = 2, error bars where visible represent the range of duplicates).

With the intention of also conserving radioisotope, preliminarily experiments showed that a working total volume of 100 µl was sufficient for the binding assays. Other research groups used similar total volumes (Inada *et al.*, 2002) or even larger volumes of 400-500 µL (Auger-Messier *et al.*, 2003; Caballero-George *et al.*, 2003). Using a smaller total assay volume provided opportunities to minimise the amounts of other assay constituents such as purified G-proteins. Therefore, the assay was set at 0.02 mg/mL or 2 µg per 100 µL assay.
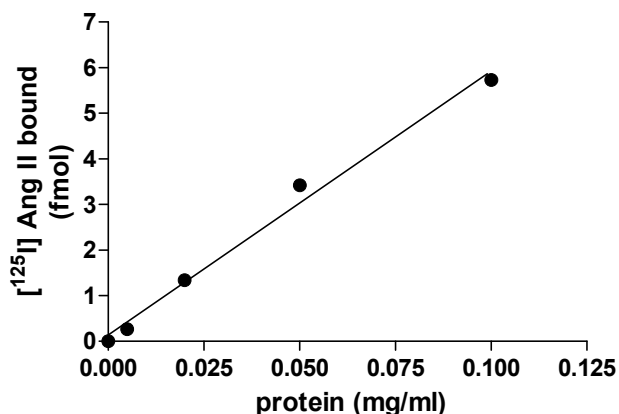
Figure 2.11: Specific [^{125}I]Ang II binding to increasing amounts of CHO cell membranes expressing the Angiotensin II receptor (AT$_{1A}$).
CHO cell membranes (amounts as indicated) stably expressing AT$_{1A}$ were incubated in binding buffer (plus protease inhibitors and 0.1% (w/v) BSA) with 2 nM [^{125}I]Ang II for 60 min at 26°C. Non-specific binding was determined using 10 μM Ang II. The reaction was terminated by filtering the total assay volume (100 μl) over GF/C filters pre-soaked with 0.1% (w/v) BSA and washing with 3x 4 mL volumes of TMN buffer with 0.1% (w/v) BSA. Specific [^{125}I]Ang II binding is shown (n = 2, error bars where visible represent the range of duplicates).

2.3.7.1. Agonist or antagonist to determine non-specific binding

Previously, non-specific binding was determined in the presence of unlabelled Ang II (human). Losartan, a non-peptide antagonist for the AT$_1$ receptor (Tamura *et al.*, 1997) was investigated in this system to compare the effectiveness of unlabelled agonist or antagonist to determine the extent of non-specific binding (data not shown). There was no significant difference between the specific binding (96%) in this receptor system. Therefore, Losartan was preferred because of its specificity to the AT$_1$ receptor and thus used in the further studies but either could be used.

2.3.7.2. Characterisation of the AT$_{1A}$ expressed on CHO cells

To further confirm that the membranes prepared from CHO cells were stably expressing the AT$_{1A}$ and to quantify expression levels, a saturation binding isotherm was performed. The specific [^{125}I]Ang II binding of non-urea treated CHO membranes is shown in **Figure 2.12**. The B$_{max}$ for this preparation was determined to be 626 fmol/mg protein and the K$_d$ was 0.85 nM. These values compared well with previously documented data from (Inada *et al.*, 2002). These researchers obtained a

B_{max} of 750 fmol/mg protein and a K_d of 0.52 nM for rat AT_{1A} expressed in COS 7 cells, suggesting that cell membrane preparations and assay conditions were adequate for the CHO cell-line stably expressing the rat AT_{1A}.
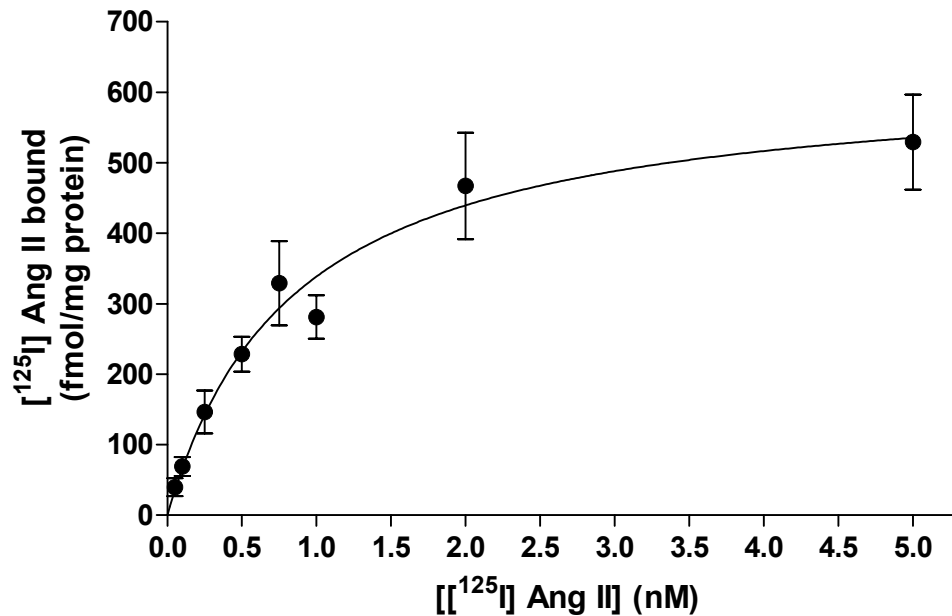

Figure 2.12: **Specific [^{125}I]Ang II binding to CHO cell membranes expressing the Angiotensin II receptor (AT_{1A}): Saturation binding isotherm.**
Assays were incubated as described in the materials and methods section ensuring the following; CHO cell membranes (0.02 mg/mL) stably expressing AT_{1A} were incubated with increasing concentrations of [^{125}I]Ang II for 60 min at 28°C. Non-specific binding was determined in the presence of 10 μM Losartan. Each data point represents n = 4 samples, mean ± SEM. Where error bars are not visible they are contained within the data point

The ability of the membranes to bind the agonist after a freeze/thaw cycle was determined. Re-freezing and thawing membranes caused a statistically significant 38% (p<0.001) reduction in the [^{125}I]Ang II binding to the AT_{1A} receptor in CHO cell membranes compared to binding to membranes that were freshly thawed (data not shown). As expected, heat denaturing the membranes abolished the binding of [^{125}I]Ang II (data not shown).

2.3.7.3. Passage number and biological variability

Further characterisation included examining the effect of passage number on the expression levels of stably transfected CHO cells. Membranes were simultaneously prepared from CHO cells harvested after 28 passages (low passage membranes) or

after 39 passages (high passage membranes). Preliminary data (not shown) suggested that high passage membranes were more effective in binding [^{125}I]Ang II suggesting a higher receptor expression level. Further investigation of this biological variation is shown in **Figure 2.13**. From this data, there was a statistically significant (67%) increase in specific [^{125}I]Ang II binding by using CHO cells that were harvested after a higher passage number (p< 0.001).
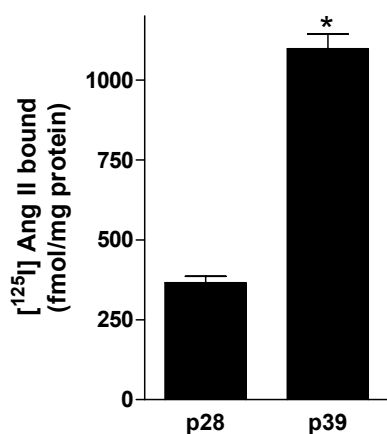

Figure 2.13: **Specific [^{125}I]Ang II binding to low or higher passage CHO cell membranes expressing the AT$_{1A}$ receptor.**
Assays were incubated as described in the materials and methods section ensuring the following; CHO cell membranes stably expressing AT$_{1A}$ were incubated with 2 nM [^{125}I]Ang II for 60 min at 26°C. Non-specific binding was determined in the presence of 10 μM Losartan. Membranes from low passage (p28) cells or higher passage (p39) cells are shown. Specific [^{125}I]Ang II binding is shown. Each data point represents n = 4 samples, mean ± SEM. Where error bars are not visible they are contained within the data point. * p< 0.001).

As mentioned, investigating the expression level of the CHO cells as passage number increases was not an objective of this thesis.

2.3.7.4.   The effect of urea treatment

CHO cells from passages between 35 and 39 were harvested and membranes were prepared and divided equally into two parts (untreated and 7M urea treated). In order to ensure that urea treatment did not affect receptor agonist binding functionality, [^{125}I]Ang II binding was determined and compared with simultaneously prepared non urea treated membranes. As previously mentioned urea treatment decreased the amount of membrane protein (Lim and Neubig, 2001) and this limited the

experiments that could be performed initially for investigation purposes. However, these preliminary results (shown in the Appendix on page 240) imply that urea treatment does not prevent or inhibit agonist binding to the receptor. Following these preliminary experiments, larger preparations of urea-treated membranes were prepared. Saturation binding using urea treated membranes from CHO cells harvested from passage 40-42 is demonstrated in **Figure 2.14**. The B_{max} was determined as 3448 fmol/mg protein which is comparatively higher than the B_{max} obtained from membranes that were prepared from non-urea treated membranes (**Figure 2.12**). The K_d was determined as 0.68 nM.
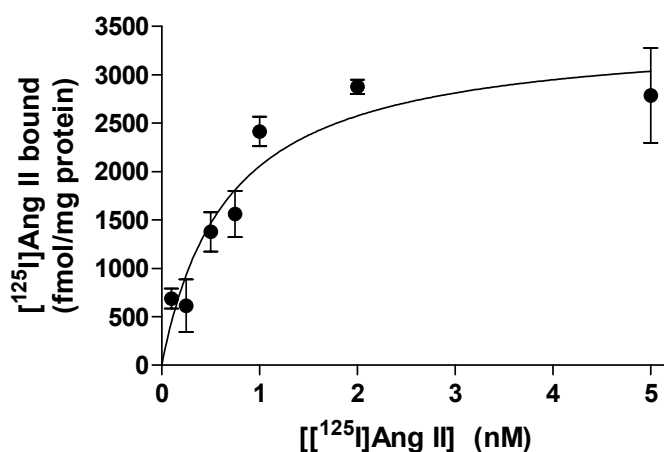

Figure 2.14: Specific [^{125}I]Ang II binding to urea treated CHO cell membranes expressing the AT_{1A} receptor: Saturation binding isotherm.
Urea treated CHO cell membranes (0.03 mg/mL) stably expressing AT_{1A} were incubated in binding buffer as described in the materials and methods section with increasing concentrations of [^{125}I]Ang II for 60 min at 28°C. Non-specific binding was determined in the presence of 10 µM Losartan. Specific [^{125}I]Ang II binding is shown. Each data point represents n = 3 samples, mean ± SEM. Where error bars are not visible they are contained within the data point.

2.3.7.5. Addition of G-proteins to non-urea treated membranes

Some receptors undergo affinity shift for agonists in the presence of excess cognate G-proteins (McIntire *et al.*, 2002) and the ability of the addition of a G-protein heterotrimer to increase the fraction of AT_{1A} receptors in the high affinity agonist bound conformation was investigated. G-proteins were added to the [^{125}I]Ang II binding assay with the intention to show that the addition of the subunits would increase the affinity for agonist binding. Upon the inclusion of different

combinations of G-proteins ($G\alpha_{i1}$, $G\alpha_q$ and $\beta_1\gamma_2$) to non-urea treated and urea-treated membranes containing the AT_{1A}, $[^{125}I]$Ang II binding was inhibited. It was important to use $G\alpha_q$ as well as $G\alpha_{i1}$ as the AT_{1A} receptor has been shown to recognise both these subunits (Sasamura *et al.*, 2000). The data suggested that there is no distinguishing feature resulting from the combination of subunits or specific subunit ($G\alpha_q$ or $G\alpha_{i1}$) thus it seems that the effect is an artefact likely due to the buffer that the G-proteins reside. Furthermore, the inclusion of G-protein buffer, buffer F, was added to the assay in the same volume that G-proteins was added as a control (data not shown) as well as heat denatured G-proteins (data not shown) also inhibited $[^{125}I]$Ang II binding. It became apparent that it was important to investigate this phenomenon further because if adding G-proteins inhibited Ang II binding then showing reconstitution in this system would fail. It was necessary to determine trends and eliminate artefacts because although important for the reconstitution, thorough understanding of the relationship was not part of the aims of this section.

2.3.7.6.   The effect of G-proteins on urea-treated samples

The effect of the addition of G-proteins on the $[^{125}I]$Ang II binding was further investigated by using membranes that were urea treated in the assay. The recovery of the high affinity state can be measured using a radiolabelled agonist at a concentration near the K_d of the high affinity state (McIntire *et al.*, 2002) and 1 nM was used to achieve this. Similarly, $[^{125}I]$Ang II binding to membranes expressing the AT_{1A} receptor was inhibited by the inclusion of $G\alpha_q$ subunit alone (condition **B**), heat denatured $G\alpha_q$ subunit (**C**) and both buffer F (**D**) and buffer E. (**E**) (**Figure 2.15**). There was a highly significant 78% ($p<0.0001$) decrease in $[^{125}I]$Ang II binding upon the addition of 150nM $G\alpha_q$. The volume of each addition was kept constant in this incubation (3.9 µL). In addition, buffer F reduced $[^{125}I]$Ang II binding by 43% in urea treated membranes ($p<0.001$). The inhibition (78%) observed

by the addition of 150 nM $G\alpha_q$ (3.9 µL) was significantly different to the decrease observed when buffer was added at the same volume. This suggests that the effect of G-proteins on the specific binding in this system is partly due to the composition of the buffer F and also an unidentified artefact. Furthermore, the addition of $\beta_1\gamma_{2(6xHIS)}$ also inhibited [125I]Ang II binding to membranes expressing AT_{1A} (Appendix page 243-244). This inhibition was less than the inhibition with the non-histidine tagged subunit. It is likely that during the purification process the histidine tagged subunit stays on the column longer and thus there is more opportunity for buffer E exchange to buffer F (with 10 fold less cholate)
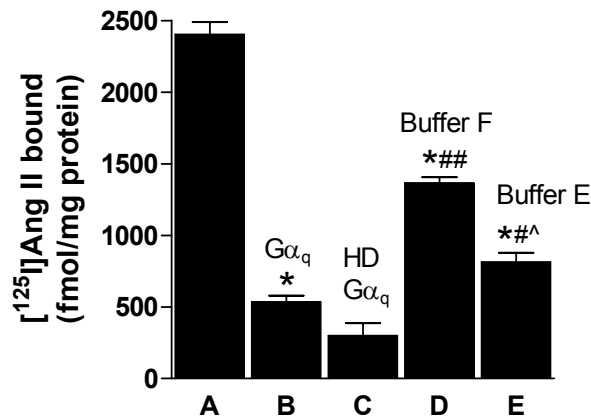

Figure 2.15: **The effect of addition $G\alpha_q$ to the specific [125I]Ang II binding to urea treated CHO cell membranes expressing the AT_{1A} receptor.**
CHO cells from passage 40-42 stably expressing the AT_{1A} were harvested and membranes were treated with 7M urea. Assays were performed as described in the methods section. CHO cell membranes (0.03 mg/mL) stably expressing AT_{1A} were incubated with 1 nM [125I]Ang II for 60 min at 26°C. Non-specific binding was determined in the presence of 10 µM Losartan. The incubation also included the addition of **A**) no G-protein; **B**) 150 nM $G\alpha_q$ (in a total volume of 3.9 µL);**C**) 150 nM heat denatured $G\alpha_q$ (in a total volume of 3.9 µL); **D**) 3.9 µl Buffer F or **E**) 3.9 µl Buffer E. Specific [125I]Ang II binding is shown and data in A (n = 9, mean ± SEM,). Data in B, D, and E (n = 3, mean ± SEM,). Data in C (n = 2, error bars represent the range of duplicates). * p<0.0001 (comparison to A); #p<0.001 and ##p<0.0001 (comparison to B); ^p<0.001 (comparison to D). Abbreviations: HD- heat-denatured)

Buffer F contains 0.1% cholate, 10mM β-mercaptoethanol and 1 µM GDP as well as HEPES buffer and magnesium and sodium salts and the detergent is most likely the compound with the most effect on the receptor. By adding a volume of 3.9 µL of buffer F corresponds to a total 0.0004% (v/v) cholate in the 100 µL assay volume. In

a preliminary experiment 0.0005% (v/v) cholate has no significant effect on specific [^{125}I]Ang II binding (data not shown). It is also possible that the β-mercaptoethanol (diluted to 0.4 mM) is having an inhibitory effect on [^{125}I]Ang II (this effect is discussed later).

It was suggested that perhaps during the production and purification process for the preparation of G-proteins, buffer E (which contains 1% (v/v) cholate) is not entirely exchanged for buffer F in dialysis (with 10 fold lower cholate). In order to determine whether buffer E was inhibiting [^{125}I]Ang II binding to the CHO cell membrane homogenates (and indirectly this also demonstrates the effect of 0.04% cholate on the system), 3.9 μL was added to the incubation (**condition E; Figure 2.15**). There was a 66% reduction in [^{125}I]Ang II binding with the addition of buffer E and this was statistically significant ($p < 0.0001$). This buffer E inhibition of specific binding was significantly different to the inhibition caused by the addition of 150 nM Gα_q ($p < 0.01$) or buffer F ($p < 0.01$).

In order to remove the excess cholate that may be contributing to the effect of G-protein addition to the inhibition of [^{125}I]Ang II binding, Centricon tubes were used to exchange buffer E for buffer F which has 10 fold less cholate. Dialysis is an effective method to remove detergent from a sample provided that the detergent in the buffer has a high critical micelle concentration (CMC). The CMC for the ionic detergent cholate is between 9-15 mM at 25 °C (manufacture's specifications). In the case of cholate in buffer E at 1% (w/v), the concentration is equivalent to a 23.25 mM solution which is greater than the critical CMC and this may imply that dialysis may not be an effective method to remove the detergent in this case. Thus, an alternative method of buffer exchange was investigated. This method involved using

Centricon tubes and centrifugation. The inhibitory effects of buffer E (i.e. cholate) on [^{125}I]Ang II binding to the membrane preparations were successfully decreased (see Appendix page 243) by using centricon tube centrifugation. However, this inhibition (artefact) still existed.

2.3.7.7.   The effect of Histidine tagged Gα_{i1} subunit.

The effect of a histidine tagged Gα_{i1} on specific [^{125}I]Ang II binding was determined. This subunit was prepared in high concentration and as a result much smaller amounts of the solution were added to the assay to achieve effective concentrations of the subunit. **Figure 2.16** shows that the addition G$\alpha_{i1(6xHIS)}$ (within the concentration range of 5-50 nM) had no statistically significant effect on decreasing specific [^{125}I]Ang II binding. Both 150 nM G$\alpha_{i1(6xHIS)}$ and heat denatured G$\alpha_{i1(6xHIS)}$ significantly inhibited specific [^{125}I]Ang II binding to the urea treated membranes expressing the AT$_{1A.}$
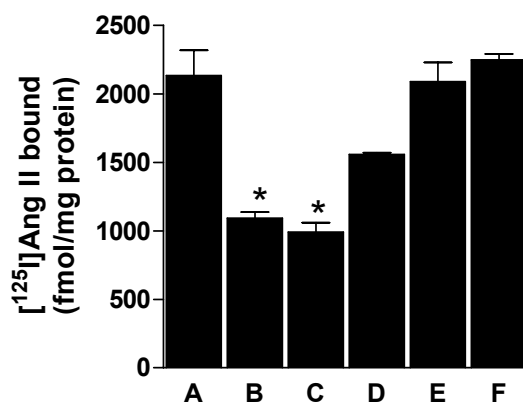

Figure 2.16:   Specific [^{125}I]Ang II binding to CHO cell membranes expressing the Angiotensin II receptor (AT$_{1A}$): effect of the addition G$\alpha_{i1(6xHIS)}$.
CHO cells from passage 40-42 stably expressing the AT$_{1A}$ were harvested and membranes were treated with 7M urea. Assays were performed as described in the methods section CHO cell membranes (0.03 mg/mL) stably expressing AT$_{1A}$ were incubated with 1 nM [^{125}I]Ang II for 60 min at 26°C. Non-specific binding was determined using 10 μM Losartan and was less than 6% of total binding. The incubation also included the addition of **A**) no G-protein; **B**) 150 nM G$\alpha_{i1(6xHIS)}$ (1.4 μL); **C**) 150 nM heat denatured G$\alpha_{i1(6xHIS)}$ (1.4 μL); **D**) 50 nM Gα_{i1} $_{(6xHIS)}$ (0.48 μL); **E**) 10 nM G$\alpha_{i1(6xHIS)}$ (0.096 μL) or **F**) 5 nM Gα_{i1} $_{(6xHIS)}$ (0.048 μL). Specific [^{125}I]Ang II binding is shown and data in A (n = 6, mean $\pm$ SEM, error bars). Data in B-F (n = 3, mean $\pm$ SEM, error bars). *p<0.001 (comparison to A).

Summary of results

In conclusion, membrane homogenates were prepared from natural tissue sources as well as recombinant expression systems. Radioligand binding analysis confirmed that the membranes expressed the GPCR of interest. Expression of the β_1AR was shown in turkey erythrocyte membranes and *Sf*9 cell membranes after infection with recombinant baculovirus using both [^{125}I]ICYP and [^{3}H]CGP-12177. AT_1 expression was demonstrated using [^{125}I]Ang II (human) (and for rat liver membranes the saralasin analog of this agonist) in membrane homogenates from both rat and turkey liver as well as from CHO-K1 cell membranes stably expressing the receptor. Finally, the baculovirus expression system was used to recombinantly express the $\alpha_{2A}AR$ as shown by radioligand antagonist binding with [^{3}H]MK-912. In addition, these membranes were treated with the chaotropic agent urea to remove/denature endogenous G-protein activity. This post–preparative treatment did not interfere with radioligand binding for all 3 receptors studied. The baculovirus expression system was used to prepare specific G-protein subunits; $G\alpha_{i1}, G\alpha_q, \beta_4, \beta_1$ and γ_2 . These G-proteins were purified using affinity chromatography techniques utilising the histidine tag on either the $G\alpha$ or $G\gamma$ subunit and purification was routinely demonstrated using gel electrophoresis. Although purifying the $G\alpha_s$ subunit was unsuccessful after many attempts, $G\alpha_{i1}, G\alpha_q$ and the dimers $\beta_1\gamma_2$ and $\beta_4\gamma_2$ were prepared. Furthermore, the potential to restore high affinity agonist binding for the AT_{1A} receptor was investigated. Several problems were encountered with this receptor system. The major challenge identified was the concentration of cholate in the buffer in which the G-protein subunits reside.

2.4. DISCUSSION

The aim of this chapter was to obtain 3 different GPCRs preparations from various sources and characterise them briefly to verify their class. Three different GPCRs, 2 from the adrenergic family, $\beta_1 AR$ and $\alpha_{2A} AR$, and the AT_1 receptor were examined. Receptors were obtained from natural sources and from cloned sources. Furthermore, several G-protein subunits were purified successfully using the baculovirus expression system. Finally, restoration of high affinity binding in the AT_{1A} receptor system was investigated.

2.4.1. Natural sources of receptors

Initially the $\beta_1 AR$ and the AT_1 receptors were obtained from natural sources with the rationale being that these sources were readily available, and other means of obtaining suitable material for receptors (i.e. cell culture) were not established in this laboratory at the time work for this thesis was initiated. This allowed for development and optimisation of the various radioligand binding assays for each of these receptors. Preparation of membranes from natural sources such as a single membrane system (turkey erythrocytes) was simple and involved relatively few steps including differential centrifugation for separation. This technique has been well documented (Shorr *et al.*, 1981; Shorr *et al.*, 1982) and the membranes obtained showed specific binding to both adrenoreceptor radioligands that were tested. Conversely, using membranes from rat heart (known to have high $\beta_1 AR$ expression levels (McMurchie *et al.*, 1987) did not prove as reliable a source of receptor as turkey erythrocyte membranes.

In tissue such as liver tissue (and rat heart), plasma membrane is a very low proportion perhaps only 5% of the total membrane. Thus, when using these tissues

sources, enrichment of receptor-containing membranes is necessary. Density gradient

separation using inert Percoll was chosen to enrich liver tissue in plasma membrane

for examining AT_1 receptor expression. Secondary messenger signalling and receptor

activation regulation might not be as complicated in single membrane system thus

less likely to interfere with ligand binding. The difference in receptor enrichment

between the single membrane system and the heart tissue membranes is a

demonstration of the factors contributing to the move towards a recombinant

expression system.

2.4.2. β_1AR receptor characterisation: natural and expressed

Radioligand binding assays are a reliable and robust method to quantify and qualify

the GPCRs (Shorr *et al.*, 1982; de Gasparo *et al.*, 2000). It was important to

investigate GPCR ligand binding capabilities to optimise conditions for reducing

non-specific binding, minimising artefact generation, and conservation of protein

(especially important when using natural receptor sources and stably expressed

mammalian sources). Initial studies on the β_1AR used an iodinated ligand

($[^{125}I]$ICYP) because of higher specific activity (commonly used when density of the

receptors is low) and ease of counting (Bylund and Toews, 1993). This radioligand

has reliably been used with this receptor system previously (Shorr *et al.*, 1982).

Results showed low, non-specific binding to the β_1AR membranes and an

incubation/reaction time of 10 min that was comparable with the literature (Shorr *et*

al., 1981). Data obtained using a second radioligand ($[^3H]$CGP-12177) and the

antagonist, Propranolol, correlated well with the literature (De Lean *et al.*, 1982) for

this receptor. Saturation binding using $[^{125}I]$ICYP was not demonstrated. Short

comings of saturation and competition experiments can be overcome by performing a

'mixed' experimental protocol where lower concentrations of ligand are obtained by using increasing amounts of labelled ligand while higher concentrations are obtained by adding increasing amounts of unlabelled ligand. However, the estimated K_d could be affected by a difference in affinity between the labelled and unlabelled ligands (Rovati, 1998). Extensions of the classical protocol are reviewed in (Bylund and Murrin, 2000). Nonetheless, membranes treated with [^{125}I]ICYP showed a higher B_{max} (1037 fmol/mg protein) than those treated with [^{3}H]CGP-12177 (621 fmol/mg protein) and the latter value compared well with the literature values for this receptor (Shorr *et al.*, 1982). In membrane homogenates, vesicles are usually a mixture of right-side out and inside-out vesicles (Mardon *et al.*, 1998). [^{3}H]CGP-12177 was shown to be more selective for cell surface βAR than lipophilic agents such as [^{3}H]dihydroalprenolol and [^{125}I]ICYP because it shows little non-specific binding to intact cells (Riva and Creese, 1989; Staehelin and Hertel, 1983). [^{3}H]CGP-12177 has high selectivity for β_1AR receptors (Kuznetsov *et al.*, 1995). A lipophilic radioligand, such as [^{125}I]ICYP, may be capable of binding to total cellular binding sites (externalized membrane-bound, internalized membrane-bound, and cytosolic), and may not enable the detection of a shift in receptors between different cellular compartments (Mardon *et al.*, 1998). This may suggest that [^{3}H]CGP-12177 binding parameters represent a more accurate reflection of cell surface exposed receptors and, in the case of determining externally exposed receptors, it would be the preferred radioligand. In a natural system, prolonged agonist exposure leads to β_1AR receptor desensitisation via internalisation (Lefkowitz *et al.*, 1983). However, membrane homogenates are unlikely to contain machinery to be able to initiate and sustain agonist-induced receptor internalisation.

2.4.3.   AT$_1$ receptor characterisation: natural

The AT$_1$ receptor was studied using membranes prepared from rat liver and turkey liver by using agonist radioligand binding. The rat AT$_1$ receptor maintained rank order potency for Ang II (human), Ang II (saralasin) and Losartan as was reported previously by (Inada *et al.*, 2002). [^{125}I]Ang II incubation with membranes from turkey liver showed very low non-specific binding. However, the agonist binding was non saturable in this receptor preparation. Interestingly, the addition of non-labelled agonist did not displace the radioligand. This incomplete displacement was also seen in rat liver membranes and turkey erythrocyte membranes (data not shown). One possible explanation for this effect (to the AT$_1$ receptor system) could be that upon agonist stimulation, there is a degree of receptor internalisation in the membrane vesicles that are formed (Mardon *et al.*, 1998). The unlabeled agonists may not be able to diffuse through the membranes to displace these receptors and can only displace labelled agonists on the externalised receptors.

The turkey liver AT receptor showed some inconsistencies with the rat receptor. Primarily, the rank order potency evaluation demonstrated that this receptor lacked affinity for Losartan and saralasin. This is consistent with the observation of (Ji *et al.*, 1994) who showed that mammalian AT$_1$ receptors can bind biphenylimidazole antagonists (such as Losartan) with high affinity, whereas the opposite is true for AT$_2$ receptors and non-mammalian receptors. The amphibian and avian receptors are functionally similar to the mammalian AT$_1$ receptor in that they activate the phospholipase C and calcium signal transduction pathway in response to Ang II. However, they do not recognise Losartan and other non-peptide antagonists. This unique feature of non-mammalian AT$_1$ receptors has been applied to the identification of amino acid residues involved in the binding epitopes for non-peptide

antagonists such as Losartan (Ji *et al.*, 1994). The AT_1 receptor from turkey adrenal gland shares approximately 75% identity with mammalian AT_1 (Murphy *et al.*, 1993) and turkey adrenal membranes have a K_d of 0.17 nM for $[^{125}I]$Ang II and 0.45 nM for saralasin (Murphy *et al.*, 1993).

2.4.4. $α_{2A}$AR receptor characterisation

High expression levels of $α_{2A}$AR were routinely achieved using the baculovirus/insect cell expression system. Mammalian expression systems have also been utilised for this receptor including CHO cells expressing the $α_{2A}$AR (Wade *et al.*, 1999). These receptors showed B_{max} values of approximately 19 pmol/mg protein, which was in the range obtained from the insect expressed receptors in this study. Slightly higher K_d values of 7.5 nM were seen in receptors isolated from mammalian cells compared to those prepared using the baculovirus expression system as shown in the results section of this thesis.

2.4.5. A comparison of expressed and natural sources

Low natural abundance of the receptors of interest has lead to the development of recombinant expression of GPCRs (Massotte, 2003). The implementation of recombinant expression technology as a strategic decision in the evolution of the project at CSIRO HSN, precluded further investigation into natural sources of receptors for this study. In addition, cell culture techniques for the expression of GPCRs and G-protein subunits allowed for a great improvement in the quality and speed at which the desired proteins could be obtained. Much work was put into the optimisation and development for receptor production and, hence, this is documented in more detail in the Appendix. This approach also provides a more flexible assay system for examining the interactions of G-proteins and receptors in cell-free, reconstituted signalling assays. The chosen expression systems required relatively

minor laboratory alterations, and tools for cell culture and protein production was commercially available making the system attractive for the introduction into a biochemical laboratory. Choosing to use the baculovirus expression system did not need extensive biosafety measures because the virus has a narrow host cell range which is restricted to insect cells (Massotte, 2003).

2.4.6.   Receptor purification

To date the experiments described have been performed using isolated (impure) cell membrane preparations containing the GPCR of interest. As such this receptor would be expected to be amongst many types of integral membrane proteins associated with the cell membranes, and indeed other contaminating subcellular membranes. Receptor solubilisation and subsequent lipid reconstitution is one way of purifying GPCRs (and other integral membrane proteins) (Meenagh *et al.*, 2001; Sen *et al.*, 1983; Shorr *et al.*, 1982) and this strategy may have benefits in examining cell-free models of GPCR signalling. Solubilisation of both turkey erythrocyte β_1AR and Turkey AT$_1$ receptors was investigated in pilot studies (data not shown). The main problem associated with detergent solubilisation was the establishment of a binding assay so that solubilisation could be measured. Various methods to establish a soluble receptor binding assay were investigated including a G50 Sephadex column (Shorr *et al.*, 1982), polyethyleneimine coated GF/B filters (Meenagh *et al.*, 2001) and dextran coated charcoal (Sen *et al.*, 1983). No conclusive data was obtained and this investigation was terminated as it was seen as tangential to the aims of this thesis. The solubilisation of the α_{2A}AR and M$_2$ receptors has been more recently investigated by another student in this laboratory (Ms Amanda Aloia, CSIRO). Recently, simple and robust, large scale, automated purification of receptors and their crystallisation has been reported using the neuromodulatory GPCR, the

neurotensin receptor (its cognate ligand is a 13-amino acid long peptide) (White *et al.*, 2004).

2.4.7. Urea treatment

Urea treatment of β_1AR and $\alpha_{2A}AR$ containing insect cell membranes did not attenuate antagonist binding (Lim and Neubig, 2001). However, the B_{max} in these membrane preparations was increased likely due to the significant removal of peripheral membrane proteins as reported by others (Lim and Neubig, 2001). Agonist binding to CHO cell membranes stably expressing AT_{1A} was similarly unaffected by urea treatment and did show an enhancement of receptor number. In other membrane receptor preparations, agonist or partial agonist affinity was markedly decreased after urea extraction (Lim and Neubig, 2001). However after the addition of myristoylated $G\alpha_{i1}$, the high affinity agonist binding was restored which demonstrates that the urea treatment does in fact remove endogenous G-proteins but does not abrogate receptor activity. In that study the receptors' ability to functionally bind agonist was restored upon the addition of purified G-proteins (Lim and Neubig, 2001).

2.4.8. G-proteins

The baculovirus expression system was used to achieve expression of a range of membrane associated G-protein subunits that were further detergent solubilised and purified by affinity techniques (Kozasa and Gilman, 1995). Despite numerous attempts and modification of approaches, insect cell expressed $G\alpha_s$ was unable to be prepared. Similar problems associated with the expression of this class of subunit has been experienced by other researchers (Dr Michelle Glass, personal communication) (Kozasa and Gilman, 1995). The lower affinity of $G\alpha_s$ to $\beta_1\gamma_{2(6xHIS)}$ appeared to be the major contributing factor (Kozasa and Gilman, 1995) and perhaps the positioning of the histidine tag was a problem. Creating a chimera of $G\alpha_s$ might enable expression

of the protein. Maintaining the expression of such a protein in the eukaryotic expression system may facilitate coupling to GPCRs that signal through the $G\alpha_s$ pathway. As both $G\alpha_q$ and $G\alpha_{i1}$ have been both successfully expressed in the baculovirus system, these would be the likely backbone subunits to molecularly merge with $G\alpha_s$. As Walker *et al,* (2005) has shown the length of the C-terminal tail must be greater than 5 $G\alpha_s$ residues to couple to a $G\alpha_s$ signalling GPCR (Walker *et al.*, 2005). A degree of promiscuity can also be incorporated into the backbone of the protein which has been shown to improve the signal detection of the chimeric subunit (Walker *et al.*, 2005). These workers designed and compared two $G\alpha_q$ chimeras, concluding that the chimera with the more promiscuous *C.elegans* $G\alpha_q$ backbone, significantly increased the fold stimulated response compared to the human $G\alpha_q$ chimera in almost all of the receptor systems investigated.

Interestingly, higher yields of the histidine tagged G-protein subunits were observed. Histidine tagged subunits were eluted from the column with imidazole whereas the non histidine tagged proteins are removed via AlF_4^-. Upon further investigation, it was also observed that the anticipated purity of histidine tagged proteins was not as high as expected. It was suggested that the AlF_4^- reaction was not completed and so the histidine tagged proteins were always contaminated with non-histidine tagged proteins when they were co-infected. Conversely, non-histidine tagged proteins were never contaminated with the other subunit. To obtain pure samples of histidine tagged subunits, a single infection was performed.

2.4.9.   Insect cell culture versus mammalian cell culture

Previously outlined were some difficulties and challenges that must be considered when choosing an expression system to obtain the receptor of interest (**Table 2.1**).

Although the β_1AR and $\alpha_{2A}AR$ were successfully expressed using the baculovirus system, AT_{1A} expression in the baculovirus expression system was unsuccessful. Even though the recombinant AT_{1A} baculovirus infected the insect cells, the membrane preparation failed to demonstrate Ang II binding (data was not shown). Perkin Elmer Life sciences provide membrane preparations containing the human AT_1 receptor expressed in *Sf*9 cells (technical literature[12]) indicating it may be possible to produce *Sf*9-AT_1 receptors. This approach was not undertaken due to the high price of purchase.

Post-translational modifications (palmitoylation, myristoylation for $G\alpha$ subunits and prenylation and carboxyl methylation for γ subunit) of heterotrimeric G-protein have been extensively studied in *Sf*9 cells with no differences being observed when compared to mammalian cells for certain receptors (Massotte, 2003). However, it has previously been well documented that a major limitation regarding the production of proteins in this insect cell system is the lack of complex-type N-glycans, resulting in incomplete glycosylation (Hang *et al.*, 2003). This may partly explain the lack of Ang II receptor expression in our baculovirus/*Sf*9 cell system. N-glycosylation has been shown to be important in the proper trafficking of AT_1 receptors to the plasma membrane (Lanctot *et al.*, 1999). Glycoslated AT_{1A} is greater than 66kDa as opposed to the unglycoslated form which is approximately 40 kDa (Guo *et al.*, 2001), and the removal of all 3 glycosylation sites in the AT_1 receptor was shown to decrease cell surface expression of this receptor by 80% (Lanctot *et al.*, 1999). Thus, when choosing to express this receptor recombinantly, this glycosylation requirement must be considered. Glycosylation also plays a role in surface expression and dimerisation of the β_1AR (Xu *et al.*, 2003). The construction of an expression cassette (enabling

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[12]http://las.perkinelmer.com/content/TechnicalInfo/6110121.pdf

expression of the receptor as well as three key glycosyltransferases from the mammalian biosynthetic pathway for complex-type N-glucans) (Hang *et al.*, 2003) may improve the universality of the baculovirus expression system and at the same time avoid establishing a new labour intensive cell line.

Another possibility could be that Ang II (agonist) binding was in the low affinity state because of the lack of endogenous G-proteins in the insect cell system (Massotte, 2003). To rule out this possibility, an antagonist ligand would need to be purchased. The lack of literature regarding AT_1 expression in insect cells deterred further investigation and even the Neubig lab (Department of Pharmacology, university of Michigan) could not answer specific questions regarding the baculovirus clone they provided (personal communication). Perhaps if the restoration of high affinity Ang II binding could be established in a mammalian receptor preparation using a combination of G-proteins, this combination could be added to the insect cell membranes rendering the establishment of binding.

2.4.10. CHO cell culture

CHO cell culture was established in this laboratory and CHO cells donated by Dr Walter Thomas (Baker Institute, Melbourne Vic) were grown successfully. These cells showed stable expression of the rat AT_{1A} receptor using whole cell binding assays (Dr Thomas, personal communication). An isotonic buffer was successfully used to prepare membranes compared to a hypotonic buffer which dramatically reduced receptor binding possibly due to peripheral protein loss from membranes (Prpic *et al.*, 1984). Membranes were prepared by homogenisation to facilitate the retention and integrity of membrane proteins so that membranes retained their full ligand binding activity. Membranes could be prepared from fresh or frozen cells

without compromising ligand binding ability. However, $[^{125}I]$Ang II was reduced after re-thawing membrane preparations.

Non-specific binding was determined in the presence of either unlabelled Ang II or the non-peptide antagonist Losartan. This also assisted with the verification of the subtype of the receptor as Losartan affinity is specific to AT_1 receptors (Tamura *et al.*, 1997). Further characterisation enabled the determination of receptor expression in the membrane preparation and saturable ligand binding was observed. The rat AT_{1A} has been shown to have one binding site as opposed to human AT_1 which has two (Inada *et al.*, 2002). Preliminary data suggested that membranes prepared from cells from a higher passage were more efficient at binding $[^{125}I]$Ang II indicating receptor expression enhancement may be directly proportional to the age of the cell line. As characterisation of this cell line was not the direct aim of this chapter, this was not further investigated. However such an observation raised an interesting point. Similarities can be seen using the baculoviral expression system. Other researchers in the lab as well as myself have noticed that the B_{max} increase for particular receptors as the virus is re-amplified. Additionally it has been reported that in cell-based assays the responses of cell cultures vary with age and culture, however assays that measure binding (i.e. ligand receptor responses) are less variable (Robinson, 2003).

2.4.11. High affinity binding

The measurement of affinity shift activity is useful in assessing G-protein functionality and regulation of stoichiometry as well as the specificity of G protein /receptor interactions (Graber *et al.*, 1992). Disadvantages include the use of radio-nucleotides and that this type of assay can not readily be applied to high throughput platforms (Cabrera-Vera *et al.*, 2002). Researchers have used such assays routinely

in many GPCR systems whereby a radioliganded agonist is available and the introduction of G-protein subunits (in the GDP-liganded state) has been shown to restore high affinity binding for *Sf*9 expressed bovine adenosine receptor system (McIntire *et al.*, 2002), serotonin receptors (Graber *et al.*, 1992), as well as for rat liver AT_1 receptors (Graber *et al.*, 1992). A 100 fold decrease in K_d upon subunit addition has been shown for the *Sf*9 expressed bovine adenosine receptor system (McIntire *et al.*, 2002).

A decrease in agonist affinity was not seen after urea treatment of CHO cell membranes over expressing rat AT_{1A}. This may be due to the dramatic purification that such a treatment enables by the removal of peripheral membrane proteins (Lim and Neubig, 2001). Alternatively, the denaturation of endogenous G-proteins in the mammalian system (that may or may not couple to the over expressed receptor) may be reversible as urea treatment does not physically remove G-protein subunits which are anchored to the membrane via prenylation and myristoylation (Lim and Neubig, 2001). In contrast, the addition of guanine nucleotides (GTPγS) has been shown to reduce agonist binding in rat hepatic receptors (Graber *et al.*, 1992; de Gasparo *et al.*, 2000) and recombinant COS-7 cells (Auger-Messier *et al.*, 2003).

The inhibition of $[^{125}I]$Ang binding upon the addition of G-protein to membranes (urea treated and non-urea treated) was unexpected. This effect was likely due to the addition of an unidentified artefactual constituent either in the buffer in which the subunits resided or subunits themselves. Alkoids such as sanguinarine has been shown to inhibit the binding of the specific radioligands to AT_1 receptor (Caballero-George *et al.*, 2003). AT_1 receptor has shown sensitivity to DTT possibly due to the destruction of one of the disulphide bridges located in the extracellular domain of the

receptor (Chang *et al.*, 1982; Guo *et al.*, 2001). The AT_1 receptor has two pairs of disulphide bridges identified with 4 cysteine residues located in the extracellular domain (Dinh *et al.*, 2001). The transmembrane domain and extracellular loop play an important role in Ang II binding (Hunyady *et al.*, 1996) and it may be necessary to understand how these other substances affect these regions to fully understand this observed inhibition. Perhaps the addition of G-protein subunits affected the biological activity of the Ang II. The biological activity of Ang II is highly dependent on the conservation of its Phe 8 C-terminal residue and in contrast the N-terminal residues of the hormone are important for receptor binding and the duration of the action of Ang II (de Gasparo *et al.*, 2000). Thus a detailed chemical and structural analysis would have to be completed to determine what was happening in this system.

Conclusion

This initial study dealt with the choice, establishment and validation of certain GPCR and G-protein material which could be subsequently used in studies related to cell-free, reconstitution of signalling complexes. The baculovirus/insect cell expression system was certainly the method of choice for some of this material and considerable characterisations of the expressed products was undertaken. However, this method was not amenable for all materials that were required and alternative methods had to be investigated. Overall this chapter represents the groundwork that was done in establishing and producing the material that was significantly characterised for latter studies in the more directed, reconstitution studies.

Chapter 3.

Measurement of functional G-protein signalling activity using the [^{35}S]GTPγS binding assay

"The important thing in science is not so much to obtain new facts as to discover new ways

of thinking about them"

William Lawrence Bragg

3.1.   INTRODUCTION

This section deals with the reconstitution of GPCRs and G-proteins and measuring this signalling complex (termed a transductosome) as an indication of functional activity. Measuring functional GPCR reconstitution can be used to study the specificity of the interaction between receptors and G-proteins (Lim and Neubig, 2001). Assays which rely on the principle of monitoring GTP exchange with the use of GTP analogues (radioactive and non radioactive in nature) have been used quite routinely for many years. By measuring the activation of G-proteins themselves, the first step in the traditional signalling cascade, some of the issues related to downstream signalling can be avoided (Windh and Manning, 2002).

G-proteins, functioning as regulatable, molecular switches undergo an inactivation/activation cycle also called the GTPase cycle (see **Figure 1.2**) that can be broken into three main events: displacement of GDP from the Gα subunit, GTP binding and the hydrolysis of bound GTP (Offermanns, 2003). The overall reaction rate is referred to as k_{cat} and is fast *in vivo* and typically slow *in vitro* (Berman and Gilman, 1998). This rate is the overall sum of the rates for the three events k_{dissoc}, k_{assoc}, k_{hydro} whereby, in the natural system, GDP dissociation ($k_{disssoc}$) is the rate limiting step (Mukhopadhyay and Ross, 2002). See below.

$$\text{G-GDP} \xrightarrow{k_{diss}} \text{G} \xrightarrow{k_{assoc}} \text{G-GTP} \xrightarrow{k_{hydrol}} \text{G-GDP} + \text{P}_i$$
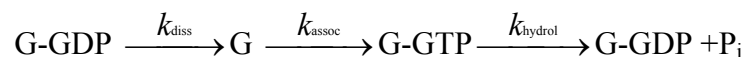
Direct assessment of G-protein activation (by measuring GTPase activity) can be typically accomplished by either receptor-induced increases in the rate of GTP hydrolysis by the α subunit or by measuring the exchange of GDP for non-hydrolysable GTP analogues on the α-subunit (Windh and Manning, 2002). Note that a time course using GTP analogues can give you the rate of dissociation of GDP and

[^{32}P] assays can give you rate of hydrolysis. This measurement can be used for comparative purposes and can also be used to determine receptor mediated responses (Kleuss *et al.*, 1994).

3.1.1. [^{35}S]GTPγS binding

GTPγS analogues radiolabelled with [^{35}S] are widely used to follow GTP binding. This non-hydrolysable analogue ensures GTP remains bound to the α subunit. Therefore the progression of an irreversible activation signal can be measured rather than steady state activation/deactivation cycles (Harrison and Traynor, 2003) (**Figure 1.2**). GTP will bind to the α subunit if there is no (or minimal) GDP present as GDP dissociation is the rate-limiting step (Kleuss *et al.*, 1994). If GDP is present in the assay, receptor induced GTP binding is required. GPCR functionality can be measured using the [^{35}S]GTPγS assay with the radioactive nucleotide measuring the level of G-protein activation following agonist induction of the GPCR. The major advantage of this approach is that the first step in the receptor: G-protein interaction can be measured (McIntire *et al.*, 2002). In the assay, [^{35}S]GTPγS replaces endogenous GTP or GDP and binds to the Gα subunit following activation of the receptor to form a Gα-[^{35}S]GTPγS species. The amount of [^{35}S]GTPγS bound determines the amount of signal observed. Since the γ-thioester bond is resistant to hydrolysis by the GTPase of Gα, G-protein is prevented from reforming a heterotrimer and thus [^{35}S]GTPγS labelled Gα subunits accumulate and can be measured by counting the amount of [^{35}S] (a relatively high β-emitter (Ferrer *et al.*, 2003)) label incorporated. As the Gα-[^{35}S]GTPγS species remains associated with the membrane, a heterogenous separation filtration assay can be used to simply and effectively count the radioactivity. (Harrison and Traynor, 2003)

3.1.1.1.   Assay variations

Specific G-protein antibodies used in conjunction with [^{35}S]GTPγS allow for identification of activation within a particular class of subunit (Harrison and Traynor, 2003). These immuno-capture techniques can overcome the limitations of the conventional [^{35}S]GTPγS assay (Milligan, 2003). By such immuno-enrichment [^{35}S]GTPγS can be used for subunits which exhibit lower affinity for [^{35}S]GTPγS and slower GDP dissociation rate (Milligan, 2003). Recently methodologies have been developed whereby separation of bound from free radioactivity is not required (Harrison and Traynor, 2003). The Flashplate technique (Perkin Elmer Life Sciences) utilises 96 or 384 well plates permanently coated with a polystyrene based scintillant and wheat germ agglutinin which captures the glycosylated receptor (Technical notes[13]). This brings the [^{35}S]GTPγS label in close proximity to the scintillant resulting in the generation of a light signal (Harrison and Traynor, 2003). Scintillation proximity beads (SPA) rely on the same principle and both these assays are amenable to high throughput screening (HTS) (Leifert *et al.*, (a) 2005).

3.1.2.   Fluorescent approaches

Fluorescence approaches are more favourable than radioactive assays as they are more amenable to high throughput screening. Unlike radioactive assays, they do not produce expensive and environmentally unfriendly waste. Some fluorescent methods involve homogeneous assays so that tedious physical separation is not required and they can be used to study fast reaction in real time (McEwen *et al.*, 2002). Other advantages and challenges can be seen in **Table 3.1** and these techniques were reviewed recently (Leifert *et al.*, (a) 2005).

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[13] http://las.perkinelmer.com/Content/RelatedMaterials/SMP%20Post7.pdf

3.1.3.   Measuring GTPase activity

The assays that measure GTPase activity revolve around measuring the release of inorganic phosphate commonly tracked using radioactivity. In this way, GTPase activity can be measured after preloading the G-proteins with [^{32}P]GTP (single turnover studies) or in the continued presence of [γ^{32}P]GTP (steady-state). The assay can be employed to determine the rate of hydrolysis (k_{hydro}) of GTP (Kleuss *et al.*, 1994) and hence detect changes in this rate in the presence of substances that effect GTP hydrolysis. Excess unlabelled GTPγS can be added at certain points in the assay to determine the rate of dissociation. The fractional steady state occupancy of the guanine nucleotide binding site on the subunit by GTP can be determined (i.e. it is equal to dissociation of GDP/(rate of hydrolysis + rate of disassociation) (Kleuss *et al.*, 1994). Accelerating the high throughput efficiency of this type of assay may include the development of a fluorescently labelled inorganic phosphate binding protein in much the same way as (Thulin *et al.*, 2001) used a bacterial binding protein to measure ATPase activity in human muscle fibres. Recently, Invitrogen have advertised a fluorescent assay to detect inorganic phosphate and nucleoside diphosphates, which may be used to measure GTPase activity (technical data[14]). Alternatively, to measure GTPase activity, other researchers have utilised an enzymatic approach to measure inorganic phosphate production (Robillard *et al.*, 2000).

3.1.4.   Assessing assay optimisation

Screening assay quality has typically been loosely attributed to the signal to noise ratio (S/N) or the signal to background (S/B) ratio (Zhang *et al.*, 1999). Both of these expressions are not comprehensively useful to methodically assess assay robustness and optimisation as neither takes into account both the variability in the sample and

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[14] www.invitrogen.com/downloads/F-13281_Fluor_Sensor_Protein_Poster.pdf

background (S/N only) plus the signal dynamic range (S/B only) (Zhang *et al.*, 1999). These ratios are classically defined as follows (and are sometimes loosely interchanged):

$$S/N = \frac{\text{mean signal - mean background}}{\text{SD of background}}$$

$$S/B = \frac{\text{mean signal}}{\text{mean background}}$$

Technique	Usefulness/Advantages	Challenges/Disadvantages
GTP Analogs [^{35}S]GTPγS	• Functional assays as well as G-protein quantification. • Immunoprecipitation-linking to an antibody to determine activation of a specific subunit. • SPA and Flashplates-homogenous, amendable to HTS. • High affinity for Gα subunit. • High specific activity of [^{35}S].	• Separation required. • Optimisation required to reduce high background. • Radioactive waste.
Early fluorescent approaches intrinsic tryptophan	• Universal (i.e. all Gα have this amino acid). • Homogenous/ non radioactive. • Can use natural GTP ligands or non-hydrolysable analogs.	• Impractical for anything apart from purified proteins because of high background binding. • Expensive equipment i.e. quartz cuvettes not amendable to HTS.
MANT fluorescence	• Homogenous/non-radioactive. • G-protein quantification.	• Excitation spectra in UV range i.e. quartz cuvettes necessary.
Europium GTP	• Functional assays as well as G-protein quantification. • Large stokes shift enables low background and high signal to noise ratio. • Time resolved. • Highly sensitive assay.	• Separation required. • Expensive analog.
BODIPY GTP	• Homogeneous. • Measure GTP binding to G-proteins. • Quantification of G-protein .	• Not effective (high background) in whole cells or membrane samples to show agonist induced binding.
GTPase activity Radioactive	• Natural GTP analogs can be used. • Using low temperatures slows down hydrolysis rate (i.e. RGS).	• Separation required. • Difficult to determine rates when compounds that enhance hydrolysis (RGS) are used. • Radioactive waste.

Table 3.1: **Advantages and disadvantages of assays targeting the GDP/GTP cycle (Level 3 assays) (Frang *et al.*, 2003; Higashijima *et al.*, 1987; Lan *et al.*, 2000; McEwen *et al.*, 2001; McEwen *et al.*, 2002).**

Fold stimulation and S/B are used interchangeably throughout this thesis as they refer to the same value. Moreover, a screening window coefficient called the Z factor, was determined and is commonly used to assess assay robustness and indicate assay quality (Zhang *et al.*, 1999). This factor is defined as:

$$Z = 1 - \frac{3\text{SD of sample} + 3\text{SD of control}}{\left| \text{mean of sample} - \text{mean control} \right|}$$

A dimensionless quantity, a sufficiently large Z factor (i.e. above 0.5 and the closer to 1 the better) is a tangible indicator of optimal assay conditions (Zhang *et al.*, 1999). Although not determined in this study, the Z factor concept has been utilised in many areas of assay development, validation and in drug screening (Ferrer *et al.*, 2003; Zhang *et al.*, 1999).

This chapter deals with the optimisation of [^{35}S]GTPγS in various formats (test tube verus 96-well plate) to demonstrate functional α_{2A}AR reconstitution. Specifically the chapter deals with;

1) Optimisation of the [^{35}S]GTPγS assay for α_{2A}AR reconstituted with Gα_{i1} and $\beta_1\gamma_2$.

2) The development and subsequent optimisation of a higher throughput (96-well plate format)[^{35}S]GTPγS binding assay.

3) The issue of biological variability

4) Adapting the assay for other receptors and other reconstitution combinations.

3.2.   MATERIALS AND METHODS

Additional methods are described below. Refer to previous chapter on methods describing membrane preparation etc.

3.2.1.   [^{35}S]GTPγS binding assay for reconstitution

Initial experiments were based on the work by (Lim and Neubig, 2001). Optimisation of the assay was required and for clarity the specific methods relating to each outcome are described in the results section. Described here, is a general method utilising [^{35}S] guanosine 5'-O-(3-thiotriphosphate) (GTPγS) binding. [^{35}S]GTPγS was purchased from Perkin Elmer Life Sciences (Boston, MA, USA) and diluted to a working stock of 40 nM with 50 mM Tris, 100mM NaCl, 10 mM MgCl$_2$ pH 7.6 aliquoted and re-frozen. Membranes expressing α$_{2A}$AR were diluted to 2-3 fold with ice cold reconstitution buffer TMND (50 mM Tris, 100mM NaCl, 10 mM MgCl$_2$ and freshly added 1 mM dithiothreitol (DTT) pH 7.6[15]) (to a total protein of 0.1 mg/mL - 0.4 mg/mL). Varying concentrations of G–protein subunits (in buffer F[16]) were added to the α$_{2A}$AR containing membranes and this reconstitution mix was incubated on ice for 60 min. Note indicated concentrations of GDP, AMP-PNP, AMP, ATP or GTPγS (Sigma-Aldrich, St. Louis, MO, USA) were also added to the reconstitution mix. Following this incubation, [^{35}S]GTPγS was added to a final concentration of 0.2 nM and incubated for 5 min at room temperature. The addition of either agonist or agonist/antagonist mix or buffer was added to start the reaction. Agonists used were Clonidine (prepared in TMND buffer), UK-14304 (17 mM stock prepared in DMSO), epinephrine and norepinephrine (prepared fresh in TMND with 1 mM ascorbic acid). Antagonists used were Yohimbine (5 mM prepared in 50% (v/v)

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[15] DTT is added fresh on the day of use.
[16] Buffer F defined in Chapter 2 (20 mM HEPES, 3 mM MgCl$_2$, 10 mM NaCl, 10mM β-mercaptoethanol, 1 μM GDP (or 5 μM for Gα$_q$) and 0.1% (w/v) sodium cholate, pH 8.0)

ethanol) and Rauwolscine (10 mM in 50% (v/v) ethanol) All agonists and antagonists were purchased from Sigma-Aldrich, St. Louis, MO, USA. The reaction (200 μL) was incubated in 5 mL polypropylene tubes in a shaking water bath (28°C) for an indicated time. Three x 50 μL aliquots (from same incubation)[17] were rapidly filtered through glass fibre filters (GF/C; Whatman, Kent, UK) using a manifold apparatus (Millipore Australia, North Ryde, NSW) terminated the reaction. Filters were washed (3 x 4 mL) with STOP buffer (100 mM Tris, 125 mM $MgCl_2$, 500 mM NaCl, pH 7.4). The filters were air dried and placed in opaque pico-pro vials with 4 mL Ultima Gold[TM] scintillation cocktail (Perkin Elmer Life Sciences, Boston, MA, U.S.A). The amount of $[^{35}S]GTP\gamma S$ (dpm) bound was determined by liquid scintillation counting using a Wallac 1410 Liquid Scintillation counter. (Pharmacia, Turku, Finland).

3.2.2. $[^{35}S]GTP\gamma S$ assay in 96-well plate format

Similarly, these experiments were performed as detailed in the results section since there were many variations of the assay which were necessary to optimise and validate the procedures in 96-well format. However, the basic protocol is described below. Membrane preparations ($\alpha_{2A}AR$ and M_2[18] from *Sf*9 cells or AT_{1A} from CHO cells) and G-protein preparations ($G\alpha_q$, $G\alpha_{i1}$, $G\alpha_{i(6xHIS)}$, $\beta_1\gamma_2$, $\beta_1\gamma_{2(6xHIS)}$ or $\beta_4\gamma_2$) were thawed from -80°C storage. Membranes and G-proteins (concentrations as indicated) were reconstituted in ice-cold TMND buffer along with 10 μM AMP-PNP and 5 μM GDP (unless otherwise indicated). The reconstitutions were mixed and added directly to 96-well MultiScreen[TM] filter plates containing a GF/C filter on a 0.65 μm Durapore® membrane in opaque plates (Millipore Australia, North Ryde, NSW) or were incubated separately in a V-shaped 96-well incubation plate (polypropylene)

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[17] these are termed filter replicates.
[18] The M2 was prepared by Amanda Aloia (CSIRO) using a similar method to produce $\alpha_{2A}AR$ expressed membranes.

and then transferred to the pre wet[19] MultiScreen^{TM} filter plate. The radionucleotide [^{35}S]GTPγS was then added at a final concentration of 0.2 nM (unless indicated otherwise). The signalling was commenced by the addition of the agonist; Ang II (human) (AT$_{1A}$ receptor), UK-14304 (α_{2A}AR) or carbachol (M$_2$ receptor). The 96-well plates were incubated with mixing (500 rpm) using a plate shaker at 28°C for 90 min. When using a separate V-shaped incubation plate, the total volume of incubation was 40-60 μL (as indicated) and 25 μL of this incubation was transferred to the appropriate well in the MultiScreen^{TM} filtration plate. Where indicated, filter plates were pre-coated with either 0.1% (w/v) polyethyleneimine (PEI), 0.1% (w/v) polyethylene glycol (PEG) or 0.5% bovine serum albumin (BSA) (w/v) for 2 hours shaking at 4°C and then washed with TMN buffer prior to use. Plates were washed 4 times with 200 μL TMN to remove unbound (free) [^{35}S]GTPγS whilst [^{35}S]GTPγS bound to the activated Gα remained bound. The MultiScreen^{TM} filter plates were air dried and the bottom was removed and a solid base fixed under the filters. A total volume of 40 μL of Microscint^{TM}20 (Perkin Elmer Life Sciences, Boston, MA, USA) was added to each well and the amount of [^{35}S]GTPγS bound (cpm) was determined by reading with a Packard Top Count Microplate Scintillation counter B99041V1 (formerly Packard Biosciences, now Perkin Elmer Life Sciences, Boston, MA, USA) using a count time of 1 minute. This counter has two detectors. Optimisation experiments included incubation time course studies, G-protein and GPCR concentration dependence, agonist dose response curves and cross over studies (2 receptors per incubation).

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[19] The MultiScreen^{TM} filter plates were pre-wet with 200 μL TMN buffer and excess liquid was removed by absorbent paper

3.2.3. [^{35}S]GTPγS binding assay for G-proteins

[^{35}S]GTPγS binding was used to verify Gα production. Subunit preparations (Gα$_q$, Gα$_{i1}$) were freshly thawed and diluted with TMND buffer as indicated. Freshly thawed aliquots of the radionucleotide were then used for binding experiments. [^{35}S]GTPγS (0.5 nM) was combined with the diluted G-protein to start the reaction. Assays were incubated at 28°C for 90 min in a V-shaped incubation plate. The total volume of the incubation was 40 μL. Following incubation, 25 μL of the assay was transferred to 96-well Millipore MultiScreen^{TM} filter plates pre-wet with TMN buffer. The liquid was vacuum filtered and filters were washed 4 times with 200 μL of TMN buffer to remove unbound [^{35}S]GTPγS. Once dried, 40 μL of Microscint^{TM}20 scintillant was added to each well in the filter plate and the radioactivity was determined by liquid scintillation counting (as described above).

3.2.4. G-protein purification

G-proteins were prepared as previously described in Chapter 2 with the exception that the buffer E contained 0.1% (w/v) cholate instead of 1% (w/v) cholate. Protein concentrations of subunits were confirmed using the Bradford assay and laser densitometry using a BSA standard control.

3.2.5. Data analysis

Data was analysed using Prism^{TM} (GraphPad Software Inc., San Diego CA, USA). Data are presented as mean and +/- SEM where (n) is greater than or equal to three and n is equal to the number of samples (separate incubations) unless indicated[20]. Where error bars are not visible they are hidden within the data point symbol. When n = 2, error bars, where visible, represent the range of duplicates. The half saturation

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[20] Where (n) represents filter replicates this is clearly stated.

point, $t_{1/2}$ and B_{max} were calculated in Prism^{TM} using non-linear regression analysis for one site binding. The effective concentration at 50% (EC_{50}) or the inhibitory concentration at 50% (IC_{50}) were calculated in Prism using sigmoidal dose response. Statistical analysis (Students unpaired t-test) was performed using Prism^{TM}.

3.3.   RESULTS

The $[^{35}S]GTP\gamma S$ assay was chosen in order to confirm and evaluate the functionality of the reconstituted $\alpha_{2A}AR$ to assess it suitability for the future development of a comparable preassembled prototype. This prototype would contain the respective assay components which could be integrated into a format which allowed a potential increase in assay throughput. Several parameters were varied in order to optimise the fold stimulation, referred to as the signal to background ratio (S/B), represented by the agonist induced binding divided by the basal binding (i.e. non agonist induced binding or basal activity of the receptor). Once the $[^{35}S]GTP\gamma S$ assay was established and validated for the $\alpha_{2A}AR$ complex, the assay was integrated into a 96-well plate format enabling further investigation into other reconstituted complexes, in particular the AT_{1A} receptor.

3.3.1.   Optimisation of the $[^{35}S]GTP\gamma S$ binding assay

Receptor enhanced GDP-$[^{35}S]GTP\gamma S$ exchange on the G-protein α subunit is the basis of this binding assay. Considerable GDP-$[^{35}S]GTP\gamma S$ exchange in the absence of ligand can markedly reduce the S/B ratio and even mask the effects of the ligand (Windh and Manning, 2002). Therefore, optimisation of the reconstitution assay focusing on decreasing the basal binding, that is the binding of $[^{35}S]GTP\gamma S$ which occurred in the absence of agonist, is one of the best ways to improve signal strength (Windh and Manning, 2002). Preliminary experiments showed a 2 fold increase from basal binding to agonist induced binding (data not shown). By decreasing the background basal binding, whilst retaining the value of the agonist induced $[^{35}S]GTP\gamma S$ binding, an increase in S/B was achieved. Throughout this section, the

experiments conducted with the aim of assay optimisation will be described in the data that follows[21].

The most critical aspect of the [^{35}S]GTPγS binding assay is the suppression of binding of the radionucleotide to membranes in the unstimulated state, that is in the absence of the agonist (referred to as basal binding) (Ferrer et al., 2003). To reduce non-specific binding in the system various concentrations of GDP, AMP and ATP were investigated in the system (**Figure 3.1**). The rationale being that these nucleotides were structurally similar to GTP. The concentration of GDP in the [^{35}S]GTPγS binding assay is particularly important for obtaining the optimal signal by reducing the rate and magnitude of [^{35}S]GTPγS binding through competitive binding for the Gα target. Fold stimulations of 2.7, 3.0 and 3.1 units were calculated from assays containing 0.5, 5 and 10 μM GDP, respectively. Additions of ATP (10 μM) and AMP (10 μM) in reconstituted system incubations, gave fold stimulations of 1.5 and 1.23 units respectively. To see whether a combination of nucleotides would produce an additive effect, GDP and ATP were added to the assay together. This combination was not effective in enhancing the fold stimulation producing a value of only 1.28 units. In striking contrast, the combination of 5 μM GDP (which equates to a GDP/[^{35}S]GTPγS ratio of 25,000) and 10 μM AMP gave the most enhanced fold stimulation (3.7 units) and thus was used in subsequent experiments. Using a similar reconstituted system, (Lim and Neubig, 2001) used 1 μM GDP. GDP-[^{35}S]GTPγS exchange (independent of receptor) had no effect (data not shown) thus indicating that constitutive G-protein activity was not likely in this system (Windh and Manning, 2002).

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[21] Blanks were not subtracted from these results because it was agreed for the purpose of this thesis that the raw (relatively) unmodified data is shown. The purpose of this research is of a strategic nature and to show effects/trends that can be developed further for commercial benefit.

At this point the importance of $[^{35}S]GTP\gamma S$ concentration was investigated. Increasing the amount of the $[^{35}S]GTP\gamma S$ from 0.2 nM to 1 nM (**Figure 3.1**) reduced the fold stimulation from about 2.7 units to 1.6 units suggesting that excess concentrations of $[^{35}S]GTP\gamma S$ leads to higher non-specific binding. This emphasises the importance of using a concentration of radioisotope that is sufficient to be detected whilst not increasing the background binding unnecessarily. Furthermore it was also more cost efficient to use less radioisotope. Others researchers chose to use 0.4 nM with this system (Lim and Neubig, 2001).

In addition, the potency (ability to increase S/B) of available agonists was assessed. The partial agonist Clonidine produced fold stimulations of 2.7 units (100 μM) and 2.9 units (1 μM). The $\alpha_{2A}AR$ agonist, UK-14304 (commonly used by (Lim and Neubig, 2001)), gave increased fold stimulations of 3.8 units (100 μM) and 3.5 units (1 μM) (see **Figure 3.1 B**). Thus, in this instance, UK-14304 was marginally more effective in increasing the fold stimulation. Therefore, this agonist was used when experimenting with other parameters.

All assays were incubated for 60 min and from a time dependent study (data not shown) the $t_{1/2}$ (reaction half time) was determined to be approximately 40 min.
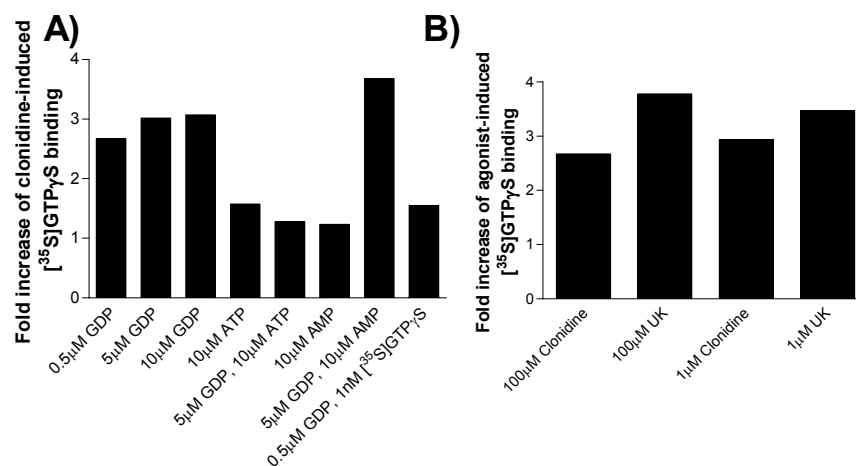
Figure 3.1: **Optimisation of the [^{35}S]GTPγS reconstitution assay**
Assays were incubated as described in the materials and methods section ensuring the following: Gα$_{i1}$ (52 nM) and β$_1$γ$_2$ (3.1 nM) were combined with 0.4 mg/mL urea treated membranes expressing α$_{2A}$AR and 0.2 nM [^{35}S]GTPγS (unless otherwise indicated). The reaction was initiated either by the addition of buffer (Basal [^{35}S]GTPγS binding) or agonist. The fold stimulation was determined as a ratio of agonist induced binding: basal binding on the mean of two filter replicates. **A)** The agonist in this set of experiments was 100 μM Clonidine (agonist induced [^{35}S]GTPγS binding) and the other additions are as indicated. **B)** The reconstitution mix contained 0.5 μM GDP and the agonist used to begin the reaction is indicated on the x-axis.

As shown previously (**Figure 3.1 A**), the combination of AMP (10 μM) and GDP (5 μM) showed enhanced fold stimulation. This was further extended by investigating [^{35}S]GTPγS binding induction by UK-14304 or clonidine in conjunction with using the more stable form of the nucleotide, AMP-PNP (10 μM) (**Figure 3.2**). After induction, similar fold stimulations were recorded in the presence of AMP-PNP or AMP and these stimulations were not statistically different. Therefore it was decided to use AMP-PNP because it was a more stable analogue and was equally as effective as AMP in reducing basal [^{35}S]GTPγS. Similar to previous observations, UK-14304 was more potent in inducing [^{35}S]GTPγS binding compared to clonidine which is a partial agonist (**Figure 3.2**). The concentration of β$_1$γ$_2$ was increased to 20 nM in an attempt to increase the fold stimulation (the concentration dependence of this dimer is further characterised in section 3.3.1.2).
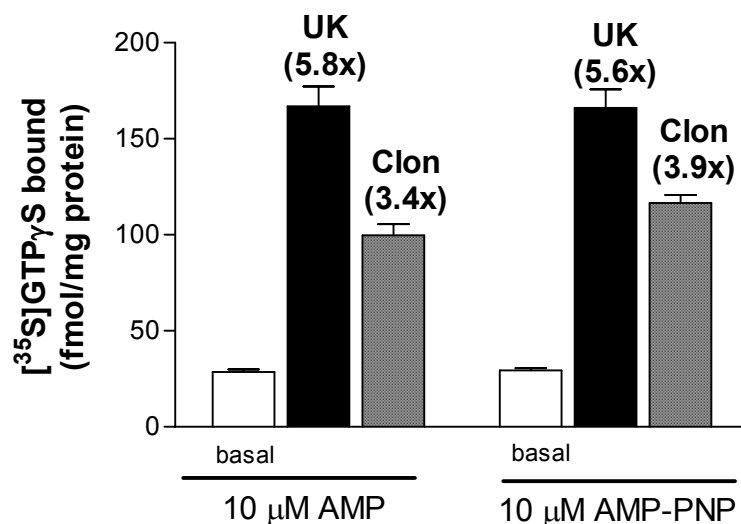
Figure 3.2: **The effect of AMP or AMP-PNP on α_{2A}AR activated [^{35}S]GTPγS binding.**
Assays were incubated as described in the materials and methods section ensuring the following: Gα_{i1} (52 nM) and $\beta_1\gamma_2$ (20 nM) were combined with 0.4 mg/mL urea treated membranes expressing α_{2A}AR, 5 μM GDP and 0.2 nM [^{35}S]GTPγS. The reaction initiated either by the addition of buffer (Basal [^{35}S]GTPγS binding) or 100 μM UK-14304 (potent agonist) or 100 μM Clonidine (partial agonist) represented by the white bars, black bars and checked bars respectively. The fold stimulation is shown above the bars (n = 3 filter replicates, mean $\pm$ SEM). Each data point represents n = 2, error bars where visible represent the range of duplicates.

3.3.1.1. Choice of agonist

To further characterise the effects of UK-14304 on the reconstitution system a concentration dose curve was performed (**Figure 3.3**). As the concentration of the agonist was increased the expected sigmoid dose response curve was obtained with an effective concentration at 50% (EC$_{50}$) of approximately 32 nM. The maximal agonist promoted [^{35}S]GTPγS binding was achieved with concentrations of 1 μM UK-14304 or more suggesting that this level of concentration is the minimum required to induce the maximal [^{35}S]GTPγS binding correlating to maximal receptor stimulation. UK-14304-induced [^{35}S]GTPγS binding was inhibited at every agonist concentration when a final concentration of 500 μM Rauwolscine, a potent α_{2A}AR antagonist, was also included in the assay. This reflected the antagonistic effects of Rauwolscine and confirmed the pharmacological response of this artificially assembled system was as expected. Rauwolscine did not stimulate [^{35}S]GTPγS above basal levels. The selective α_{2A}AR antagonist Yohimbine (500 μM) also antagonised

agonist-stimulated [^{35}S]GTPγS binding (data not shown). The concentration of β$_1$γ$_2$ was reduced to conserve protein until the minimum required was determined.
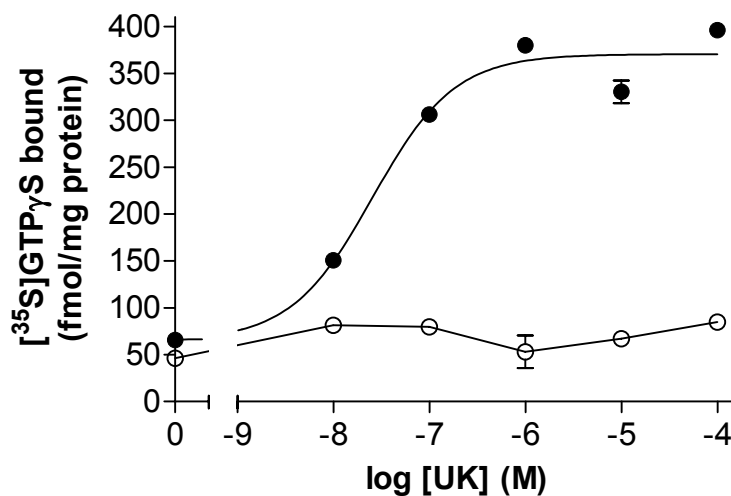

Figure 3.3: **Effects of UK-14304 and Rauwolscine on α$_{2A}$AR activated [^{35}S]GTPγS binding.** Assays were incubated as described in the materials and methods section ensuring the following: Gα$_{i1}$ (52 nM) and β$_1$γ$_2$ (3.1 nM) were combined with 0.4 mg/mL urea treated membranes expressing α$_{2A}$AR, 5 μM GDP; 10 μM AMPNP and 0.2 nM [^{35}S]GTPγS. The reaction was initiated by the addition of various concentrations of the agonist UK-14304 (concentrations as indicated). UK-14304 stimulated [^{35}S]GTPγS binding in the absence (represented by unfilled circles (○)) or presence of the potent α$_{2A}$AR antagonist 500 μM Rauwolscine (represented by filled circles (●)) is shown. The EC$_{50}$ was determined to be 31.6 nM for UK-14304 stimulation. Each data point represents n = 3 filter replicates, mean ± SEM. Where error bars are not visible they are contained within the data point.

3.3.1.2. Concentration dependence of G-protein subunits

To further enhance the S/B ratio and optimise the assay system for adaptation to a higher throughput format, the G-protein concentration dependence was investigated. In order to assess the [^{35}S]GTPγS binding capacity of an activated non-histidine tagged G-protein, β$_1$γ$_2$ concentration was kept constant (at 3.1 nM) along with the other reconstitution parameters. Interestingly, there was a non-saturable concentration dependent elevation in [^{35}S]GTPγS binding upon agonist induced Gα$_{i1}$ activation. The highest fold stimulation was obtained with a subunit concentration of 52nM and this concentration was used in subsequent experiments with this particular protein subunit. The [^{35}S]GTPγS binding kinetics of the data in **Figure 3.4** (the non-histidine tagged subunit) shows a more gradual increase to the maximal achievable binding and that this binding is not saturable suggesting this subunit preparation had

more non-specific binding sites since the non agonist stimulated basal binding also increased as the protein increased. The concentration of membranes expressing $\alpha_{2A}AR$ was halved to conserve protein and reduce non-specific binding.
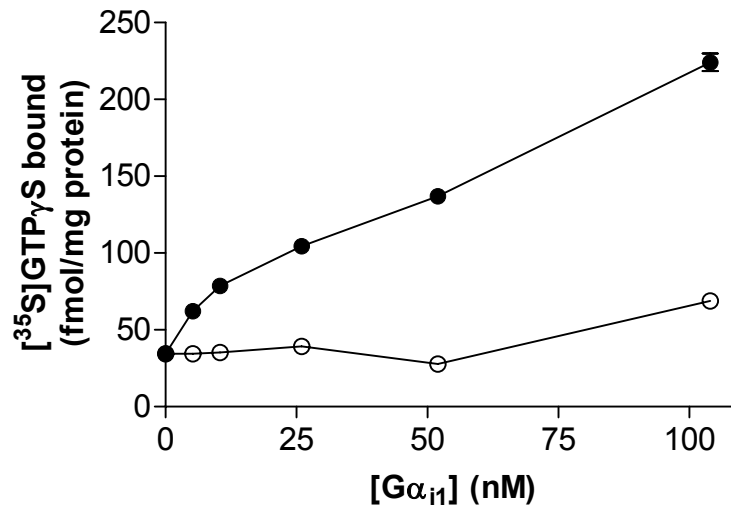

Figure 3.4: **The effect of $G\alpha_{i1}$ on $\alpha_{2A}AR$ activated [^{35}S]GTPγS binding.**
Assays were incubated as described in the materials and methods section ensuring the following: $G\alpha_{i1}$ and 3.1 nM $\beta_1\gamma_2$ were combined with 0.2 mg/mL urea treated membranes expressing $\alpha_{2A}AR$. Basal [^{35}S]GTPγS binding is represented by unfilled circles ($\circ$) and 100 μM UK-14304 induced [^{35}S]GTPγS binding is represented by filled circles ($\bullet$). The half saturation point and B_{max} upon UK-14304 stimulation were determined to be 35.6 nM an 276 fmol/mg protein respectively. Each data point represents n = 3 filter replicates, mean $\pm$ SEM. Where error bars are not visible they are contained within the data point.

The addition and concentration dependence of $\beta_1\gamma_2/\beta_1\gamma_{2(6xHIS)}$ was also investigated (**Figure 3.5 A**) by titration in the reconstitution assay ensuring that the other parameters (including $G\alpha_{i1}$) were kept constant. The half saturation point of approximately 2 nM was obtained suggesting the dimer does play an important role in the stability of the activated $G\alpha_{i1}$ subunit so its inclusion in the assay is justified. This value compares favourably with data published in (Lim and Neubig, 2001), in which the authors obtained a half saturation point of 10 nM. This low value is particularly advantageous as less protein was required to improve the signal and allowed for the conservation protein. In the absence of $\beta_1\gamma_2$, [^{35}S]GTPγS binding was minimal (**Figure 3.5**) reflecting the importance of the obligatory dimer in potentiating the agonist activated signal. The requirement for a stoichiometric addition of the 3 subunits for subunit stability (Lim and Neubig, 2001), proper

processing and membrane targeting has been clearly established (Evanko *et al.*, 2001).

Assembly of the GPCR-G-protein transductosome complex with a histidine tagged G-protein dimer was investigated. Indeed, when $\beta_1\gamma_{2(6xHIS)}$ was titrated in the following experiment (**Figure 3.5 B**) while the $G\alpha_{i1}$ subunit concentration was kept constant (along with the other parameters) a half saturation point of approximately 8.7 nM was obtained.
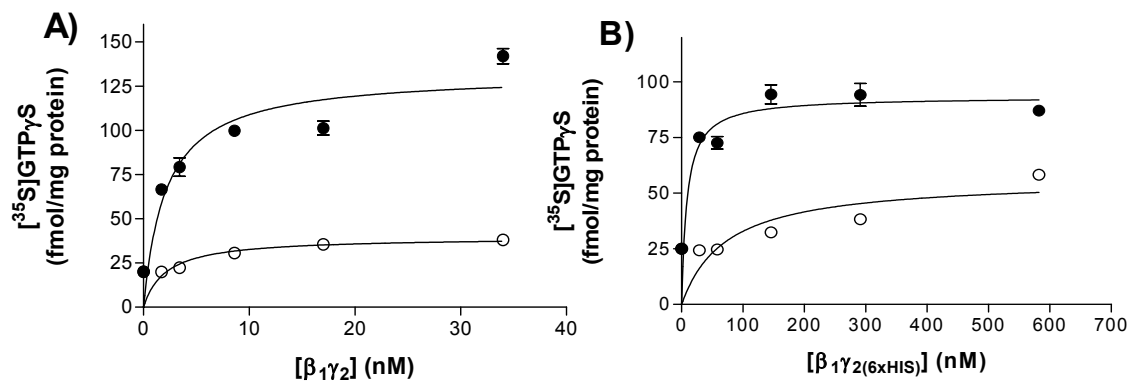

Figure 3.5: The effect of $\beta_1\gamma_2$ on $\alpha_{2A}AR$ activated [^{35}S]GTPγS binding.
Assays were incubated as described in the materials and methods section ensuring the following: $\beta_1\gamma_2$ (A) or $\beta_1\gamma_{2(6xHIS)}$ (**B**) were combined with 52 nM $G\alpha_{i1}$ with 0.2 mg/mL urea treated membranes expressing $\alpha_{2A}AR$. Basal [^{35}S]GTPγS binding is represented by unfilled circles (○) and 100 μM UK-14304 induced [^{35}S]GTPγS binding is represented by filled circles (●). **A)** The half saturation point and B_{max} upon UK-14304 stimulation were determined to be 2.1 nM and 132 fmol/mg protein respectively. **B)** The half saturation point and B_{max} upon UK-14304 stimulation were determined to be 8.7 nM an 93.3 fmol/mg protein respectively. Each data point represents n = 3 filter replicates, mean ± SEM. Where error bars are not visible they are contained within the data point.

As shown above, the dose response curve of [^{35}S]GTPγS binding to the $G\alpha_{i1}$ subunit following ligand activation of the $\alpha_{2A}AR$ was observed to differ with respect to the $\beta_1\gamma_2$ subunit concentration depending on whether this dimer was expressed with or without a histidine tag motif. Therefore, the $G\alpha_{i1(6xHIS)}$ concentration effect on [^{35}S]GTPγS binding was also considered (**Figure 3.6**). The fold stimulation was highest (above 3) at a $G\alpha_{i1(6xHIS)}$ concentration of 50 nM which corresponded to the half saturation point. This further confirmed that a histidine tag does not interfere with the receptor activated [^{35}S]GTPγS binding. The fact that the inclusion of

histidine tag did not hinder the activation of the subunit and its ability to bind the radioisotope was important. Recently, it was reported that histidine tagged G-protein subunits bound GTPγS unhindered (Simons *et al.*, 2003; Simons *et al.*, 2004) as detected by flow cytometry. A concentration of 20 nM $\beta_1\gamma_2$ was chosen because it was above the half saturation point[22].
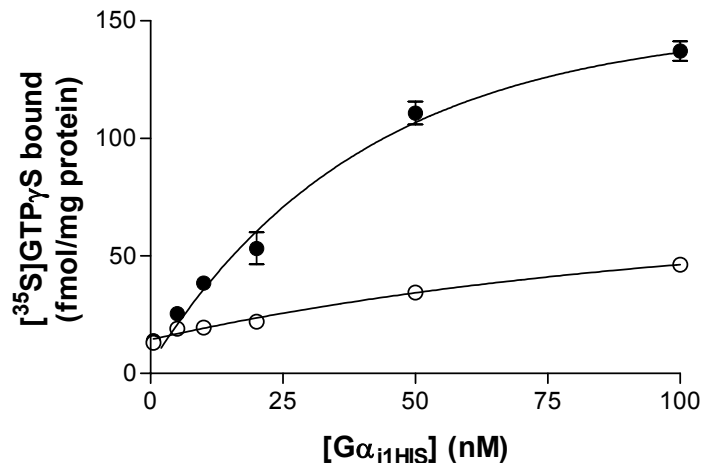

Figure 3.6: **The effect of $G\alpha_{i1(6xHIS)}$ concentration on $\alpha_{2A}AR$ activated [^{35}S]GTPγS binding.** Assays were incubated as described in the materials and methods section ensuring the following: $G\alpha_{i1(6xHIS)}$ and 20 nM $\beta_1\gamma_2$ were combined with 0.2 mg/mL urea treated membranes expressing $\alpha_{2A}AR$. Basal [^{35}S]GTPγS binding is represented by unfilled circles (○) and 100 μM UK-14304 induced [^{35}S]GTPγS binding is represented by filled circles (●). The half saturation point and the B_{max} were determined to be 49 nM and 207 fmol/mg protein respectively upon UK-14304 stimulation. Each data point represents n = 3 filter replicates, mean ± SEM. Where error bars are not visible they are contained within the data point.

3.3.1.3.   Concentration dependence membrane protein

The concentration dependence of urea treated membranes expressing $\alpha_{2A}AR$ was examined for optimisation (see **Figure 3.7**). Concentrations of 0.1 mg/mL and 0.2 mg/mL gave the best fold stimulations of 7.4 and 7.2 units respectively for this particular membrane preparation. The other fold stimulations were calculated as 5.5 and 2.0 for 0.4 and 0.8 mg/mL of membrane protein. The half saturation point was calculated to be 0.19 mg/mL. Obviously, it was important to keep the concentration for future assays in this lower range in order to potentiate the fold stimulation. As

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[22] In addition, the expression of this subunit was increased in the baculovirus expression system by amplifying the virus titre thus amount of protein was less concerning.

demonstrated, adding unnecessarily large amounts of proteins increased the basal binding.
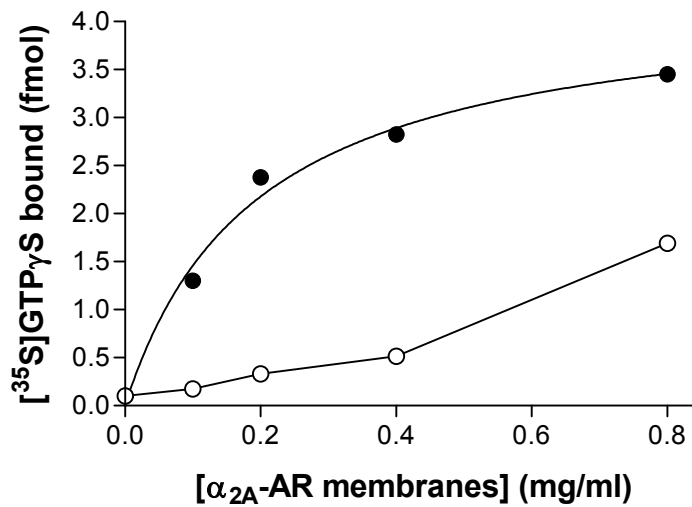

Figure 3.7: **Effect of urea treated *Sf*9 membranes expressing α_{2A}AR on α_{2A}AR activated [^{35}S]GTPγS binding.**
Assays were incubated as described in the materials and methods section ensuring the following: Various concentrations of urea treated membranes expressing α_{2A}AR were combined with 20 nM $G\alpha_{i1(6xHIS)}$ and 20 nM $\beta_1\gamma_2$. Basal [^{35}S]GTPγS binding is represented by unfilled circles ($\circ$) and 100 μM UK-14304 induced [^{35}S]GTPγS binding is represented by filled circles ($\bullet$). Each data point represents n = 3 filter replicates, mean $\pm$ SEM. Where error bars are not visible they are contained within the data point.

To further characterise the system, a study was performed to compare reconstitution of α_{2A}AR with $G\alpha_{i1}$ and $\beta_1\gamma_2$ (gold standard for comparative purposes); α_{2A}AR with $G\alpha_{i1}$ and $\beta_1\gamma_{2(6xHIS)}$; α_{2A}AR with $G\alpha_o$ and $\beta_1\gamma_{2(6xHIS)}$ and β_1AR with $G\alpha_{i1}$ and $\beta_1\gamma_2$ (**Figure 3.8**). Reconstitution with the subunit $G\alpha_o$ at an initial concentration of 50 nM was not capable of agonist promoted [^{35}S]GTPγS binding and this assay configuration was not pursued any further in this thesis. Other researchers in the laboratory did achieve α_{2A}AR signalling through the $G\alpha_o$ pathway with much higher concentrations of subunits (data not shown) although the total amount of [^{35}S]GTPγS bound was very low compared with using $G\alpha_{i1}$. Finally, a membrane preparation from insect cells infected with a baculovirus encoding the β_1AR were reconstituted and used with the [^{35}S]GTPγS assay. This receptor was previously shown to be

functional with respect to ligand binding (see chapter 2)[23]. The agonist, isoproterenol did not induce signalling through this receptor as anticipated since this receptor has not previously been shown to signal through the $G\alpha_{i1}$ pathway in such a system. However it is known to signal through the $G\alpha_s$ pathway (Parker *et al.*, 1991). Nevertheless, this data helps to demonstrate the sensitivity and specificity of this assay system.
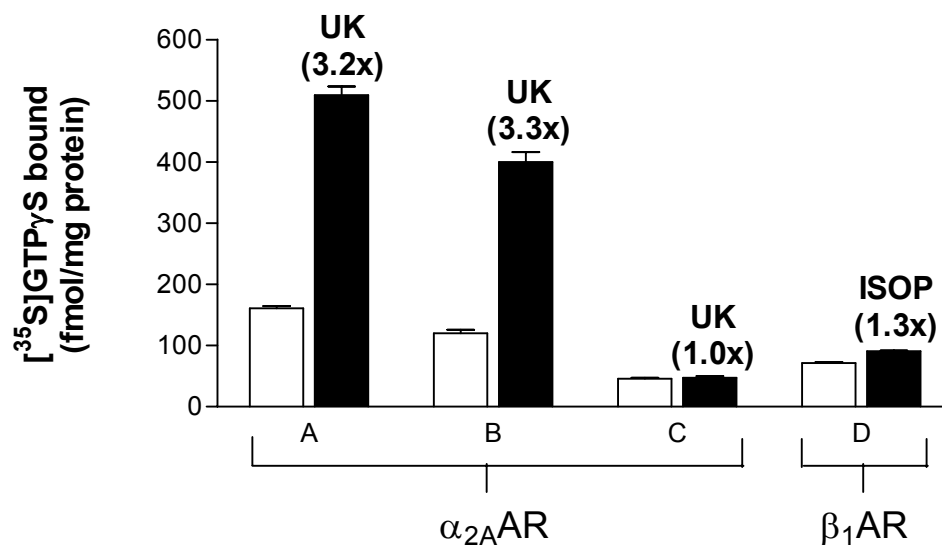

Figure 3.8: **Reconstitution of α_{2A}-AR or β_1AR with $G\alpha_{i1}$ or $G\alpha_o$ + $\beta_1\gamma_2$ or $\beta_1\gamma_{2(6xHIS)}$ in suspension.**
All incubations contained 5 μM GDP, 10 μM AMP-PNP and 0.2 nM [35S]GTPγS along with the following combinations of membranes and G-proteins; **A)** $G\alpha_{i1}$ (50nM), $\beta_1\gamma_2$ (50 nM) and urea treated *Sf*9 membranes over expressing α_{2A}AR (0.2 mg/mL). **B)** $G\alpha_{i1}$ (50nM), $\beta_1\gamma_{2(6xHIS)}$ (50 nM) and urea treated *Sf*9 membranes over expressing α_{2A}AR(0.2 mg/mL). **C)** $G\alpha_o$ (50nM), $\beta_1\gamma_{2(6xHIS)}$ (50 nM) and urea treated *Sf*9 membranes over expressing α_{2A}AR(0.2 mg/mL). **D)** $G\alpha_{i1}$ (50nM), $\beta_1\gamma_2$ (50 nM) and urea treated *Sf*9 membranes over expressing β_1AR (0.2 mg/mL). Basal [35S]GTPγS binding) is represented by the white bars or 100 μM UK-14304 or 50 μM isoproterenol (agonist induced [35S]GTPγS binding) is represented by black bars. The fold stimulation is shown above the bars (n = 3 filter replicates, mean ± SEM).

The [35S]GTPγS reconstitution assay was chosen to measure the functionality of the reconstituted α_{2A}AR transductosome. The main focus of this section was to optimise and with regard to the S/B ratio so as to allow further development of the assay into a format which would allow higher throughput without the loss of sensitivity. In addition and as opposed to methods indicated in (Lim and Neubig, 2001), it was

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[23] Ideally a positive control for this experiment would include the functional reconstitution of the β_1AR with $G\alpha_s$. However, this subunit was unable to be purified as previously discussed.

determined that pre incubating the reconstitution mix (receptor and G-proteins) was not required and that a 5 min incubation with [^{35}S]GTPγS before the addition of the agonist did not make a difference to the amount of [^{35}S]GTPγS upon agonist induction (data not shown).

3.3.2. Moving towards a high throughput [^{35}S]GTPγS assay.

The aim of this section was to adapt the single tube [^{35}S]GTPγS based signalling assay to one which made use of the 96-well plate format which would allow greater latitude in terms of sample replication[24]. In the past Millipore 96-well plates with a GF/C filter base have been used for radioligand binding (Harms *et al.*, 2000). Indeed these MultiScreen[TM] filter based methods have long been used for receptor assays because of their high sensitivity and sample rates (Janssen *et al.*, 1999). There were however some technical considerations in using such a format. Firstly, the volume of Microscint[TM]20 (which allowed the beta ray emission in the detection machinery) was determined. Microscint[TM]20 was used at 40 μL as this was shown to give the most reproducible data (data not shown) and it correlated with the literature on this product (Janssen *et al.*, 1999). The other important consideration was that according to the manufacturer's guidelines assay convenience is increased if the filters were pre-wet. Thus, each individual well can be used for assay incubation thereby making the assay less cumbersome than a test tube assay whereby sample must be transferred after incubation to the filter (product specifications). However, it was immediately apparent that the MultiScreen[TM] plates would not support the incubation step required for the [^{35}S]GTPγS binding assay unlike the specification where it serves as both an incubation platform and a platform for filtration/separation as was described

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[24] Note previously assays were performed as filter replicates and this was not optimal. From this point forward n= number of samples that is separate incubations.

for the Europium-GTP (Frang *et al.*, 2003). This problem was circumvented by using the MultiScreen[TM] plates only as a filter interface. Agonist-promoted [^{35}S]GTPγS binding was compared using the 2 technical procedures (**Figure 3.9**); "all-in-one" represented the use of the MultiScreen[TM] plate only (for both assay incubation and termination by filtration), while "separate" represented the use of a separate incubation plate (for the assay incubation) followed by transference of the sample to the MultiScreen[TM] plate for assay termination via the filtration step. A V-shaped polypropylene 96-well plate was shown to be effective as an "incubation plate" and polypropylene was chosen because it had minimal protein binding. Despite this requirement for assay sample transfer, the 96-well format still provided advantages with regard to convenience, time required to carry out multiple assays as well as the amount of data obtained when compared to the original test tube assay.
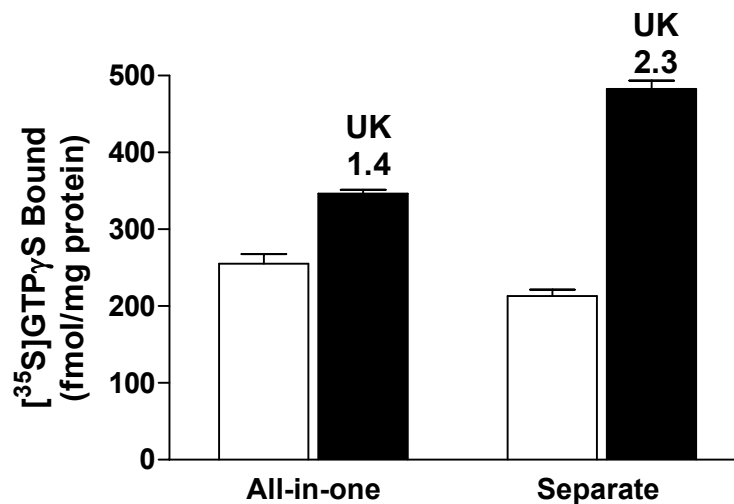

Figure 3.9:   **Effect of separate incubation plate on α$_{2A}$AR activated [^{35}S]GTPγS binding in 96-well plate.**
Assays were incubated as described in the materials and methods section ensuring the following: Gα$_{i1(6xHIS)}$ (50 nM) and β$_1$γ$_2$ (50 nM) were combined with 0.2 mg/mL urea treated membranes expressing α$_{2A}$AR. Basal [^{35}S]GTPγS binding is represented by the white bars while 100 μM UK-14304 induced [^{35}S]GTPγS is represented by black bars. The fold stimulation is shown above the bars. Incubations (25 μL) were performed all-in-one on the filter plate (Millipore MultiScreen[TM] 96-well plate with GF/C filters) (n = 6, mean ± SEM) or incubated in a separate polypropylene tube and transferred to the filter plate after 60 min. Data represents n = 3 samples, mean ± SEM. The plate was washed 3 times with 200 μl ice-cold TMN.

To further assess the possibility of using the MultiScreen^{TM} plate as both an incubation plate and a filtration plate, pre-coating the well was investigated. The technical literature (Millipore technical notes) describes coating plates with 0.1% (w/v) polyethyleneimine (PEI) or BSA to reduce non-specific binding in MultiScreen^{TM} GF/C assays. These methods were not effective in the present assay. Hence from this point forward incubations were performed in a separate incubation 96-well plate in a total volume of 40 μL and 25 μL of this sample was transferred to the Millipore 96-well MultiScreen^{TM} plate (containing GF/C filters) for filtration and subsequent counting of bound [^{35}S]GTPγS. It was necessary to determine the amount and number of washes required to adequately minimise background [^{35}S]GTPγS binding. The maximum wash volume (well volume) was 250 μl and 4 washes were chosen. Other researchers suggest that 2 washes are sufficient (Frang *et al.*, 2003).

3.3.2.1. Concentration dependence of membrane protein

The relationship between the amount of membrane protein used and the extent of [^{35}S]GTPγS binding is shown in **Figure 3.10**. Total [^{35}S]GTPγS under basal or UK-14304-stimulated conditions increased linearly with increasing protein concentrations up to 0.2 mg/mL. Additional increments in protein concentration did not produce further increases in total [^{35}S]GTPγS binding but did increase the background binding. The half saturation point was determined to be 0.04 mg/mL which is over 4 fold less than the half saturation point calculated in the [^{35}S]GTPγS manifold assay. This indicates that less protein may be used in this higher throughput format.
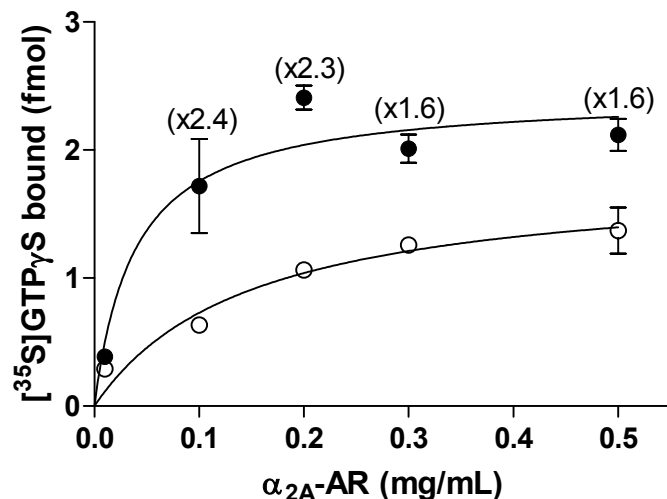
Figure 3.10:   **Effect of membranes expressing $\alpha_{2A}AR$ on $\alpha_{2A}AR$ activated [^{35}S]GTPγS binding in 96-well plate.**
Assays were incubated as described in the materials and methods section ensuring the following: Various concentrations of urea treated membranes expressing $\alpha_{2A}AR$ were combined with 50 nM $G\alpha_{i1(6xHIS)}$, 50 nM $\beta_1\gamma_2$. Basal [^{35}S]GTPγS binding is represented by unfilled circles ($\circ$) and 100 μM UK-14304 induced [^{35}S]GTPγS binding is represented by filled circles ($\bullet$). The half saturation point upon UK-14304 stimulation was determined to be 0.04 mg/mL. The fold stimulation is displayed above each point. Each data point represents n = 3 samples, mean $\pm$ SEM. Where error bars are not visible they are contained within the data point.

3.3.2.2.   Concentration dependence of G-protein subunits

The 96-well format for the [^{35}S]GTPγS binding assay was used to determine the concentration dependence of the G-protein subunit combinations to ensure that the results were consistent between the two assay formats (single test tube and 96-well format). Furthermore, such optimisation was required to ascertain the concentration range of subunits for maximal signal generation. In the first instance the concentration of $G\alpha_{i1(6xHIS)}$ was monitored with respect to agonist promoted [^{35}S]GTPγS binding (**Figure 3.11**). The fold stimulation at each concentration of $G\alpha_{i1(6xHIS)}$ ranged from x1.9 (100 nM $G\alpha_{i1(6xHIS)}$) up to x4.0 at 20 nM $G\alpha_{i1(6xHIS)}$ with this concentration used in subsequent assays. It is likely that this effect can be attributed to the lower basal binding at these lower concentrations of $G\alpha_{i1(6xHIS)}$ thereby enhancing the S/B ratio by a reduction in the level of background binding. Interestingly the addition of G-protein subunit was saturable, as opposed to the non-

saturable receptor activated [^{35}S]GTPγS binding shown using the manifold assay on
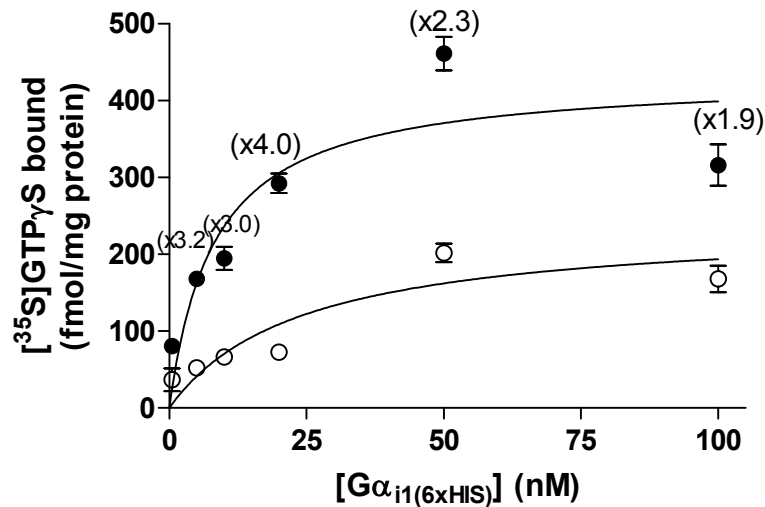
addition of Gα$_{i1}$.

Figure 3.11: **Effect of Gα$_{i1(6xHIS)}$ on α$_{2A}$AR activated [^{35}S]GTPγS binding in 96-well plates.**
Assays were incubated as described in the materials and methods section ensuring the following
Various concentrations of Gα$_{i1(6xHIS)}$ and 50 nM β$_1$γ$_2$ were combined with 0.2 mg/mL urea treated
membranes expressing α$_{2A}$AR. Basal [^{35}S]GTPγS binding is represented by unfilled circles (○) and
100 μM UK-14304 induced [^{35}S]GTPγS binding is represented by filled circles (●). The half
saturation point upon UK-14304 stimulation were determined to be 8.1 nM. Each data point
represents n = 3 samples, mean ± SEM. Where error bars are not visible they are contained within the
data point.

Concentration-dependent elevation of agonist-stimulated [^{35}S]GTPγS binding was

also monitored with the obligatory dimer, β$_1$γ$_2$, at the constant concentration of 20

nM Gα$_{i1(6xHIS)}$. Dimer concentration was not a limiting factor in the reaction after a

concentration of 20 nM was reached (**Figure 3.12**). The lower dimer concentrations

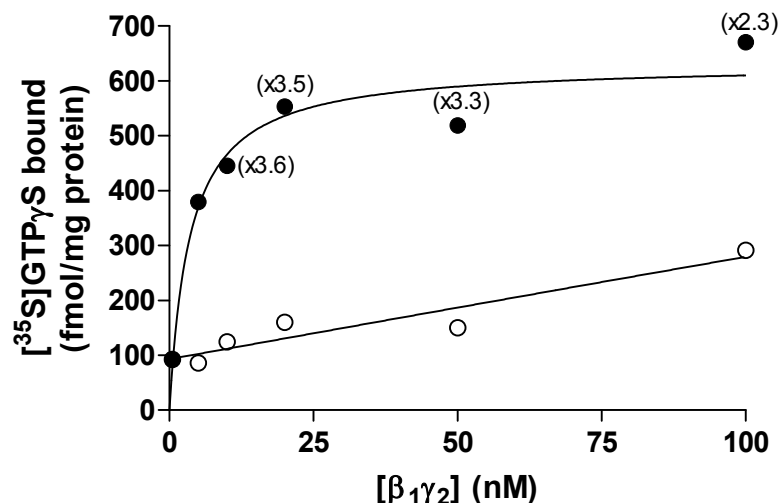of 5, 10 and 20 nM gave the larger fold stimulations.

Figure 3.12: **Effect of $\beta_1\gamma_2$ on α_{2A}AR activated [^{35}S]GTPγS binding in 96-well plates.**
Assays were incubated as described in the materials and methods section ensuring the following Various concentrations of $\beta_1\gamma_2$ and 20 nM G$\alpha_{i1(6xHIS)}$ were combined with 0.1 mg/mL urea treated *Sf*9 membranes expressing α_{2A}AR. Basal [^{35}S]GTPγS binding is represented by unfilled circles ($\circ$) and 100 μM UK-14304 induced [^{35}S]GTPγS binding is represented by filled circles ($\bullet$). The half saturation point for UK-14304 stimulation, was determined to be 3.5 nM. The fold stimulation is shown. Each data point represents n = 3 samples, mean $\pm$ SEM. Where error bars are not visible they are contained within the data point.

3.3.2.3. Concentration dependence of UK-14304

The concentration dependence of the α_{2A}AR to its agonist UK-14304 was examined in the preassembled transductosome complex to further confirm the feasibility of using the 96-well configured [^{35}S]GTPγS binding assay. The dose response curve of the α_{2A}AR/G-protein complex to UK-14304 as measured by the [^{35}S]GTPγS binding assay performed in 96-well format is shown in **Figure 3.13**. The EC$_{50}$ was determined as 82 nM and this is similar to the EC$_{50}$ from the test tube assay (32 nM) thus indicating that the complex is functioning in a manner similar to that of the single tube assay. The selectivity of the receptor complex was verified by the addition of the antagonist (Rauwolscine) concurrently with all concentrations of UK-14304. Upon this addition, the agonist promoted [^{35}S]GTPγS was reduced.
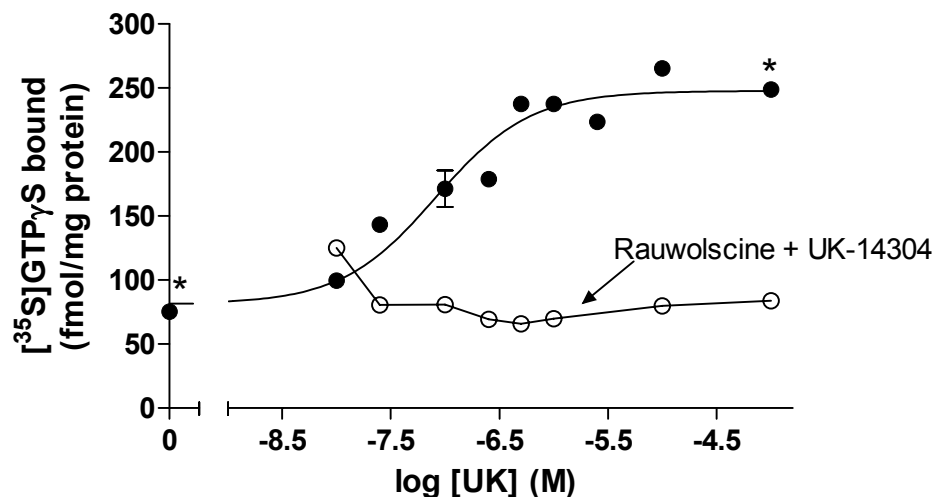
Figure 3.13:   **Effect of UK-14304 on α_{2A}AR activated [^{35}S]GTPγS binding in 96-well plates.** Assays were incubated as described in the materials and methods section ensuring the following: Urea treated *Sf*9 membranes expressing α_{2A}AR were combined with 20 nM G$\alpha_{i1(6xHIS)}$, and 20 nM $\beta_1\gamma_2$. The reaction initiated by the addition of 100 μM UK-14304 (agonist induced [^{35}S]GTPγS binding, represented by filled circles ($\bullet$) or 100 μM UK-14304 with 500 μM Rauwolscine represented by unfilled circles ($\circ$). The EC$_{50}$ was determined to be 82 nM. Each data point represents n = 3 samples, or n = 6 (indicated by *) mean $\pm$ SEM. Where error bars are not visible they are contained within the data point.

3.3.3.   Biological variability

Earlier, biological variability was observed between different $\beta_1\gamma_2$ subunits with respect to whether they were expressed with or without a histidine tag motif (**Figure 3.5**). The data suggests that there was a difference in receptor activated [^{35}S]GTPγS binding between the two different preparations of subunits. After noticing this variability early in the assay optimisation studies, an internal "gold standard" control was deemed necessary for each assay undertaken. To further investigate the biological variability within the membrane preparations and preparations of $\beta_4\gamma_2$ the following comparison was performed (**Figure 3.14**). The transductosome was reconstituted with $\beta_4\gamma_2$ instead of $\beta_1\gamma_2$ because experiments showed that the former dimer increased the fold stimulation slightly when compared to $\beta_1\gamma_2$ (data shown later). As a consequence $\beta_4\gamma_2$ was used in preference to $\beta_1\gamma_2$ in the rest of these 96-

well format $[^{35}S]GTP\gamma S$ assays[25]. The agonist stimulated $[^{35}S]GTP\gamma S$ binding was compared in 2 separate reconstitutions. All protein concentrations were kept the same throughout. The amount of UK-14304-induced $[^{35}S]GTP\gamma S$ was statistically different in each of the preparations indicating the validity of internal assay standardisation. The conservation of protein becomes an issue in the replication of experimental parameters. That is, due to the biological variability in the production of the expressed proteins each preparation is slightly different in yield and functional proteins. This makes comparison between experiments that used different subunits very difficult to interpret. In addition, a problem was identified later which related to biological activity of frozen $[^{35}S]GTP\gamma S$ (see Appendix page 246) that made it impossible to compare all data sets (see appendix page 235).
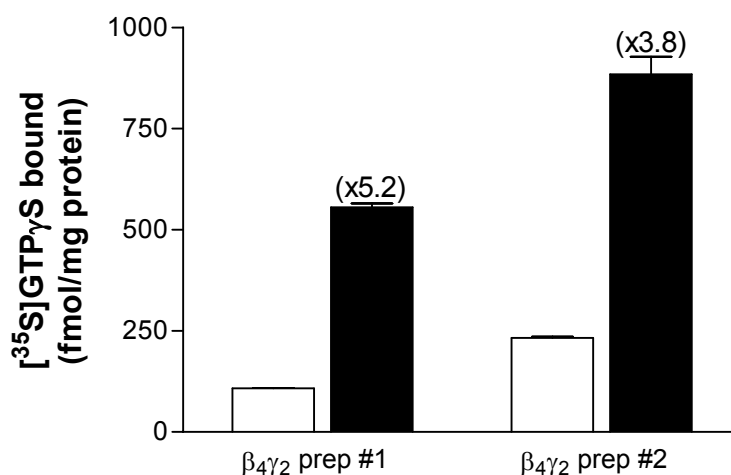

Figure 3.14: **Effect of biological variability of samples in the $[^{35}S]GTP\gamma S$ binding assay.**
Assays were incubated as described in the materials and methods section ensuring the following. The concentrations of the proteins were the same in each assay i.e. urea treated *Sf*9 membranes over expressing $\alpha_{2A}AR$ (0.1 mg/mL), 20 nM $G\alpha_{i1(6xHIS)}$ and 20 nM $\beta_4\gamma_2$. However, 2 different preparations of $\beta_4\gamma_2$ (#1 and #2) were compared: Basal $[^{35}S]GTP\gamma S$ binding) is represented by the white bars or 10 μM UK-14304 induced $[^{35}S]GTP\gamma S$ binding is represented by black bars. Each bar represents n = 4 samples, mean $\pm$ SEM.

3.3.4. Extension of the $[^{35}S]GTP\gamma S$ assay

Following the initial characterisation studies of the signalling assay using the 96-well assay format, further characterisation was performed. To begin with $\alpha_{2A}AR$ receptor

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[25] Note $\beta_4\gamma_{2(6xHIS)}$ could not be prepared at this time and reason behind this were inconclusive.

system's repertoire of subunit recombinations was extended. The diversity of the subunits in the G-protein heterotrimer can have important functional consequences (Fletcher *et al.*, 1998). Previously the optimisation assays utilised the reconstitution of $\alpha_{2A}AR$ with $G\alpha_{i1}$ and $\beta_1\gamma_2$. In **Figure 3.8** recombination with $G\alpha_o$ was briefly examined. Here, the $G\alpha_q$ subunit as well as the $\beta_4\gamma_2$ dimer was investigated with the $\alpha_{2A}AR$.

In order to quantify the functionality of $\alpha_{2A}AR$ transductosome reconstituted with various combinations of G-proteins, the fold stimulations upon UK-14304 induction were calculated. These values are represented above each of the pairs of binding (**Figure 3.15**). For all three $G\alpha$ subunits examined, the higher fold stimulation was achieved when the subunit was reconstituted with $\beta_4\gamma_2$ compared to $\beta_1\gamma_2$ suggesting that the $\beta_4\gamma_2$ dimer enhances the agonist induced $[^{35}S]GTP\gamma S$ conformation above the latter dimer. Surprisingly the $\alpha_{2A}AR$ also signalled to a small extent through the $G\alpha_q$ pathway in this artificial system. As a control, Ang II was used instead of UK-14304 in the $\alpha_{2A}AR/G\alpha_q/\beta_1\gamma_2$ transductosome and no agonist induced $[^{35}S]GTP\gamma S$ was evident (data not shown).

In a similar fashion, both the M_2 and AT_{1A} receptors were investigated for agonist promoted $[^{35}S]GTP\gamma S$ binding when using different sets of G-protein subunits. The M_2 receptor, which is reported to signal through the $G\alpha_{i/o}$ pathway (Krejci *et al.*, 2004), was reconstituted with $G\alpha_{i1\ (6xHIS)}$ or $G\alpha_q$ and $\beta_4\gamma_2$ or $\beta_1\gamma_2$[26] The M_2 activated $[^{35}S]GTP\gamma S$ using the $G\alpha_{i1}$ subunit but not when $G\alpha_q$ was used.(data not shown). On the contrary, the AT_{1A} receptor has been shown to signal through both the $G\alpha_{i1}$ and

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[26] The characterisation of this receptor transductosome was performed by other researchers in the lab lead by Dr Wayne Leifert (CSIRO HSN).

$G\alpha_q$ pathways (Sasamura *et al.*, 2000) and so both G-protein subunits were investigated in this study (**Figure 3.15**). Non-urea treated membranes from CHO cells stably expressing the AT_{1A} receptor were used because endogenous G-proteins were expected to support any receptor mediated signalling activity. Agonist-promoted [^{35}S]GTPγS binding was not observed in any of the combinations investigated.
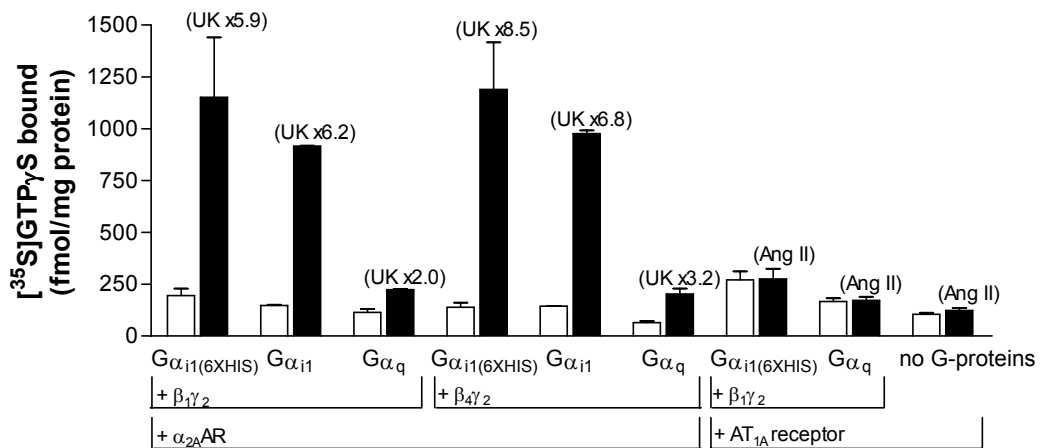

Figure 3.15: **Reconstitution of α_{2A}-AR with $G\alpha_{i1(6xHIS)}$/$G\alpha_{i1}$/$G\alpha_q$ + $\beta_1\gamma_2$/$\beta_4\gamma_2$ in suspension.**
Assays were incubated as described in the materials and methods section ensuring the following: Urea treated *Sf*9 membranes over expressing α_{2A}AR (0.1 mg/mL) or non-urea treated CHO cell membranes stably expressing AT_{1A} (0.2 mg/mL)were reconstituted with one of the following combinations of purified G-proteins (20 nM of each subunit); $G\alpha_{i1(6xHIS)}$/$G\alpha_{i1}$/$G\alpha_q$ and $\beta_1\gamma_2$/$\beta_4\gamma_2$. Basal [^{35}S]GTPγS binding is represented by the white bars and induced [^{35}S]GTPγS binding (10 μM UK-14304 or 10 μM Ang II) is represented by black bars. (n =2; error bars represent the range of duplicates) and AT_{1A} data (n=3, mean $\pm$ SEM).

To further explore the apparent activation of $G\alpha_q$ by agonist stimulated α_{2A}AR, the concentration dependence of $G\alpha_q$ on the α_{2A}AR transductosome system was investigated (**Figure 3.16**). Agonist promoted [^{35}S]GTPγS as well as basal binding increased as the concentration of $G\alpha_q$ was increased in the assay. The highest fold stimulation (x2.3) obtained was at a concentration of 50 nM. Thus this data indicates that α_{2A}AR can be functionally reconstituted with $G\alpha_q$ albeit with relatively lower levels of signalling activity compared with $G\alpha_{i1}$ subunits. This subunit has been shown to have lower affinity for [^{35}S]GTPγS (Milligan, 2003) which may reflect the lower agonist promoted [^{35}S]GTPγS seen here. The other possibility is that the

receiver under study is not efficient at coupling to this subunit as it is to $G\alpha_{i1}$. Also, to verify that the $G\alpha_q$ baculovirus did in fact contain the cDNA for $G\alpha_q$ the DNA was sequenced with the help of Dr Richard Glatz (CSIRO HSN) which showed that the DNA was in fact $G\alpha_q$. An alterative approach to sequencing could be to use a selective $G\alpha_q$ inhibitor (GP2A) (McKillop *et al.*, 1999) although this has not be used in a cell-free system.
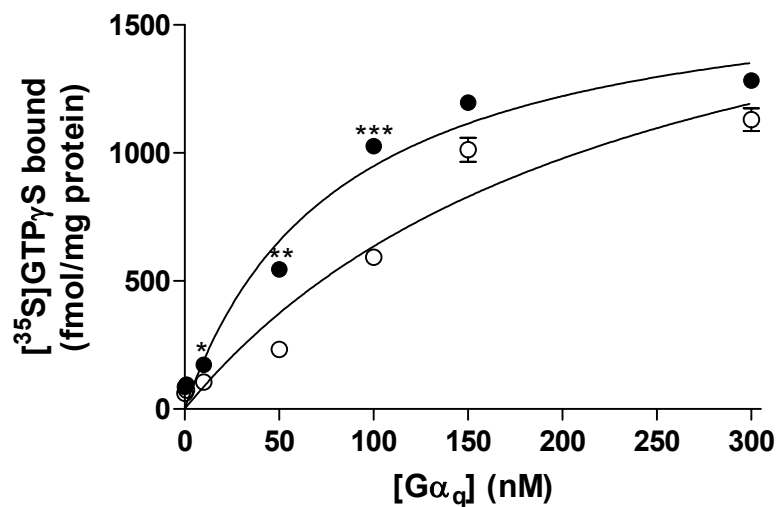

Figure 3.16: **Effect of $G\alpha_q$ on $\alpha_{2A}AR$ activated [^{35}S]GTPγS binding in 96-well plates.**
Assays were incubated as described in the materials and methods section ensuring the following: Urea treated *Sf*9 membranes over expressing $\alpha_{2A}AR$ receptor (0.1 mg/mL) were combined with increasing concentration of $G\alpha_q$ and 20 nM $\beta_4\gamma_2$. Basal [^{35}S]GTPγS binding is represented by open circles (o) and 10 μM UK-14304 [^{35}S]GTPγS binding is represented by black circles (●).Each data point represents n = 2 samples, mean ± SEM. Where error bars are not visible they are contained within the data point. $p < 0.05$; $p < 0.01$; $p < 0.0001$.

3.3.4.1.   AT$_{1A}$ reconstitution

Various approaches were taken to investigate possible agonist induced [^{35}S]GTPγS binding using the AT$_{1A}$ receptor in CHO cell membranes. The membrane concentration was decreased to 0.1mg/mL to reduce non-specific [^{35}S]GTPγS binding. Urea treated membranes were used in the assay and the concentration of $G\alpha_q$ was raised to 150 nM. GDP and AMP-PNP concentration were also manipulated. However no trends indicative of signalling activity with the AT$_{1A}$

receptor were evident (data not shown). Before signalling assays were attempted, [^{125}I]Ang II binding to these membranes was apparent (described in Chapter 2).

In order to systematically attempt to demonstrate signalling using the reconstituted AT$_{1A}$ receptor and the [^{35}S]GTPγS binding assay, it was decided to initiate a cross over study, that is, start with a system that is functional (α_{2A}AR system) and add to this system. In the previous chapter, it was shown that [^{125}I]Ang II binding to CHO cell membranes was hindered by the presence of the detergent cholate, in the semi-purified subunits. In the present study the G$\alpha_{i1(6xHIS)}$ subunit was used in a highly concentrated (i.e. 10.4 μM stock) form thus limiting the amount of cholate added to the assay. As previously shown, concentration of this subunit up to and including 50 nM did not inhibit specific [^{125}I]Ang II binding (Chapter 2).

Using the "gold standard" transductosome system (see **Figure 3.17 A**) over a 3.7 fold stimulation was observed upon UK-14304 induction. As expected, the agonist Ang II had no effect on this system. This fold stimulation was decreased to 3.1 upon the addition of 20 nM Gα_q (**B**) to the assay and this decrease is likely due to the increase in basal binding by the addition of another subunit (and [^{35}S]GTPγS binding sites). As seen previously, when G$\alpha_{i1(6xHIS)}$ was replaced by Gα_q, UK-14304 promoted [^{35}S]GTPγS binding was reduced significantly suggesting that either functional coupling to this G-protein subunit is limited, or that Gα_q has reduced affinity for [^{35}S]GTPγS or both. The total protein was increased 2 fold in the assay with the addition of membranes expressing AT$_{1A}$ and was accounted for in the fmol/mg protein which is shown. The gold standard (**A**) was repeated with the CHO membrane addition (**D**). Interestingly, UK-14304 fold stimulation was dramatically decreased in assay **D** which is likely due to the excess protein blocking the

transduction in the α_{2A}AR system. Ang II, the AT$_{1A}$ receptor agonist, caused a slight increase in [^{35}S]GTPγS bound above basal. However this was not significant. The membrane only control (**E**) was added to determine non-specific binding levels and ensure that no endogenous G-protein activity was having an effect.
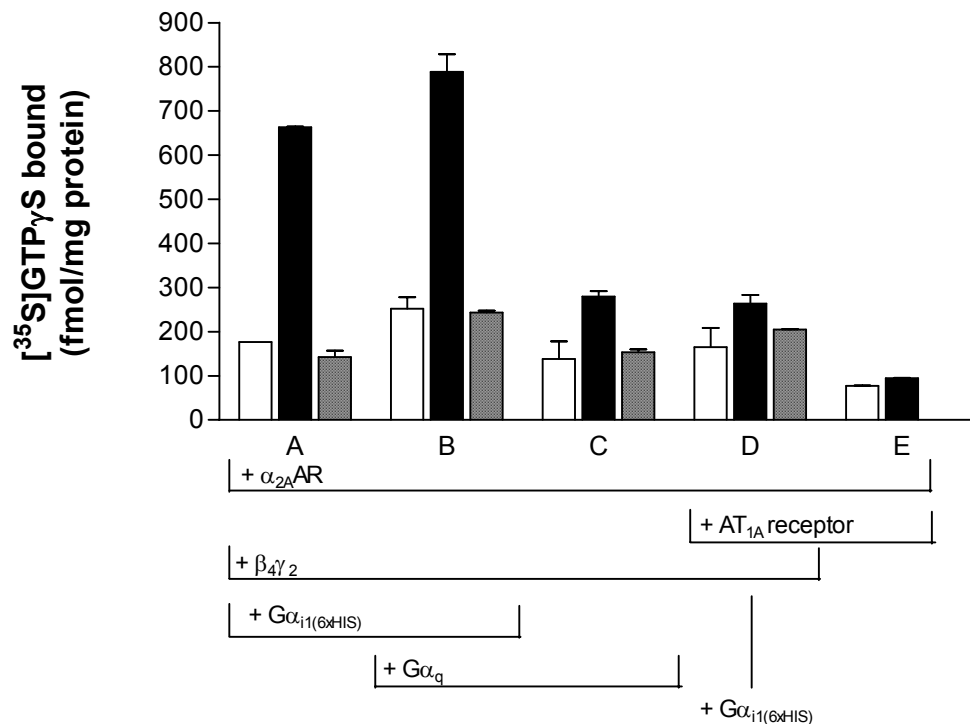

Figure 3.17: **Reconstitution cross over analysis using 0.2 nM [^{35}S]GTPγS.**
Assays were incubated as described in the materials and methods section ensuring the following: Urea treated *Sf*9 membranes over expressing α_{2A}AR receptor (0.1 mg/mL) along with the following: **A**) 20 nM G$\alpha_{i1(6XHIS)}$ and 20 nM $\beta_4\gamma_2$ **B**) 20 nM G$\alpha_{i1(6XHIS)}$, 20 nM $\beta_4\gamma_2$ and 20 nM Gα_q **C**) 20 nM $\beta_4\gamma_2$ and 20 nM Gα_q **D**) 20 nM G$\alpha_{i1(6XHIS)}$, 20 nM $\beta_4\gamma_2$ and 0.1 mg/mL CHO cell membranes expressing AT$_{1A}$ **E**) 0.1 mg/mL CHO cell membranes expressing AT$_{1A}$. Basal [^{35}S]GTPγS binding is represented by the white bars, 10 μM UK-14304 induced [^{35}S]GTPγS is represented by black bars and 10 μM Ang II induced [^{35}S]GTPγS is represented by checked bars (n =2; error bars represent the range of duplicates).

To rule out the possibility that [^{35}S]GTPγS concentration was limiting the signal, the cross over study was repeated with a 5 fold higher concentration of [^{35}S]GTPγS (1 nM). As expected the fold stimulation was decreased due to higher basal binding (Windh and Manning, 2002). However the same trends were observed (

Figure 3.18). Similarly, $G\alpha_q$ activation by $\alpha_{2A}AR$ was not significant; however a trend was evident. In this study, although the UK-14304 induced [^{35}S]GTPγS binding in the presence of membranes expressing AT_{1A} was much reduced (**D**) it was significantly ($p < 0.05$) different to the basal binding in this assay. Furthermore, Ang II induced [^{35}S]GTPγS binding was increased above basal binding. However this increase was not significant and it was not significantly different to the UK-14304 promoted binding in this assay.
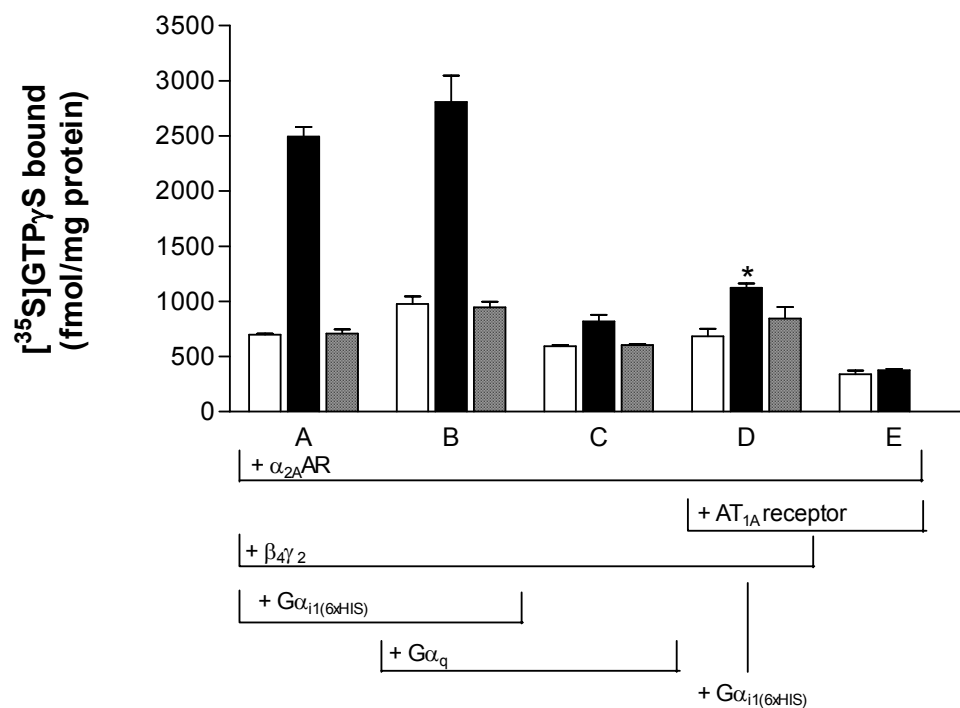

Figure 3.18:   Reconstitution cross over analysis using 1 nM [^{35}S]GTPγS.
Assays were incubated as described in the materials and methods section ensuring the following: Urea treated *Sf*9 membranes over expressing $\alpha_{2A}AR$ receptor (0.1 mg/mL) along with the following: **A**) 20 nM $G\alpha_{i1(6XHIS)}$ and 20 nM $\beta_4\gamma_2$ **B**) 20 nM $G\alpha_{i1(6XHIS)}$, 20 nM $\beta_4\gamma_2$ and 20 nM $G\alpha_q$ **C**) 20 nM $\beta_4\gamma_2$ and 20 nM $G\alpha_q$ **D**) 20 nM $G\alpha_{i1(6XHIS)}$, 20 nM $\beta_4\gamma_2$ and 0.1 mg/mL CHO cell membranes expressing AT_{1A} **E**) 0.1 mg/mL CHO cell membranes expressing AT_{1A}. Basal [^{35}S]GTPγS binding is represented by the white bars, 10 μM UK-14304 induced [^{35}S]GTPγS is represented by black bars and 10 μM Ang II induced [^{35}S]GTPγS is represented by checked bars (n =2; error bars represent the range of duplicates).* $p < 0.05$.

3.3.4.2.   Purified G-proteins with reduced cholate.

The rationale behind this purification was to reduce the cholate in the G-protein preparations as it is likely to be causing a decreased affinity of [^{125}I]Ang II to the receptor (see last chapter). Thus, $G\alpha_q$ and $G\alpha_{i1}$ were both prepared using the

conventional purification method (1% cholate (w/v) in buffer E) as well as using buffer E with 10 fold less cholate (0.1% (w/v) referred to as the unconventional purification strategy). When $G\alpha_{i1}$ was eluted from the Ni(NTA) column using AlF_4^- (in buffer E containing 0.1% (w/v) cholate), half as much of this protein was present compared to the elution using the conventional method (see **Table 3.2**). Thus, the rest of this protein was seen un-dissociated to the $\beta_1\gamma_{2(6xHIS)}$. In contrast, $G\alpha_q$ (purified using 0.1% (w/v) cholate in buffer E) did not dissociate from the histidine tagged $\beta_1\gamma_{2(6xHIS)}$ after AlF_4^- elution as opposed to the $G\alpha_q$ prepared simultaneously using the conventional methodology. The un-dissociated heterotrimeric protein concentration (mixed sample) was determined (using the Bradford protein assay) to be 6.0 μM. Thus, using 10 fold less cholate in buffer E reduced the ability of dissociation of subunits on the column and the total amount (protein concentration) of the subunits when compared to conventional purification methods. In the mixed samples the individual concentrations of the subunits were determined using laser densitometry.

Cholate in buffer E (% (w/v))	G-protein subunit(s)	Concentration (μM)
1	$G\alpha_q$	5.7
0.1	$\beta_1\gamma_{2(6xHIS)}$ (+$G\alpha_q$)	6.0
1	$G\alpha_{i1}$	12.4
0.1	$G\alpha_{i1}$	6.7
0.1	$\beta_1\gamma_{2(6xHIS)}$ (+ $G\alpha_{i1}$)	8.2

Table 3.2: **G-protein purification and concentration determination with 0.1% (w/v) cholate in Buffer E and 1% (w/v) cholate in buffer E.**
Protein concentrations were determined using the Bradford assay.

The functionality of α subunits can be assessed by investigating their ability to bind GTP using [^{35}S]GTPγS (Manning, 2002). The extent of high affinity [^{35}S]GTPγS binding to Gα subunits has been shown to be dependent on the nature of the subunit under investigation (Manning, 2002), and this methodology has been used to directly compare different G-protein subunits. The heterotrimeric units described above were

analysed for [^{35}S]GTPγS binding ability (**Figure 3.19**). The binding of [^{35}S]GTPγS (in fmol) was measured over a concentration range of 0-300 nM of Gα$_{i1\ (6xHIS)}$ (prepared using the conventional method, i.e. buffer E contained 1% (w/v) cholate) and Gα$_{i1}$ purified with 10 fold less cholate in buffer E. At the final concentration of 300 nM there was a significant 46% increase in the amount of [^{35}S]GTPγS bound using the conventionally purified subunit preparation (**Figure 3.19 A**).

The affinity of Gα$_q$ and the reduced cholate prepared β$_1$γ$_{2(6xHIS)}$(Gα$_q$) to [^{35}S]GTPγS was also investigated (**Figure 3.19 B**). The conventionally prepared (1% (w/v) cholate) Gα$_q$ showed a higher affinity for [^{35}S]GTPγS than the Gα$_q$ subunit in the mixed heterotrimer preparation. There was more protein added in the assays containing the mixed sample because of the β$_1$γ$_{2(6xHIS)}$ subunit and the data is represented in fmol bound. However, 700 nM β$_1$γ$_{2(6xHIS)}$ did not bind [^{35}S]GTPγS (data not shown) which suggests that higher protein in the mixed samples would not interfere with [^{35}S]GTPγS binding. The affinities of Gα$_{i1(6xHIS)}$ and Gα$_q$ were compared and the former subunit had a statistically significant 2.4 fold higher affinity (p<0.0001) for the radionucleotide. As indicated in the literature, demonstrating [^{35}S]GTPγS binding to Gα$_q$ is difficult and has been discussed (Chidiac *et al.*, 1999; Hepler *et al.*, 1993). As expected, the addition of 10 μM GDP to Gα$_q$ completely abolished all [^{35}S]GTPγS binding. Others reported a 20 fold acceleration in [^{35}S]GTPγS binding to Gα$_q$ when 100 mM (NH$_4$)$_2$SO$_4$ was incubated with Gα$_q$ (Chidiac *et al.*, 1999). This addition had no statistically significant effect in the present study, however, there was a trend to show that it decreased binding at 150 nM Gα$_q$ (data not shown).
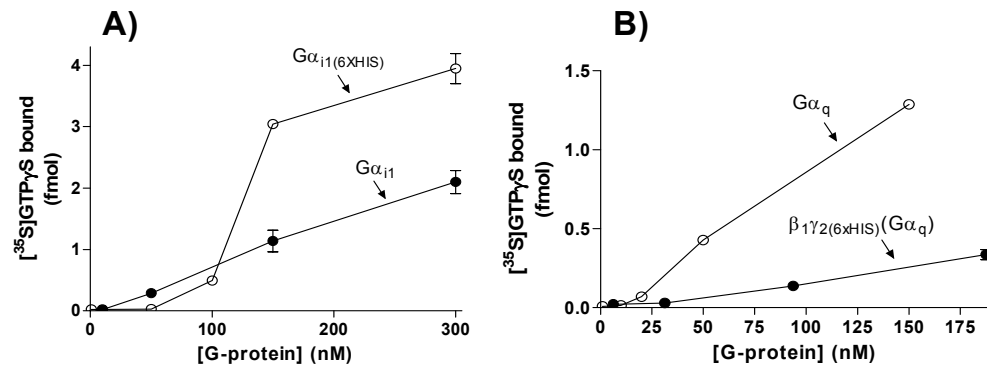
Figure 3.19: Affinity of [^{35}S]GTPγS to Gα$_{i1}$, Gα$_q$ and β$_1$γ$_{2(6xHIS)}$(Gα$_q$). **Effect of reducing cholate concentration in Buffer E.**
Assays were incubated as described in the materials and methods section ensuring the following: All incubations contained 0.5 nM [^{35}S]GTPγS along with increasing concentrations of G-protein diluted in TMND buffer. Incubations (40 μL) were performed in a separate plate and were for 60 min. Then 25 μL was transferred to a Millipore 96-well MultiScreen^{TM} GF/C filter plate. The plate was washed with 4 x 200 μL with ice-cold TMN buffer. **A)** The G-proteins used were Gα$_{i1(6xHIS)}$ (o) or Gα$_{i1}$ (●), this latter subunit was purified using 0.1% (w/v) cholate in buffer E. **B)** The G-proteins used were Gα$_q$ (o) or β$_1$γ$_{2(6xHIS)}$(Gα$_q$) (●), this latter subunit was purified using 0.1% (w/v) cholate in buffer E. Each data point represents n = 3 samples, mean ± SEM. Where error bars are not visible they are contained within the data point.

Next the ability of these unconventionally purified G-proteins to couple to the α$_{2A}$AR was determined. Functional G-protein subunit coupling was determined in the [^{35}S]GTPγS assay (**Figure 3.20**). Agonist induced [^{35}S]GTPγS binding of the system reconstituted with purified β$_1$γ$_{2(6xHIS)}$(Gα$_q$) (**B**), Gα$_{i1}$ and β$_4$γ$_2$ (**C**) and β$_1$γ$_{2(6xHIS)}$(Gα$_{i1}$) (**D**) was compared to binding in the "gold standard" (α$_{2A}$AR + Gα$_{i1(6xHIS)}$ + β$_4$γ$_2$) reconstituted receptor system (**A**). The "gold standard" reconstitution exhibited an 8.8 fold UK-14304 stimulated increase in [^{35}S]GTPγS binding compared to basal, while fold stimulation was 2.1 for (**B**), 5.7 (**C**) and 5.1 (**D**). In all cases the agonist induced [^{35}S]GTPγS bound was reduced compared to the "gold standard". However, this reduction was not significant in incubation **C** suggesting that although this subunit had demonstrated reduced GTP affinity, its ability to functionally couple to the α$_{2A}$AR was not significantly hindered. On the other hand, the mixed heterotrimeric preparations were not as effective.
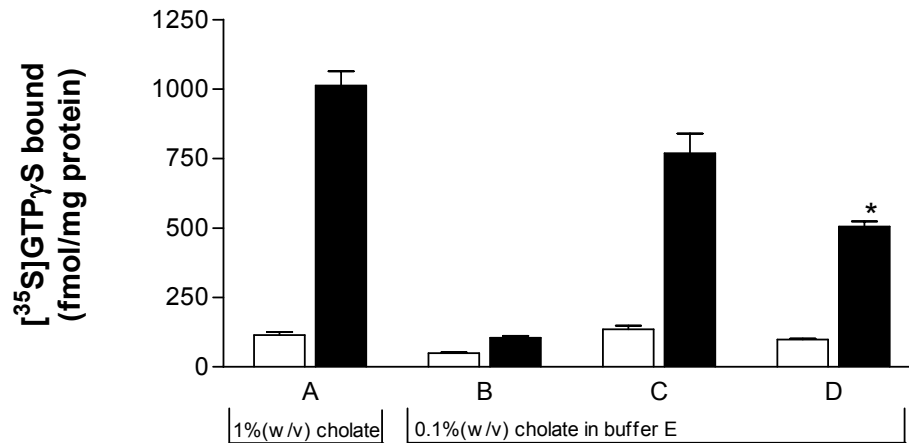
Figure 3.20: **Reconstitution cross over analysis using G-protein subunits that were prepared in buffer E containing 0.1% (w/v) cholate.**
Assays were incubated as described in the materials and methods section ensuring the following. Urea treated *Sf*9 membranes over expressing $\alpha_{2A}AR$ receptor (0.1 mg/mL) along with the following: **A)** 50 nM $G\alpha_{i1(6xHIS)}$ and 20 nM $\beta_4\gamma_2$ (gold standard prepared by conventional method using 1% (w/v) cholate in buffer E **B)** 50 nM $\beta_1\gamma_{2(6xHIS)}(G\alpha_q)$ **C)** 50 nM $G\alpha_{i1}$ and 20 nM $\beta_4\gamma_2$ **D)** 50 nM $\beta_1\gamma_{2(6xHIS)}(G\alpha_{i1})$. Basal [^{35}S]GTPγS binding is represented by the white bars and 10 μM UK-14304 induced [^{35}S]GTPγS binding is represented by black bars (n =2; error bars represent the range of duplicates). *p<0.05.

Subsequently, the unconventionally (0.1% (w/v) cholate in buffer E) purified G-protein subunits were used in the reconstitution cross over study with the AT_{1A} receptor (**Figure 3.21**). Six different incubations were conducted in this study, whereby 3 combinations of subunits were added; $\beta_1\gamma_{2(6xHIS)}(G\alpha_q)$ (**A** and **B**), $\beta_1\gamma_{2(6xHIS)}(G\alpha_{i1})$ (**C** and **D**) and $G\alpha_{i1}$ (**E**). In incubations **B**, **D** and **E**, 20 nM $\beta_4\gamma_2$ was also included. UK-14304 stimulated [^{35}S]GTPγS binding was only significant in incubations **B**, **C** and **D** indicating that functional reconstitution of $\alpha_{2A}AR$ was achieved in those incubations. Likewise, Ang II induced a significant increase in the amount of [^{35}S]GTPγS bound above basal in incubations **A** and **B** indicating that this receptor couples the $G\alpha_q$ more effectively than $G\alpha_{i1}$ even though the amount of the subunit is diluted (as it is in a mixed subunit preparation). Interestingly the inclusion of $\beta_4\gamma_2$ in assays seemed to increase the amount of agonist induced [^{35}S]GTPγS binding which is likely due to the dimers ability to stabilise the conformation (Fletcher *et al.*, 1998).
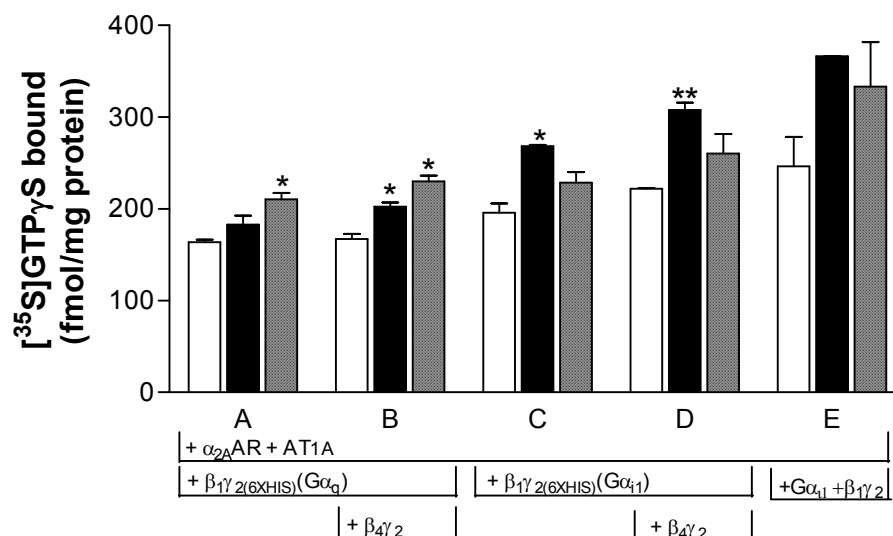
Figure 3.21: **Reconstitution cross over analysis with G-protein subunits that were prepared in buffer E containing 0.1% (w/v) cholate.**
Assays were incubated as described in the materials and methods section ensuring the following: Urea treated $Sf9$ membranes over expressing $\alpha_{2A}AR$ receptor (0.1 mg/mL) and CHO cell membranes (0.2 mg/ml) expressing AT_{1A} along with the following: **A)** 50 nM $\beta_1\gamma_{2(6XHIS)}(G\alpha_q)$ **B)** 50 nM $\beta_1\gamma_{2(6XHIS)}(G\alpha_q)$ and 20 nM $\beta_4\gamma_2$ **C)** 50 nM $\beta_1\gamma_{2(6XHIS)}(G\alpha_{i1})$ **D)** 50 nM $\beta_1\gamma_{2(6XHIS)}(G\alpha_{i1})$ and 20 nM $\beta_4\gamma_2$ **E)** 50 nM $G\alpha_{i1}$ **F)** 50 nM $G\alpha_{i1}$ and 20 nM $\beta_4\gamma_2$. Basal [^{35}S]GTPγS binding is represented by the white bars or 10 μM UK-14304 induced [^{35}S]GTPγS binding is represented by black bars or 10 μM Ang II induced [^{35}S]GTPγS is represented by checked bars (n =2; error bars represent the range of duplicates) and* $p<0.05$ ** $p<0.01$ (basal binding to agonist binding).

3.3.4.3. Increasing the Ang II induced [^{35}S]GTPγS signal

To improve the chances of signal detection, a reconstitution assay without the addition of membranes containing the $\alpha_{2A}AR$ was constructed, with the aim of reducing non-specific binding (**Figure 3.22**). The amount of CHO cell membranes expressing the AT_{1A} was increased from 0.1 mg/mL – 0.5 mg/mL which equates to 2.5 μg to 12.5 μg protein per assay. To rule out the possibility that the signal propagated by Ang II was not an artefact, the antagonist Losartan was also added to the assay. Receptor catalysed [^{35}S]GTPγS binding to G-protein subunits was significantly increased above basal binding in incubations **C** and **D** and this reflected the largest amount of protein added to the assays. The addition of the antagonist blocked the signal to a level comparable to basal levels suggesting that the AT_{1A} receptor signalling was functional. A membrane only control (**E**) was included to show the high levels of basal binding that were present.
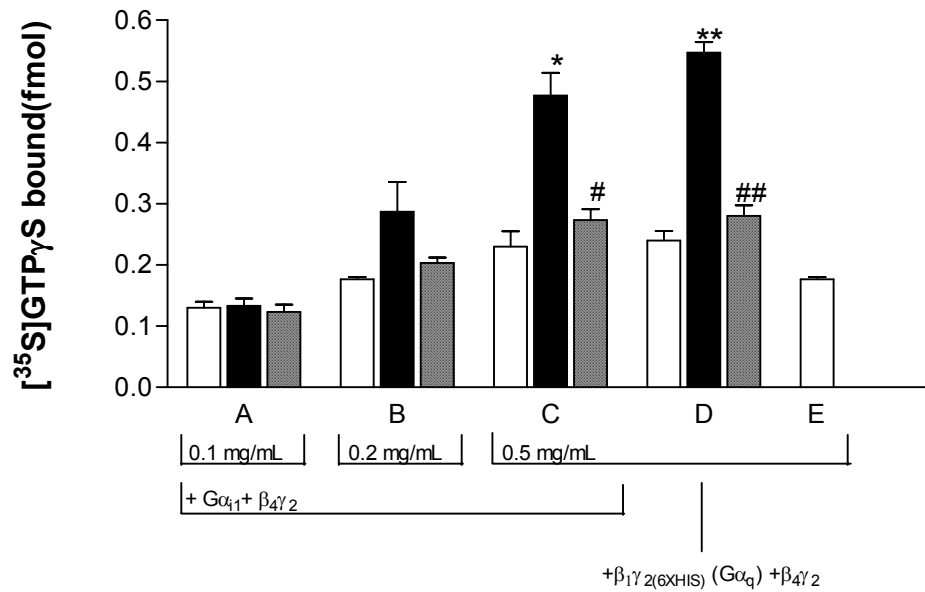
Figure 3.22: **Effect of protein concentration dependence of membranes expressing AT$_{1A}$ on Ang II stimulated [^{35}S]GTPγS binding.**
Assays were incubated as described in the materials and methods section ensuring the following. Urea treated CHO membranes over expressing AT$_{1A}$ receptor were added to the incubations in the following concentrations **A)** 0.1 mg/mL **B)** 0.2 mg/mL and **C-E** 0.5 mg/mL. The incubations also included 20 nM β$_4$γ$_2$ and the following (purified using 0.1% (w/v) cholate in buffer E): **A-C** 50 nM Gα$_{i1}$; **D)** 50 nM β$_1$γ$_{2(6XHIS)}$(Gα$_q$)and **E)** No G-proteins. Basal [^{35}S]GTPγS binding is represented by the white bars or 1 μM Ang II induced [^{35}S]GTPγS is represented by black bars or 1 μM Ang II/100 μM Losartan represented by checked bars (n = 3, mean ± SEM). *p<0.01, **p<0.001 (for white bars compared to black bars) and #p<0.01, ##p<0.001 (for black bars compared to grey bars).

Further investigation of this system would be required as the agonist induced [^{35}S]GTPγS binding was much lower than levels seen in other receptor systems, despite a greater amount of membrane protein being used. This is clearly reflected in **Figure 3.23**.
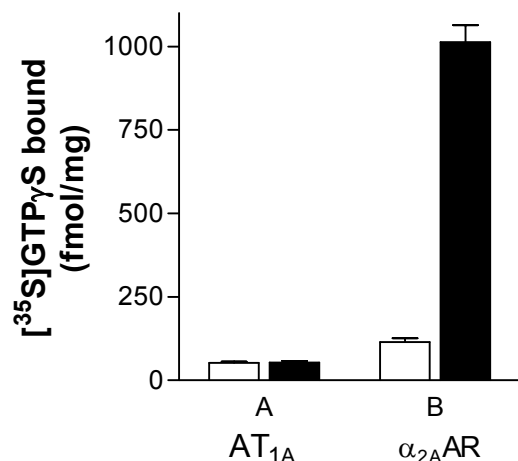
Figure 3.23: **Comparison of AT$_{1A}$–activated and α_{2A}AR-activated [^{35}S]GTPγS binding.**
Assays were incubated as described in the materials and methods section ensuring the following: **A**). Urea treated CHO membranes over expressing AT$_{1A}$ receptor (0.1 mg/mL) along with included 20 nM $\beta_4\gamma_2$ and 50 nM Gα_{i1} (purified using 0.1% (w/v) cholate in buffer E). Data represents n = 3, mean ± SEM. **B**) Urea treated *Sf*9 membranes over expressing α_{2A}AR receptor (0.1 mg/mL) along with the 50 nM G$\alpha_{i1(6xHIS)}$ and 20 nM $\beta_4\gamma_2$ (gold standard prepared by conventional method using 1% (w/v) cholate in buffer E. Data represents n =2; error bars represent the range of duplicates. Basal [^{35}S]GTPγS binding is represented by the white bars and agonist (1 μM Ang II or 10 μM UK-14304) induced [^{35}S]GTPγS binding is represented by black bars.

Summary of results

In conclusion, the functionality of the reconstituted signalling complex was established using the [^{35}S]GTPγS binding assay. In the present study, functionality refers to the activation of the G-protein signalling complex (as shown by the non-hydrolysable binding of [^{35}S]GTPγS to Gα subunit) upon induction by the incubation with the specific agonist. The receptor activated [^{35}S]GTPγS binding was optimised and demonstrated in a single test tube format for urea treated *Sf*9 cell membrane homogenates expressing the α_{2A}AR reconstituted with Gα_{i1} and $\beta_1\gamma_2$. Basal binding (that is [^{35}S]GTPγS binding in the absence of the agonist) was reduced by the addition of a combination of nucleotides; GDP and AMP-PNP. The full α_{2A}AR agonist, UK-14304, was shown to be effective in stimulating the [^{35}S]GTPγS binding. G-protein concentration dependence, agonist sensitivity and receptor concentration dependence were optimised for this system. Moreover, the assay was

modified to incorporate a 96-well format which enabled further investigations into $\alpha_{2A}AR$ activated binding of [^{35}S]GTPγS. This receptor was shown to function upon reconstitution with other G-protein combinations such as $G\alpha_q$ and $\beta_4\gamma_2$. Furthermore, reconstitution of the recombinant AT_{1A} with either $G\alpha_{i1}$ or $G\alpha_q$ was investigated. After several attempts to establish AT_{1A} activated [^{35}S]GTPγS binding, a degree of stimulation was shown. This stimulation could be inhibited upon incubation with the AT_{1A} antagonist Losartan. However the level of the AT_{1A} activated [^{35}S]GTPγS was significantly less than that achieved with the $\alpha_{2A}AR$.

3.4. DISCUSSION

The [^{35}S]GTPγS binding assay was used to measure the functional reconstitution of the recombinantly produced α_{2A}AR and G-protein subunits in a cell-free environment. Initially this assay was optimised using a low throughput manifold assay commonly utilised for such a purpose (Lim and Neubig, 2001). A higher throughput technique was employed and optimised using 96-well MultiScreen^{TM} plates. The routine use of these plates has not been previously reported for this specific assay using the separate reconstituted receptor and G-protein preparations used here. After this assay was established, various other combinations of receptors and G-proteins were investigated with some interesting observations revealed. The biological variability of reconstituted signalling complex was also noted and will be discussed.

3.4.1. Optimisation of [^{35}S]GTPγS assay

Employing a characterised assay using agonist stimulated [^{35}S]GTPγS binding to membranes expressing α_{2A}AR(Lim and Neubig, 2001), various assay parameters, conditions and constituents were optimised. As discussed throughout the literature the amount of protein determines the maximal or saturable [^{35}S]GTPγS binding while the ratio of GDP to GTP determines agonist sensitivity in a manner similar to modulation of B_{max} and K_d parameters in receptor binding assays (Fletcher *et al.*, 1998). The optimal GDP concentration in the assay is a balance between the concentration required to suppress basal activity and that permitting the detection of the exchange of GDP by [^{35}S]GTPγS upon receptor stimulation (Windh and Manning, 2002). The combination of 5μM GDP and 10μM AMP-PNP was optimal for lowering non-specific binding for this particular transductosome or reconstituted signalling system. Different receptor/G-protein transductosome assays benefit from

different combinations of nucleotides to minimise non-specific binding; M_2 receptor reconstitution (also through the $G\alpha_{i1}$ pathway, concurrently being studied in the laboratory) exhibited the best fold stimulation when 1 µM GDP was present, while for mammalian systems expressing $\alpha_{2A}AR$, 30 µM GDP was reported to be optimal (Pauwels and Tardif, 2002). This non-uniformity may be due to both quantity and quality of non-specific binding sites. In terms of the $\alpha_{2A}AR$ system discussed here, the fact that the best combination for lowering such non-specific binding involved using a specific combination of nucleotides may indicate that there are 2 structurally different non-specific binding sites.

3.4.2.   Choice of agonist

Agonist and partial agonists are characterised by their ability to activate a receptor and induce a response (Jasper *et al.*, 1998) and the rate of GDP/GTP exchange has been shown to be slower upon partial agonist induction (Lorenzen *et al.*, 1993). The agonist potency of UK-14304 (full agonist) and clonidine (partial agonist) to stimulate [^{35}S]GTPγS binding was directly related to their ability to compete with [^{3}H]MK-912 binding as shown by (Jasper *et al.*, 1998). Transductosome composition (i.e. which G-proteins are coupled to the $\alpha_{2A}AR$) did not change the affinity of UK-14304 to the receptor, while certain combinations have been shown to elicit better second messenger effects (Chabre *et al.*, 1994). In addition to transductosome composition induced agonist selectivity, a degree of species selectivity specifically between human and rodent $\alpha_{2A}AR$ (Cockcroft *et al.*, 2000) and host cell selectivity between various host cell lines using the human calcitonin receptor Type II (Watson *et al.*, 2000) has been shown. These factors will need to be considered when attempting to adapt this *in vitro*, reconstituted signalling assay to one which may be suitable for biosensing applications.

CHO cells expressing human α_{2A}AR demonstrated an EC_{50} of 52 nM for UK-14304 (Peltonen *et al.*, 1998) which compares well to the EC_{50} obtained in the present study using a reconstituted system.

3.4.3. G-protein subunits and Histidine tagging

G-protein concentration dependence and in particular, the activation and subsequent GTP binding to the $G\alpha$ subunit provided the tangible parameter by which functional reconstitution was measured (Harrison and Traynor, 2003). Certainly, $\beta_1\gamma_2$ dimer is required for heterotrimeric stability and for significantly improving the sensitivity of the system, in some cases without this dimer agonist induced activity is not seen (Gales *et al.*, 2005). As Gales and colleagues (2005) have shown, there were indeed differences in the sensitivity of the BRET-based signal observed from one subunit to another. In addition the phosphorylation state of the receptor has been shown to preferentially regulate G-protein engagement (Lefkowitz *et al.*, 2002).

The purification of G-proteins using affinity capture techniques resulted in the preparation of non –histidine tagged subunits as well as histidine tagged subunits. The importance of this initial characterisation of histidine tagged G-protein subunits is discussed in the next chapter. In the present study, it was established that the inclusion of the histidine tag did not hinder [^{35}S]GTPγS binding or the heterotrimer association or stability. The histidine tag is usually constructed on the amino terminus of G-proteins[27] (Kozasa and Gilman, 1995; Simonovic *et al.*, 1998). However, it has been reported that this terminus plays complex roles in the signal transduction (Preininger *et al.*, 2003) and subunit dissociation (Medkova *et al.*, 2002)

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[27] Bioclone markets purified G-proteins which are N-amino terminal histidine tagged (see the website http://www.bioclon.com/recombinant-protein-G-Protein-Glycobiology.html)

of $G\alpha_i$ which likely involve conformational changes in the amino terminus of $G\alpha_i$. Nevertheless, structural and functional studies suggested that the amino terminus of the γ subunit could be modified without interfering with ternary complex assembly (McIntire *et al.*, 2001).

3.4.4.   Moving towards high throughput

One of the aims of this study was to reconstitute a functional, cell-free signalling assay comprising GPCR and appropriate G-proteins and to consider the challenges in adapting such as assay as a prototype for biosensing. As such these considerations would include as a minimum, the surface display of such an assay system and the increase in the throughput rate for assay completion. A first step was to modify this assay from single tube to 96-well format. Work described in this chapter shows that adaptations can be made to the radioligand $[^{35}S]GTP\gamma S$ binding assay so that it can be carried out in 96-well plates. Such an assay was not described in the literature for this particular transductosome system although (Frang *et al.*, 2003) used very similar technology with $\alpha_{2A}AR$ expressed in CHO cells using a cell harvester. There are commercially available technologies that utilise a 96-well format for the $[^{35}S]GTP\gamma S$ assay (Harrison and Traynor, 2003), however, these technologies were not considered because of expense, the additional complexity of the assay and the observed unfavourable results from initial pilot experiments with SPA beads. This latter research approach using SPA beads was subsequently terminated. The major problem encountered using the described higher throughput assay was with regard to the use of a separate incubation plate and the transferring of incubations to the filtration plate. Pre-coating filtration plates with PEI and BSA was not effective in this study in allowing only a single (filter containing) 96-well plate to be used. Other researchers have used Tween as a coating to prevent solution binding and they

indicated this coating solution was non-ionic and yielded lower backgrounds when compared with the more common coating reagents (PEI and BSA) (Scott *et al.*, 1995).

As indicated in the introduction, considerably more expensive fluorescent based techniques were more amendable to higher throughput (Sarvazyan *et al.*, 2002) than techniques which relied on radioactive procedures. Although preliminary experiments with BODIPY-GTP showed good sensitivity (2-3 fold increase in fluorescence polarisation) upon binding to semi-purified $G\alpha_{i1}$ and the time course that compared well to others (data not shown), there were intrinsic problems associated with high background fluorescence when using membrane preparations in this study, as was also encountered by (McEwen *et al.*, 2001). Nonetheless, radioactivity based detection in higher throughput formats should be feasible with the advent of newer high resolution instrumentation (Fang *et al.*, 2002).

3.4.5. Biological variability and use of a "gold standard" assay

Biological variation and $[^{35}S]GTP\gamma S$ stability[28] were critical factors that impacted significantly on the reproducibility and reliability of the experimental data. Specifically, it was observed that variability between experiments resulted from the use of different receptor preparations and G-protein preparations. This variability can be most simply explained by variations in the expression levels of the GPCR and/or variations in the levels of the functional proteins under study. The production of the proteins in the insect expression system introduces a degree of biological variation which can only be controlled to a limited extent. A "gold standard" also allows for

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[28] $[^{35}S]GTP\gamma S$ stability aspects are discussed in the Appendix.

monitoring of the assay conditions to ensure that any variability in the data is caused by the experimental parameters. A key factor in the development of higher throughput screening formats is the capability of multiplexing the assay i.e. allowing the running of many different reactions in parallel and in the same environment (Yingyongnarongkul *et al.*, 2003)

3.4.6.   Extension of the [^{35}S]GTPγS assay

Many GPCRs have been shown to exhibit a broad specificity with regard to their interactions and coupling with G-α subunit proteins and this is definitely the situation with the α_{2A}AR which has been shown to couple to Gα_i , Gα_o, Gα_s, Gα_q/Gα_{11} and Gα_z in a transient expression system measuring receptor activated second messenger effects (Chabre *et al.*, 1994). In addition, agonist promoted [^{35}S]GTPγS has been used to demonstrate chimeric Gα_q coupling to α_{2A}AR (Pauwels *et al.*, 2001), although a reconstituted transductosome assay was not undertaken by these workers. In this study, the higher throughput [^{35}S]GTPγS assay format using 96-well plates was used to demonstrate diversity of the GPCR reconstitution, and specifically, to demonstrate the functional coupling of α_{2A}AR and Gα_q. As well as the qualitative assessment of reconstitution, it is also important to determine the Gα binding to $\beta(\gamma)$ subunits. Investigating this interaction, could lead to unique functional heterotrimer combinations for example Gα_q was the only Gα subtype shown to interact with the structurally divergent β_5 subunit (Fletcher *et al.*, 1998). Although, it is not possible to use the relative increase in [^{35}S]GTPγS binding in response to a particular agonist as a measure of the preference of a receptor for different G-proteins (Windh and Manning, 2002), this assay may be extended to assess G-protein coupling qualitatively which may have exploitable outcomes.

3.4.7.   GTP binding of subunits: cholate

A modification to the standard method used to purify G-protein subunits from insect cell membranes (using buffer E with less cholate) i.e. the unconventional purification method showed $G\alpha$ subunits had a reduced ability to dissociate from the $\beta_1\gamma_{2(6xHIS)}$ dimer, as well as a reduced total protein content and reduced affinity for [^{35}S]GTPγS binding regardless of the fact that different $G\alpha$ subtypes have different affinities for GTP (Manning, 2002). One reason for this effect may be that reducing the solubility of the proteins may reduce the formation of the heterotrimer as subunits are embedded deeper into membranes (Fletcher *et al.*, 1998). Thus, non solubilised protein is not caught on the affinity column and subsequently is lost in the washing step. It is possible that non-functional subunits may be present in the G-protein preparations eluted and contributed to the protein concentration. In lower cholate it is likely that these non-functional proteins are not denatured and still bind in the trimer and are eluted off the column. The heterotrimer is disbanded by activation with negatively charged AlF_4^- (Kozasa and Gilman, 1995) and, on its own, this compound may not be able to access subunits that are relatively deeply embedded in the membrane to allow subsequent dissociation of the heterotrimer. It is likely that $G\alpha_q$ is embedded more deeply in the membrane than $G\alpha_{i1}$ (Fletcher *et al.*, 1998), and this is reflected by the limited dissociation in the former subunit. The stability of $G\alpha_q$ was reported not to be improved in the presence of cholate or other detergents (Chidiac *et al.*, 1999).

Finally, there was reduced [^{35}S]GTPγS binding when the $G\alpha_q$ subunit was used compared with $G\alpha_{i1}$ possibly due to the fact that the former subunit is reported to exchange nucleotides very slowly (Chidiac *et al.*, 1999). In contrast to other G-proteins, the ability of $G\alpha_q$ to exchange guanine nucleotides is diminished by its

solubilisation from membranes (Chidiac *et al.*, 1999) and un-liganded $G\alpha_q$ is rapidly

denatured (Chidiac *et al.*, 1999). However, this behaviour is returned to normal after

co-reconstitution with receptor and $\beta\gamma$ (Berstein *et al.*, 1992).

3.4.8. AT_{1A} reconstitution

Reconstitution, using the AT_{1A} receptor expressed in CHO cells was difficult to

assess. Initially the new receptor was introduced in a cross over study whereby the

α_{2A}-AR was present and then agonists UK-14304 (α_{2A}-AR) or Ang II(AT_{1A}) were

used for stimulation. There were a number of reasons for the inability to stimulate

Ang II induced $[^{35}S]GTP\gamma S$ binding using the AT_{1A} receptor. In this regard the

literature relating to the cell-free reconstitution of the functional AT_{1A} receptor and

restoration of signalling activity does not clearly indicate that such activity has been

demonstrated in the past. In a review (Sayeski *et al.*, 1998) the author writes "Early

studies indicated that Ang II-stimulated membranes were in fact capable of activating

heterotrimeric G-proteins as measured by the amount of $[^{35}S]GTP\gamma S$ bound to filters

when compared with unstimulated membranes". This author cited two references for

this statement (Crawford *et al.*, 1992; Inagami *et al.*, 1992) however when these

papers are examined they contain experiments that showed that agonist binding is

suppressed by GTP analogs such as $[^{35}S]GTP\gamma S$ only and thus do not contain

unequivocal data to support AT signalling. Also functional reconstitution with AT_{1A}

and $G\alpha_q$ was shown by (Hansen *et al.*, 2000) (one bar graph) with limited

experimental documentation. In an effort to gather more information on published

attempts of AT_{1A} reconstitution, email contact was made with one of the

corresponding authors of the published study (Hansen *et al.*, 2000). The information

gained related mainly to intrinsic difficulty encountered by the authors in their

attempts to reconstitute functional activity. Despite the lack of success relayed by

other researchers in the field, a considerable commitment to this goal was made and it was decided to further pursue it.

Four main points were considered germane to the understanding of the reconstitution of AT_{1A}. Firstly, was the agonist Ang II binding to the receptor preparation? In Chapter 2 it was established that Ang II competed with $[^{125}I]$Ang II suggesting that specific ligand binding at the receptor surface was occurring. This however, was not evidence that functional signalling was occurring as a consequence. The next point to consider was the origin and purity of the various G-protein subunit preparations. Receptor and G-protein origin is unlikely to be a problem as Lim and Neubig (2001) reconstituted the $\alpha_{2A}AR$ using myristoylated bacterial $G\alpha_{i1}$ and bovine brain isolated $\beta_1\gamma_2$. The quality of purified subunits is often uncertain (Francken *et al.*, 2001) and in communications with Sheikh (see above (Hansen *et al.*, 2000)), it was indicated that $G\alpha_q$ purity was paramount for AT_{1A} reconstitution (personal communication). Others have claimed that in mammalian cells, the detection of a signal upon ligand induction was limited by the receptor rather than the G-protein subunit used for reconstitution (Windh and Manning, 2002). Nonetheless, a technique that circumvents the necessity to purify G-protein subunits by fusing membranes containing the GPCR with membranes containing G-proteins has been shown to allow receptors (be they mammalian, bacterial or insect) to be reconstituted with G-proteins expressed in insect cells (Francken *et al.*, 2001).

The attempts to employ the standard $[^{35}S]$GTPγS binding assay to measure enhanced activation of the AT_{1A} receptor was frustrated by high basal binding. In mammalian cells the higher basal activity shown (compared to insect cells) is likely to reflect a combination of constitutive G-protein activity and activation by a variety of receptors

that may be present in those cells (Windh and Manning, 2002). Furthermore, significant stimulation of subunits coupled to the over-expressed receptor (AT_{1A}) may be masked by other subunits that have a higher affinity for GTP ($G\alpha_{i/o}$ subunits) (Milligan, 2003). In a recent review Milligan (2003) stated that the high basal binding in the mammalian system may be attributed to increased binding efficacy of $G\alpha_{i/o}$ subunits to GTP, which he suggested limits the conventional [^{35}S]GTPγS binding assay. However, treatment of the membranes with the chaotropic agent urea, should remove/denature endogenous G- proteins from the preparation (Lim and Neubig, 2001). Furthermore, a 25,000:1 ratio of GDP/[^{35}S]GTPγS was shown to optimise non-specific binding which was 4 fold less than the ratio used to functionally reconstitute dopamine receptors with $G\alpha_q$ in a mammalian system (Panchalingam and Undie, 2000). Nonetheless, although high levels of uncoupled AT_2 GTP binding hampered the S/B ratio, trends were observed that demonstrated AT_2 activation-enhanced GTP binding with the endogenous complement of G-proteins (Hansen *et al.*, 2000).

Finally, the intrinsic activity of an agonist can be influenced by receptor expression levels (Jasper *et al.*, 1998) and thus were considered. Higher protein concentrations (2-3 fold) are used with membrane preparations from CHO cell cultures (specifically expressing the $\alpha_{2A}AR$) compared with receptor expressed in insect cell membranes (Frang *et al.*, 2003). Low expression of serotonin receptors was attributed to the failure in demonstrating receptor mediated [^{35}S]GTPγS binding when examining signalling activity in that system (Francken *et al.*, 2001). Expression levels of the CHO expressed AT_{1A} in this work were considerably lower than those sold by

Euroscreen (Belgium)[29]. This company produces the human recombinant AT_1 receptors expressed in CHO-K1 cells and they routinely obtain a B_{max} of 18.8 pmol/mg protein and a K_d of 0.32 nM compared to B_{max} of 0.63 pmol/mg protein (or 3.4 pmol/mg protein urea treated) and a K_d of 0.85 nM (or 0.68 nM urea treated) for these membranes (see chapter 2). Their membranes are not urea treated and they recommend 0.025 mg/mL in their binding assays (personal communication, Dr Karlien Maes, Euroscreen Belgium). In any case, it was reported that receptor expression levels greater than 0.1 pmol/mg protein provided an adequate signal (although, AT_{1A} was not specifically mentioned) (Windh and Manning, 2002). Regardless of their higher expression levels, Maes (Euroscreen, Belgium), have not shown or reported that the $[^{35}S]GTP\gamma S$ binding assay may be used to functionally reconstitute the AT_{1A} membrane preparation because of the need for $G\alpha_q$ coupling and the limited effectiveness of this subunit with the assay (personal communication). The opportunity of having this research group examine our particular membrane preparation for signalling activity was discarded on cost grounds.

Robust agonist stimulation of $[^{35}S]GTP\gamma S$ binding to membranes expressing the AT_{1A} receptor would require further optimisation if it was to be included in the development phase of biosensor assay for AT_{1A} ligand concentrations. In order to do this it may be necessary to investigate the addition of other constituents in the buffer such as millimolar additions of KCl which was shown to enhance agonist induced $[^{35}S]GTP\gamma S$ binding (Panchalingam and Undie, 2000). Moreover, the inclusion of deoxycholate (in a dopamine receptor system reconstituted with $G\alpha_q$) resulted in a significant decrease in basal $[^{35}S]GTP\gamma S$ binding and a dramatic increase in agonist

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[29] Euroscreen (Belgium) market and sell membrane receptor preparations from cells over-expressing GPCRs (www.euroscreen.be)

sensitivity (Panchalingam and Undie, 2000). These authors suggest that the addition of detergent may also increase the accessibility of receptor activated G-proteins to GTP by enhancing the fluidity or mobility of the membrane (Panchalingam and Undie, 2000). Alternatively, the detergent induced changes may allow for conformational changes in the receptor necessary for association with G-proteins (Panchalingam and Undie, 2000).

Conclusion

In conclusion, the main advantage of the $[^{35}S]GTP\gamma S$ assay is that it has attributes applicable to the objectives of this project and is a reliable, well documented assay for determining functional signalling activity with certain receptors and G-protein subunits. Although it measures a functional consequence of receptor occupancy at one of the earliest receptor mediated events, it is not generic in its applications. This assay can be used to determine the degree of agonism and the potency of compounds acting at a particular GPCR. In general, the assay is experimentally more feasible and easier with $G\alpha_{i/o}$ coupled receptors since these proteins are more abundant than other families, and have higher rates of nucleotide exchange rate and, so as a consequence, a higher S/B ratio is obtained.

Chapter 4.

Capture and surface attachment of GPCRs and G-proteins

"Technology is the knack of so arranging the world that we do not experience it."

Max Frish

4.1.   INTRODUCTION

Advances in high throughput screening are clearly seen in the range of different assay techniques and platforms that are exploding rapidly into the marketplace. The trend towards miniaturisation is driving research towards thorough investigation of platform and microarray technologies. Microarrays consist of immobilised biomolecules spatially addressed on planar surfaces (microchannels, mircowells or an array of beads) (Venkatasubbarao, 2004). Those arrays that require additional processing steps such as reagent addition are classified as bioassays (Rodriguez-Mozaz *et al.*, 2004).

4.1.1.   Microplate and beaded assays

The development of protein microarrays was inspired by the DNA microarray revolution as an attempt to obtain large amounts of bio-information from small amounts of sample within shorter time frames (Fang *et al.*, 2002b). Solution/suspension based assays and 2D-surface arrays are two commonly utilised microarray formats. As a solution based assay platform, multi-well microplate technologies offer many advantages and these are highlighted in **Table 4.1**. On the other hand, bead-based assays can be used in either format and their use and development has enabled flexible methodologies to be developed to rapidly large sample sets (Yingyongnarongkul *et al.*, 2003). Conveniently customised, beads are used extensively for a broad spectrum of platforms including flow cytometry, allowing for the study of real time interactions (Edwards *et al.*, 2004; Nolan and Sklar, 2002). The most common type of bead used in bead-based assays is the polystyrene microsphere. In addition, a variety of functional microspheres for specific applications including magnetic beads are available commercially. Magnetic bead technology eliminates the need for column chromatography and centrifugation

(Yingyongnarongkul *et al.*, 2003) and magnetic technology is commonly used to separate whole cells (Safarik and Safarikova, 1999).

	Advantages	Challenges
Microplate	• Low cost. • Increased throughput (up to a 20,000 well plate working with 25 nL volume).	• Assay sensitivity. • High surface tension. • Mixing issues related to small volumes. • Physical limitations of liquid handling. • Format incompatibilities in plate reader.
Beads based	• Versatile. • Simple. • Ease of automation. • Reliable. • Uniform surface.	• High cost. • Technical problems. • Sheer force damage from centrifugation (prevented using magnetic beads).

Table 4.1: **Advantages and challenges of using microplate assays versus bead-based assays (adapted from (Yingyongnarongkul *et al.*, 2003).**

4.1.1.1. Detection techniques

A biosensor is a self-contained, small integrated device capable of providing specific quantitative and qualitative information using biological recognition (Rodriguez-Mozaz *et al.*, 2004). Surface plasmon resonance (SPR) biosensors, such as BIAcore, have found considerable commercial application (Rodriguez-Mozaz *et al.*, 2004) and such instrumentation has been used with GPCRs (Bieri *et al.*, 1999). SPR is a surface sensitive technique that involves real time measurement of biomolecules binding to appropriately modified surfaces with the change in absorbed mass being detected at the sensor surface (Mozsolits *et al.*, 2003). Mozsolits and colleagues (2003) reviewed the utility of SPR to study signalling events mediated through specific protein: membrane interactions. There are limitations of this technology in some circumstances regarding the sensitivity, the amount detectable, sample characteristics and resolution (Alves *et al.*, 2005). Flow cytometry, which has recently entered the GPCR research and discovery arena, is a sensitive technique that allows for the discrete measurements of fluorescence and light scatter from single cells, beads or molecules in real time enabling sub-second kinetic resolution (Waller *et al.*, 2004).

The major drawback for using this technique for high throughput screening is sample handling limitations (Waller *et al.*, 2003). Other techniques used to monitor the biomolecular interactions specifically involved in GPCR activation have been recently reviewed (McMurchie and Leifert, 2006).

4.1.2. Surface arrays and high affinity attachment

Surface array techniques require the immobilisation of one of the interacting partners. Biomolecules commonly immobilised on microarrays include oligonucleotides, polymerase chain reaction (PCR) products, proteins, lipids, peptides and carbohydrates (Venkatasubbarao, 2004). Chemical cross linking and high affinity interactions are some of the immobilisation strategies used to impregnate surfaces specifically or non-specifically with proteins (Martinez *et al.*, 2003) as discussed below. Ideally, the attached molecule must retain activity, remain stable and not desorb during the reaction (Venkatasubbarao, 2004). Commonly used affinity immobilisation techniques, such as biotinlyation and histidine tagging, are described below in more detail. Additionally, immobilisation techniques used for attaining "functionally-active" (able to bind to ligands) GPCRs include the FLAG system (Neumann *et al.*, 2002) and antibody capture (Mirzabekov *et al.*, 2000). The FLAG octapeptide fusion tag is used typically to purify proteins, to study protein interactions, protein structure and localisation (Buranda *et al.*, 2001). These techniques and others have been reviewed in more detail in (McMurchie and Leifert, 2006).

4.1.2.1. Biotinylation

A non-covalent biospecific interaction exists between biotin and avidin (or streptavidin) in a similar way to receptor ligand recognition. The extraordinarily high affinity binding of 1.3×10^{-15} M (Wilchek and Bayer, 1990) is aided by each

streptavidin molecule having 4 multivalent biotin binding sites. The biotin binding partner has a valeric side chain which is not involved in this binding. Thus, as the valeric acid side chain of biotin is not involved in this binding, incorporating an acylating active group such as NHS ester on the valeric acid tail can be used to bind to amino containing molecules to create a stable amide bond. The simplest biotinylating agent is NHS-biotin, which is insoluble in water. Various alternatives have been synthesised including longer chain derivatives that are soluble in water as well as derivatives with cleavable long chains (Drotleff *et al.*, 2004). Microarray surfaces can be prepared with a streptavidin surface (Venkatasubbarao, 2004). Bieri *et al.* (1999) described the functional and orientated immobilisation of the rhodopsin receptor using carbohydrate specific chemistry for biotinylation. In another novel approach, Martinez *et al.* (2003) recently immobilised minute amounts of recombinant C-terminal biotinylated GPCRs on streptavidin coated sensor surfaces. The high affinity interaction of biotin to streptavidin enabled high mechanical stability which withstood extensive washing steps (Martinez *et al.*, 2003).

4.1.2.2.　　　Histidine tagging

Nitrilotriacetic acid (NTA) can be used on microarray surfaces to bind histidine tagged biomolecules in an approach based on immobilised metal ion affinity chromatography (IMAC) (Schmid *et al.*, 1997; Venkatasubbarao, 2004). The binding of histidine–tagged proteins to the NTA is highly specific with reasonable affinities (the affinity of Ni(NTA) for the hexahistidine tag has a $K_d = 10^{-13}$ M (Kozasa and Gilman, 1995)). Others have reported that the nickel histidine affinity is buffer dependent and have demonstrated lower affinities such as 7×10^{-7}M (Nieba *et al.*, 1997). In addition, the interaction is fully reversible upon the addition of a competitive ligand such as histidine or imidazole (Schmid *et al.*, 1997). Using this

approach both GPCRs (Sklar *et al.*, 2000) and G-proteins (Simons *et al.*, 2003; Simons *et al.*, 2004) have been immobilised to nickel containing surfaces.

4.1.3.   Membrane protein attachment

It is particularly important to consider that membrane proteins typically need to be embedded in a membrane environment in order to maintain their native conformations (Fang *et al.*, 2002a). Membrane protein microarrays require immobilisation of both the target of interest (GPCR) and the associated lipid molecules (Fang *et al.*, 2002a). Fabricating GPCR microarrays (first reported by (Fang *et al.*, 2002b)) is even more challenging and three significant issues have been highlighted (Fang *et al.*, 2002c);

1. Surface chemistries must enable spatial localisation, i.e. between GPCR and lipid molecules.

2. The preservation of membrane fluidity and lateral mobility of receptors and other proteins within the membrane.

3. Accommodation of extra membrane domains of bound proteins, i.e. the inclusion of tethers or polymer cushions.

Self assembly monolayers (SAMs) in combination with affinity directed chemistries (histidine tagging) have been useful for the immobilisation of specific receptor proteins and may have an added advantage for fabricating monolayers (Sigal *et al.*, 1996). Meanwhile, others showed that the GPCR chemokine receptor (CCR5), could be reconstituted into a native lipid environment formed on the surface of paramagnetic beads by tethering the GPCR via an antibody (Mirzabekov *et al.*, 2000). However, the compromise between high mechanical stability and long-range lateral fluidity is paramount (Fang *et al.*, 2002c) and it may not be desirable to use affinity-directed immobilisation for the fabrication of biomimetic supported membranes (Fang *et al.*, 2002a). Amine containing surfaces, such as

polyethyleneimine (PEI) and γ-aminopropylsilane (GAPS), have been used to attach proteins (specifically membrane proteins) to surfaces such as glass (Fang *et al.*, 2002c). This technique was shown to be useful in the surface attachment of members of the adrenergic receptor family as well as other GPCR receptor families (Fang *et al.*, 2002a) and may provide a powerful platform for the reverse pharmacological approaches used to elucidate orphan GPCRs (Fang *et al.*, 2002a). However, others have indicated that such amine coupling may promote unfavourable receptor orientations (Karlsson and Lofas, 2002).

4.1.3.1.   Tethered lipids

Supported lipid bilayers are widely used to study the structure and function of membrane proteins and receptors with such bilayers being formed around beads to mimic a cellular environment (Wagner and Tamm, 2000). This technology has the ability to provide surface-sensitive detection while maintaining a natural environment for the immobilization of proteins under non-denaturing conditions (Heyse *et al.*, 1998; Sackmann, 1996). Tethered lipid bilayers (TLB) are one type of supported lipid bilayer that have been recently used with some success for GPCRs, namely rhodopsin (Heyse *et al.*, 1998). In a different approach, Karlsson and Löfås (2002) first solubilised the rhodopsin receptor, promoted surface attachment via amine coupling on an amphiphilic surface (hydrogel), then removed the detergent which facilitated the reconstitution of the receptor into a lipid environment. They showed that 'on-surface' reconstitution into a lipid environment facilitated the same handling of membrane protein as for soluble protein and that the ligand binding capacity of the receptor was also preserved (Karlsson and Lofas, 2002).

This final chapter aims to investigate the feasibility of preparation of a cell-free surface based assay format. Specifically the chapter deals with:

1) Deciding what to screen : receptors or ligands

2) For receptor screening: Using an attached Ang II linker to capture the AT_1 receptor.

3) For ligand screening: deciding the appropriate platform and its specificity.

4) Ensuring the functionality (determined using the [^{35}S]GTPγS binding assay) in the presence of the platform is the same.

5) Ensuring that pharmacological profile of the receptor was unhindered on the platform.

4.2. MATERIALS AND METHODS

Additional methods are described below. Refer to previous chapters on methods describing membrane preparation etc.

4.2.1. Synthesis of biotinylated Ang II

The Ang II-biotin linker was prepared using a modification of the method outlined by (Jans *et al.*, 1990). Sulfo-NHS-SS-Biotin purchased from Pierce Chemical Company, (Rockford, IL, USA) contained disulfide bond in the 24.3 Å spacer arm that can be cleaved by reducing agents and was dissolved in dimethylformide (DMF). Sulfo-NHS-SS-Biotin and Ang II (Auspep, Parkville VIC) were mixed in a 1:2 mole ratio. The reaction was incubated overnight at 30°C with constant mixing and maintained at pH 7.5.

4.2.2. Preparation of Ang II- biotin- Beads

SPHERO^{TM} streptavidin magnetic particles (2-2.9 µm) catalogue number SVM-20-10 were purchased from Spherotech Inc. (Libertyville IL, USA) (will be referred to as Spherotech beads throughout this thesis). Biotin binding capacity for 20 µl (100 µg, particle concentration was 0.5% w/v) beads was stated at 60 pmoles. Beads (300 µL) were mixed with 20 µL sample (Ang II- Biotin linker) and 20 µL PBS pH 7.5. This reaction was incubated at 30°C for 1 hour with constant mixing. All contents were transferred to a 1.5 mL eppendorf. Dynal MPC-S tool (this tool can hold 6 x 1.5 mL eppendorfs and contains a removable magnet) was used to separate magnetic beads from the liquid sample and washes. Beads were washed 3 times with 1 mL PBS and made to 90 µL with PBS.

4.2.3. [³H] Biotin assay.

In order to measure the biotin binding capacity of the Spherotech streptavidin magnetic beads a [³H] Biotin binding assay was performed. [³H]Biotin was donated from Professor John Wallace (University of Adelaide) and the specific activity was 32 Ci/mmol. D-Biotin was purchased from Pierce Chemical Company (Rockford, IL, USA). Spherotech beads (20 μl) were combined with various amounts of D-Biotin and the volume was made to 40 μl with PBS. These samples were incubated for 1 hour at 24°C with shaking and then 50 μM Biotin (which contained 300 dpm/pmol) was added. The samples were then incubated for a further 2 hours at 24°C with shaking and then beads were washed 3 times using 250 μl of PBS. Beads were removed after washes with 50 μL PBS and combined with 3 mL Ultima Gold™ scintillation cocktail (Perkin Elmer Life Sciences, Boston, MA, USA). The radioactivity was determined on a Wallac 1410 Liquid Scintillation counter (Pharmacia, Turku, Finland) with 57% counting efficiency.

4.2.4. Ni(NTA) agarose bead assay

The [³⁵S]GTPγS binding assay was modified to determine functional GPCR and G-protein activation in the presence of nickel containing agarose beads. A "reconstitution mix" was prepared on ice consisting of 0.05–0.4 mg/mL of *Sf*9 cell membranes expressing α_{2A}AR as indicated, 5 μM GDP, 10 μM AMP-PNP, and appropriate Gα and G$\beta\gamma$ proteins (± histidine tagged) at 20–100 nM, 0.2 nM [³⁵S]GTPγS in TMND buffer (50 mM Tris pH 8.0, 100 mM NaCl, 1 mM MgCl$_2$ and 1 mM DTT). Where indicated, 5-10 μL of suspended agarose beads in TMND (50% suspension, 45–165 μm diameter beads) that were pre-charged with Ni^{2+}-nitrilotriacetic acid (Ni(NTA)) (Qiagen Pty. Ltd., Clifton Hill, Vic, Australia) were included in the assay to allow for G-protein capture via the hexahistidine-tags on the

Gα subunits. The reactions (100 μl final volume) were initiated by addition of either buffer (basal condition) or 10 μM UK-14304 (unless otherwise indicated) (agonist-stimulated condition). The α_{2A}AR antagonist Rauwolscine was added (where indicated) to determine receptor-induced signalling specificity. Where shown, compounds such as 150 mM imidazole and 100 mM NiCl were used to show the hexahistidine-tagged G-protein specificity to Ni(NTA) agarose beads by competition binding experiments. In addition, Ni^{2+}-depleted beads (pre-treated with EDTA) were used where indicated to demonstrate specificity of binding. Reactions were incubated at 27°C, with orbital mixing at 300 rpm for 90 minutes (unless otherwise indicated) and were filtered over a Whatman #1 paper filter to capture Ni(NTA)-bound hexahistidine-tagged G-proteins associated with the Ni(NTA) beads. Reactions without beads were filtered over GF/C. Filters were washed with 3 $\times$ 4 ml washes of ice cold TMN buffer. Filters were dried (separately) and subjected to liquid scintillation counting to determine [^{35}S]GTPγS bound.

4.2.4.1. Assays to test GF/C filter protein binding properties.

These assays were performed to test the intrinsic protein binding properties of GF/C filters. The [^{35}S]GTPγS binding assay was performed as a filter blank (that is the incubation contained 20nM G$\alpha_{i1(6xHIS)}$, TMN buffer and 0.2 nM [^{35}S]GTPγS) in the presence of 0.2% (w/v) BSA in the wash buffer with no added receptor containing membranes. The GF/C (or pre-treated GF/C) filters were treated as above.

4.2.4.2. Centrifugation separation assay

These assays were performed to separate radioactivity (bound to Gα_{i1}) from unbound radioactivity using centrifugation as opposed to filtration. This involved using 1.5 mL test tubes to incubate the Ni(NTA)-agarose beads plus 20 nM G$\alpha_{i1(6xHIS)}$, TMN buffer and 0.2 nM [^{35}S]GTPγS for 90 min. After the reaction, the beads were

centrifuged (5 min on microcentrifuge setting high) and the beads were transferred to a picopro vial to measure radioactivity as described above.

4.2.5. Ni(NTA) agarose bead assay in 96-well format

Modifications were undertaken to adapt the single tube [^{35}S]GTPγS binding assay in the presence of Ni(NTA) agarose beads for a 96-well assay format. A 'reconstitution mix' was prepared as above and assays were performed in 96-well Millipore MultiScreen^{TM} plates containing a 1.2 μm Durapore® membrane. This mix along with Ni(NTA) beads (as indicated), 0.2 nM [^{35}S]GTPγS and UK-14304 (± antagonist) was made up to a total assay volume of 100 μL (with TMND buffer) and was incubated in the plate. Antagonists used were Yohimbine (5 mM prepared in 50% (v/v) ethanol), Rauwolscine (10 mM in 50% (v/v) ethanol), Prazosin (2 mM in 100% DMSO (v/v) and Propranolol (10 mM in TMND) and were purchased from Sigma-Aldrich (St. Louis, MO, USA). The 96-well plates were incubated with mixing (500 rpm) using a plate shaker at 28°C for 90 min. Plates were washed 4 times with 200 μl TMN to remove unbound [^{35}S]GTPγS while capturing [^{35}S]GTPγS bound to the activated Gα. The MultiScreen^{TM} filter plates were air dried and the bottom was removed and a solid base fixed under the filters, 40 μl Microscint^{TM}20 (Perkin Elmer Life Sciences, Boston, MA, USA) was added to each well. The amount of [^{35}S]GTPγS bound (cpm) was determined by reading with a Packard Top Count Microplate Scintillation counter B99041V1 (formerly Packard Biosciences, now Perkin Elmer Life Sciences, Boston, MA, USA) using a count time of 1 minute.

4.2.6. Data analysis

Data was analysed using Prism^{TM} (GraphPad Software Inc., San Diego CA, USA). Data are presented as mean and +/- SEM where (n) is greater than or equal to three and n is equal to the number of samples (separate incubations). Where error bars are

not visible they are hidden within the data point symbol. When n = 2, error bars, where visible, represent the range of duplicates. The half saturation point, $t_{1/2}$ and B_{max} were calculated in Prism^{TM} using non-linear regression analysis for one site binding. The effective concentration at 50% (EC_{50}) or the inhibitory concentration at 50% (IC_{50}) were calculated in Prism using sigmoidal dose response. Statistical analysis (Student's unpaired t-test) was performed using Prism^{TM}.

4.3. RESULTS

GPCR display could be achieved by the direct association of GPCRs with an appropriate platform through an epitope tagging scheme or indirectly, through the display of ligands, G-proteins or other binding partners (Waller *et al.*, 2004). Two opportunities were considered here for the design of an attached transductosome complex; ligand-beads or G-protein-beads (see **Figure 4.1**). Ligand beads could have applications as a diagnostic tool (receptor screen) and the G-protein bead approach could be used as a drug screening tool (ligand screen) and the applications will be discussed later.
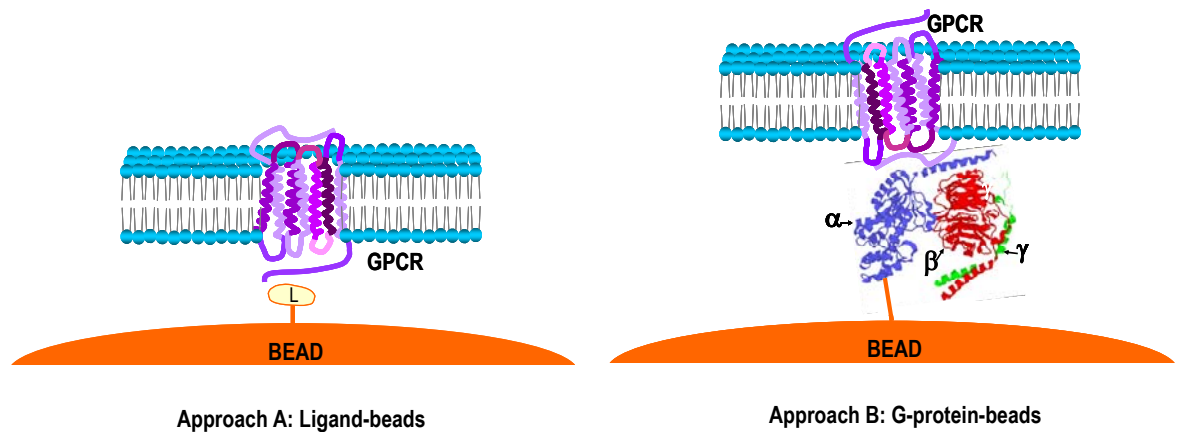

Figure 4.1: **Schematic representation of the two bead based approaches.**
Approach **A** involves attaching a ligand to the bead whereas in Approach **B** the $G\alpha$- subunit is attached to the bead.

Ligand-receptor capture for receptor screening involved the design of the appropriately attached ligand (Ang II) to 'capture' the AT_1 receptor. Ligand screening using G-protein beads was pursued further after considering both opportunities. In this case, the strategy was to attach the receptor specifically via the G-proteins. The approach is based on the immobilised metal ion affinity chromatography (IMAC) technique and the well established $[^{35}S]GTP\gamma S$ binding assay (Windh and Manning, 2002) was used to test the functionality of the transductosome complex.

4.3.1.   Receptor capture by surface-attached ligand

Initially, capturing GPCRs (for diagnostic purposes or other applications) via the ligand-bead approach was considered. Thus, for the two receptor systems that were established and partly characterised in this study (the β_1AR and AT_1 expressed in naturally occurring membranes isolates), methods for specific receptor capture were examined. Ligand capture has been used to capture solubilised GPCRs for purification (Caron *et al.*, 1979) while more recently; Simons *et al.* (2004) fabricated dihydroalprenolol (DHA) beads via a sulfhydryl activation step to capture the solubilised β_2AR[30]. For the current study, magnetic streptavidin coated Spherotech beads were the chosen surface and biotin-ligand linkers were designed. An Ang II-biotin linker was relatively simple to manufacture in house and purity was tested using high-performance liquid chromatography (HPLC)[31]. The Ang II biotin linker was equally as effective as Ang II in the competition binding experiments using AT_1 containing membranes isolated from turkey liver and [^{125}I]Ang II (data not shown). The biotin binding capacity of the beads was reported to be 60 pmol/ 20 µl beads by the manufacturer and this was confirmed in this study by using [^{3}H] biotin. In terms of separation and washing, 3 washes with 250 µL was sufficient to remove unbound radioactivity using the Dynal MPC-S tool (a magnetic device that accommodates 6 x1.5 mL Eppendorf tubes). In order to assess the affinity of the Ang II-biotin linker on the magnetic platform (beads) for the naturally occurring AT_1 receptor (from turkey liver plasma membranes), a competition binding curve was performed (**Figure 4.2**). The concentration of Ang II on the streptavidin beads was calculated from the HPLC[32] traces. Surface-attached Ang II competed with [^{125}I]Ang II as

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[30] Note the work pertaining to this particular technology was performed in 2001 and knowledge of other approaches such as Simons *et al.* (2004) was uncovered later and therefore not used at the time.
[31] An adrenaline-biotin linker was chemically difficult to manufacture in house and the cost to purchase was not warranted.
[32] HPLC was performed by Dr Wayne Leifert and Ms Camilla Dorian.

effectively as unmodified Ang II and this competition is reflected in the similar IC_{50} values obtained. The IC_{50} values can be compared to data in **Figure 2.6** (Chapter 2). The accessibility of Ang II-beads to AT_1 receptors in turkey liver membranes appears not to be as problematic, and this suggests the likelihood that such modified beads could specifically capture AT_1 receptors embedded in membranes without detectable hindrance. Simons *et al.* (2004) reported ligand affinity values in competition with the DHA beads comparable to those previously obtained from membrane preparations.
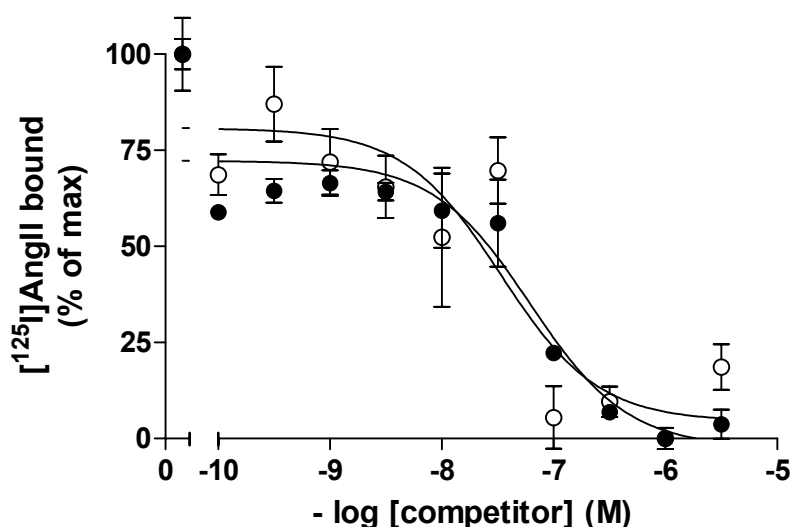

Figure 4.2:   **Competition binding with Ang II and Ang II-biotin-beads.**
Membranes from turkey liver (0.05 mg/mL protein per assay) containing AT_1 receptor binding activity were diluted in TN buffer then combined with 150 pM [^{125}I]Ang II as described in the materials and methods section of chapter 2. Radioligand binding was competitively displaced by Ang II (human) (●), or Ang II-biotin-linker on Spherotech beads (o). The IC_{50} was 60.9 nM for Ang II (human) and 33.7 nM for Ang II-biotin-beads. Each data point represents n = 3, mean ± SEM.

Although from this data, the ligand bead approach specifically capturing the AT_1 receptor from turkey liver plasma membrane looks promising there were three main reasons that lead to abandoning this line of approach. Firstly, obtaining receptors from natural sources (i.e. turkey liver) was deemed not the best system to work with and access to recombinant cell culture systems had to be established in the laboratory. Also the β_1AR ligand –biotin linker was not easily manufactured and this raised the question regarding the universality of this approach for the GPCR

superfamily. Finally, the overall focus of this thesis was in obtaining a functional cell-free assay platform.

4.3.2. G-protein-receptor capture

Until recently, cell-free ligand screening (also referred to as isolated target assays) had focused on non-specific attachment (Fang *et al.*, 2002b) or specific attachment (Martinez *et al.*, 2003; Sklar *et al.*, 2000) of GPCRs only. The limitations of these non-functional isolated target based screening formats were discussed in Chapter 1. Methods of surface attaching the transductosome specifically without compromising functionality were necessary and a strategically attractive endpoint for this thesis. Indirect attachment of GPCRs via G-proteins was the obvious choice because these proteins are highly specific to receptor and ligand (Fay *et al.*, 1991). Yet the events that occur during G-protein activation (i.e. the GTPase cycle) are characteristic of GPCR activation and potentially providing a simple, generically exploitable detection avenue. Moreover, in this study it has been shown that the histidine tag did not hinder the subunit functionality in the [^{35}S]GTPγS binding assay enabling affinity driven surface attachment. Other investigators have utilised the histidine tag on G-proteins to investigate at protein-protein interactions using flow cytometry (Simons *et al.*, 2003) and, more recently, have investigated GPCR assemblies for the development of drug discovery screens (Waller *et al.*, 2003). The work described here pertaining to GPCR attachment via the G-protein subunit was undertaken in 2002 and a provisional patent was filed on this research as well as extensions to the technology, the latter is not discussed in this thesis.

4.3.2.1. G-protein capture onto a nickel surface

There are many conditions for the successful functioning on a surface such as; the minimisation of non-specific interactions leading to non-controlled absorption; the

surface must display high specificity for the biomolecule; and the immobilised protein must retain its native conformation and function (Schmid *et al.*, 1997). In order to immobilise the transductosome to a surface via the histidine tag located on either the $G\alpha_{i1(6xHIS)}$ subunit or the $\beta_1\gamma_{2(6xHIS)}$ dimer, a nickel containing platform was required. Furthermore, it was necessary for the platform to have a large surface area so that binding could be investigated. Spherotech magnetic beads were considered. However, Ni(NTA) magnetic beads were considered too expensive and there were some intrinsic problems after several attempts to manufacture a biotin NTA linker (to be used with Spherotech streptavidin beads). The methods used to prepare the biotin-NTA linker were reported by (McMahan and Burgess, 1996) however, NMR[33] analysis of the linker were inconclusive. Nickel coated agarose beads were used successfully in the purification of the G-protein subunits (Kozasa and Gilman, 1995). Unlike the Spherotech beads, these beads were cheaper, easy to manipulate and separation did not require expensive equipment. Washing the Ni(NTA) agarose beads was accomplished by a 5 sec pulse on a micro centrifuge unlike the Spherotech beads that did not sediment as easily. In addition, the Ni(NTA) beads would sediment within 5 min and thus assays were incubated with orbital mixing at 300 rpm to maintain the bead suspension.

4.3.3. Assay of α_{2A}AR signalling activity in the presence of Ni(NTA) beads

The [35S]GTPγS binding assay was optimised and improved for the reconstituted α_{2A}AR transductosome system as described in chapter 3. The inclusion of Ni(NTA) beads introduced additional complexities to the [35S]GTPγS assay and adaptations were necessary to discriminate between agonist induced [35S]GTPγS binding that occurred at captured transductosomes (i.e. on beads) from non-specific binding that

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[33] Performed by Rachel Campbell Department of Chemistry, Flinders University.

occurred off the platform and from excess radiolabel. Previously GF/C filters were used to separate radioactivity bound to the activated transductosome complex from excess radiolabel as proteins (such as the transductosome complex as well as $G\alpha_{i1}/[^{35}S]GTP\gamma S$) were preferentially retained on this filter.

To assess whether the protein binding properties of the GF/C separation filter could be reversed; filters were pre-soaked with 0.2% BSA. This concentration of the inactive protein BSA is commonly used to displace protein radio labels in receptor binding assays (Auger-Messier *et al.*, 2003). There was no difference in the $[^{35}S]GTP\gamma S$ bound to $G\alpha_{i1}$ with or without BSA (data not shown) suggesting that the protein binding properties of this filter could not be changed by using an inactive protein such as BSA.

Next a centrifugation separation assay was assessed for adaptability to the current system. This method was ruled out as there were intrinsic technical problems and because centrifugation is not as adaptable to high throughput (without the purchase of expensive centrifugation rotors for high throughput). The centrifugation method also introduced problems associated with adequate bead washing necessary to reduce non-specific binding and artefact production. Separation using filtration is much more amenable to effective washing.

Separation via filtration was considered the better option and thus it was imperative to find a filter interface with minimal protein binding properties that could retain the 60-160 μm Ni-agarose bead. Thus for this purpose Whatman #1 filter paper was investigated (**Figure 4.3**). The Whatman #1 paper filters captured the relatively large

Ni(NTA) beads, while all proteins and membranes that were not associated with the
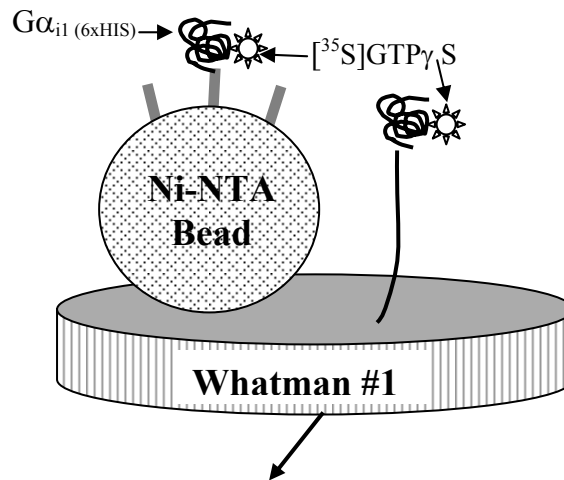
beads passed through the paper (see **Figure 4.4**)

Figure 4.3:   **A schematic showing Whatman #1 filters capturing beads but not protein.**

Two critical points need to be considered when undertaking the $[^{35}S]GTP\gamma S$ binding

assay with surface attached proteins, these being: reproduction of the assay

pharmacology on the attached platform; and optimisation on the platform to

maximise the sensitivity of the platform assay. In order to test the functionality of the

attached transductosome to promote an agonist induced signal, the $[^{35}S]GTP\gamma S$

binding assay was performed. In **Figure 4.4**, the fold stimulation above basal binding

due to the agonist is shown above each agonist bar to compare the effectiveness of

the assay after each treatment. Almost a 3 fold stimulation was observed after the

captured transductosome was exposed to the agonist UK-14304 (condition **A**). To

exclude the possibility that this increase was non-specific, separate simultaneous

incubations were prepared without G-proteins (**B**) or $\alpha_{2A}AR$ (**C**) or the platform

(beads) (**D**). It was not surprising that agonist-promoted $[^{35}S]GTP\gamma S$ binding was

prevented in each incubation in which a vital constituent was left out. This data

suggests that the beads are selectively capturing activated $G\alpha_{i1(6xHIS)}$ subunits which

subsequently are able to interact with the membrane-associated receptor to elicit a

functional signalling response. Platform selectivity for binding the Gα subunit and subsequently the receptor complex is also inferred by the fact that when the Ni(NTA) agarose beads were not included, agonist stimulated binding was not detected. Consideration was given to using either "no-G-protein" or "no receptor" or "no beads" as a blank to subtract from the basal and agonist induced binding values and thus enhance the signal to background (S/B) ratio, elevating the fold stimulation. However, to provide the raw data (unaltered) blanks were not subtracted from the data that follows unless mentioned.
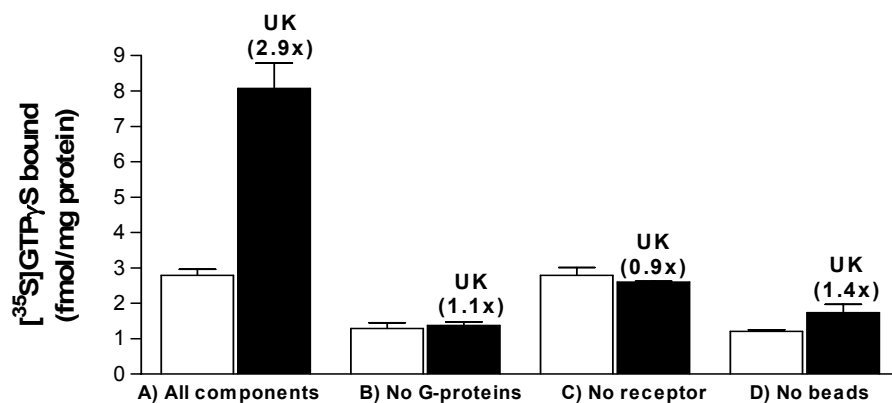

Figure 4.4:   **The specificity of reconstitution of α_{2A}AR on Ni(NTA) beads.**
Assays were incubated as described in the materials and methods section ensuring the following: G-proteins (20nM $G\alpha_{i1(6xHIS)}$ and 20 nM $\beta_1\gamma_2$) were combined with 0.4 mg/mL urea treated membranes expressing α_{2A}AR and 5 μL Ni(NTA)-agarose beads in an assay volume of 100 μL (**A**). Incubations were for 60 min. Basal [^{35}S]GTPγS binding (white bars) or agonist (UK-14304) induced [^{35}S]GTPγS binding (black bars) without: **B)** G-proteins **C)** α_{2A}AR **D)** Ni(NTA) agarose beads. The fold stimulation after UK-14304 induced [^{35}S]GTPγS binding is shown above the UK-14304 induced binding in brackets. Incubations were for 60 min. Each data point represents n = 3, mean ± SEM.

4.3.3.1.   Ni(NTA) bead specificity

Further controls were performed to demonstrate that the attachment was specific to the nickel-histidine linkage using the preassembled transductosome complex. Each experiment was internally controlled by the addition of a "gold standard[34]" (condition **A**) and in this case a fold stimulation greater than 5 was obtained (see **Figure 4.5**). Agonist-promoted [^{35}S]GTPγS binding was prevented when an excess of NiCl (100mM) was added to the incubation (**B**), thus competing with the nickel coated beads for transductosome attachment. The basal binding in this incubation

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[34] The use of a "gold standard" to internally control assays was discussed in Chapter 3.

(but not in **C** and **D**) was considerably (p<0.001) elevated compared to the "gold standard" suggesting that perhaps adding this solution prevents adequate washing. Upon the addition of the monovalent histidine competitor imidazole, the agonist induced [^{35}S]GTPγS was significantly reduced (p<0.0001) and the fold stimulation was decreased by 62% (**Figure 4.5 C**). Furthermore, when the nickel beads were depleted of Ni^{2+} by the incubation with the divalent metal chelating agent EDTA (**D**) there was a relatively low level of signalling activity (low [^{35}S]GTPγS binding and fold stimulation) indicating a low level of attachment of the Gα$_{i1(6xHIS)}$ to the beads. These Ni^{2+} depleted beads were added to the incubation represented (**D**). The volume of beads was also doubled to increase the amount of radioactivity captured (bead volume is revisited in more detail later on).
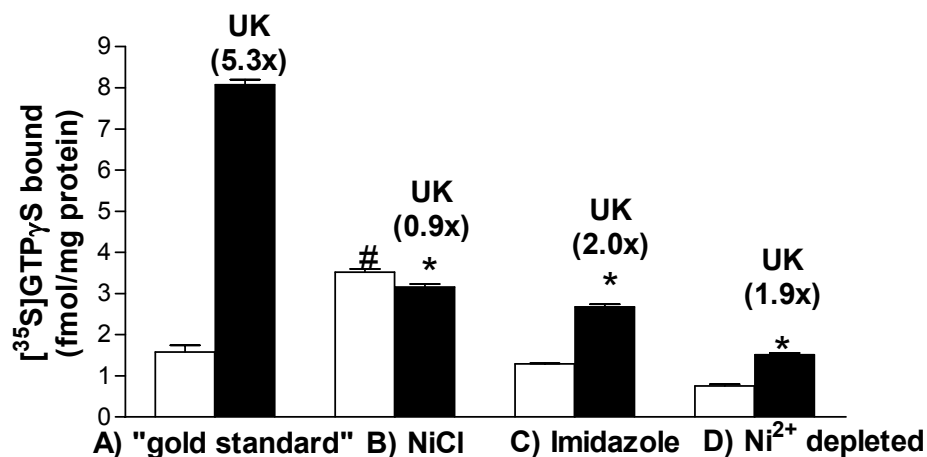

Figure 4.5:   **Reconstitution of α$_{2A}$AR with Gα$_{i1 (6xHIS)}$ and β$_1$γ$_2$ on Ni(NTA) agarose beads.**
Assays were incubated as described in the materials and methods section ensuring the following: G-proteins (20nM Gα$_{i1(6xHIS)}$ and 20 nM β$_1$γ$_2$) were combined with 0.4 mg/mL urea treated membranes expressing α$_{2A}$AR and 10 μL Ni(NTA)-agarose beads in an assay volume of 100 μL. Incubations were for 60 min. Basal [^{35}S]GTPγS binding (white bars) or 10 μM UK-14304 induced [^{35}S]GTPγS binding (black bars) and **B)** NiCl (100 mM) which was added to bind to Gα$_{i1 (6xHIS)}$ preventing the subunit from binding to the beads **C)** imidazole (150 mM) which competitively binds to Ni^{2+} **D)** Ni^{2+} depleted agarose beads were added instead. The fold stimulation after UK-14304 induced [^{35}S]GTPγS binding is shown above the bars. Incubations were for 60 min. Each data point represents n = 3, mean ± SEM. *p<0.0001 (UK-14304); #p<0.0001 (basal binding).

4.3.3.2.   Washing and bead volume

After initially determining that the Ni(NTA)-agarose beads with attached Gα$_{i1(6xHIS)}$ protein could potentially be used to attach receptor containing cell membrane

preparations to give rise to a reconstituted $\alpha_{2A}AR$ transductosome complex as a functional cell-free GPCR assay platform, further optimisation of the assay system was carried out. To begin with the number of washes that was necessary to minimise non-specific binding was determined (**Figure 4.6**). Two or more washes of 4 mL of ice cold TMN buffer was adequate to eliminate any unbound [^{35}S]GTPγS that may interfere with the S/B ratio for the assay. Routinely 3 washes were used from this point on to ensure any unbound radioactivity was removed.
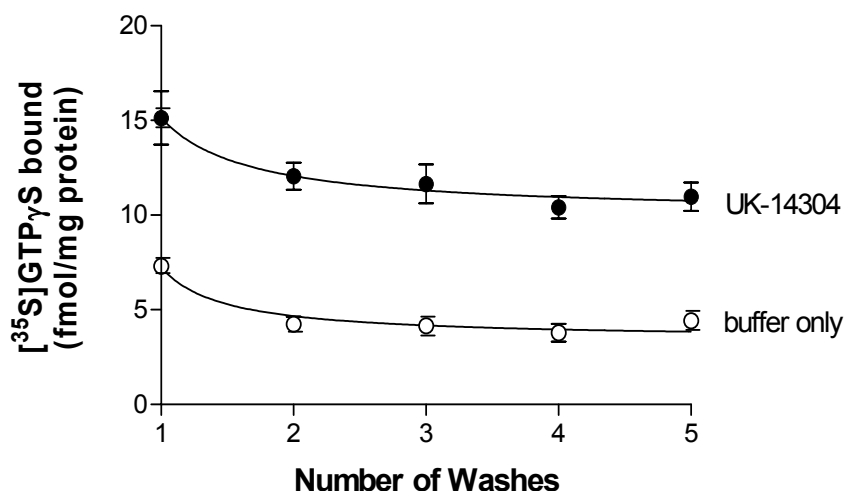

Figure 4.6: **Effect of washing with ice cold TMN Buffer.**
Assays were incubated as described in the materials and methods section ensuring the following: G-proteins (20nM $G\alpha_{i1(6xHIS)}$ and 20 nM $\beta_1\gamma_2$) were combined with 0.4 mg/mL urea treated membranes expressing $\alpha_{2A}AR$ and 10 μL Ni(NTA)-agarose beads in an assay volume of 100 μL. Incubations were for 60 min. The reaction was initiated by the addition of buffer (Basal [^{35}S]GTPγS binding, represented by unfilled circles ($\circ$)) or 10 μM UK-14304 (agonist induced [^{35}S]GTPγS binding, represented by filled circles ($\bullet$). Incubations were for 60 min. Each data point represents n = 3, mean ± SEM.

It is also important to consider the volume of beads used in the assay system because if the volume of beads is increased this will subsequently increase the sites where radioactivity accumulate can become trapped. From the data in **Figure 4.7**, 5 or 10 μL beads per 100 μL gave the best S/B ratio and the radioactivity (receptor activated $G\alpha_{i1(6xHIS)}$) was saturated above 20 μL of Ni(NTA) beads. The ideal volume of Ni(NTA) beads for protein capture was between 5-10 μL. According to the specifications reported by Qiagen the binding capacity of Ni(NTA) beads is approximately 5-10 mg/mL (protein/beads) for an approximate 20, 000 kDa protein.

Since the $G\alpha_{i1(6xHIS)}$ subunit is approximately 41,000 kDa[35] in mass we will assume that the protein binding capacity of the beads is closer to 5 mg/mL which would be equivalent to 0.05 mg of protein per 10 μL of beads. This corresponds to approximately 1.2 nmoles of $G\alpha_{i1(6xHIS)}$ subunit protein bound per 10 μL Ni(NTA) beads. It should also be noted that the background binding continued to increase and did not reach saturation which may be an indication of excess protein (receptor protein is the likely contributing factor) in the system.
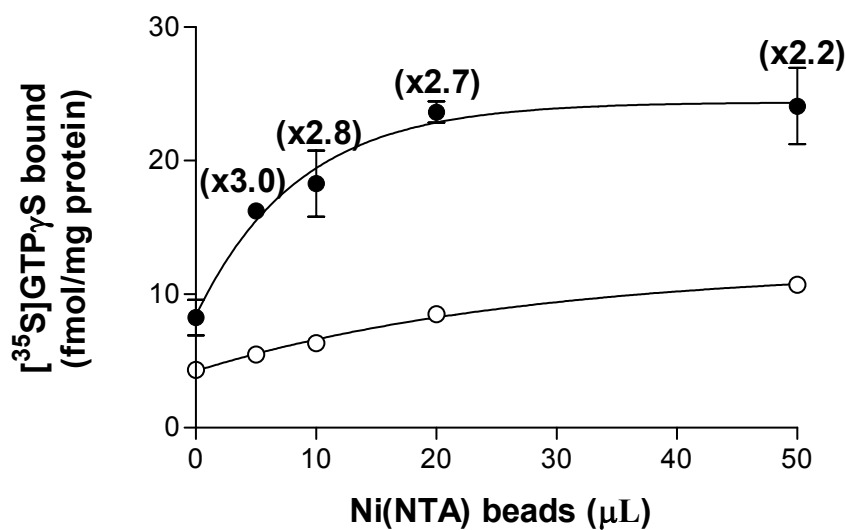

Figure 4.7:   **Reconstitution of $\alpha_{2A}AR$ with $G\alpha_{i1(6xHIS)}$ + $\beta_1\gamma_2$ on the platform: bead/platform dependence.**
Assays were incubated as described in the materials and methods section ensuring the following: G-proteins (10nM $G\alpha_{i1(6xHIS)}$ and 20 nM $\beta_1\gamma_2$) were combined with 0.4 mg/mL urea treated membranes expressing $\alpha_{2A}AR$ in an assay volume of 100 μL. Basal [35S]GTPγS binding is represented by unfilled circles (○) and agonist induced [35S]GTPγS binding is represented by filled circles (●). Ni^{2+} coated agarose beads (as indicated). Incubations were for 60 min. The fold stimulation after UK-14304 induced [35S]GTPγS binding is shown above the specific points. Each data point represents n = 2, error bars where visible represent the range of duplicates. When error bars are not visible they are contained within the data point.

The results to date demonstrate $\alpha_{2A}AR$- induced [35S]GTPγS with $G\alpha_{i1\ (6xHIS)}$ subunits when displayed on the surface of Ni(NTA) beads via the hexahistidine tag. This assay format was further characterised by investigating the effects of time, G-

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[35] The website www.signalling-gateway.org/molecule provides up-to-date data regarding molecular sizes and other properties of G-proteins among other proteins.

protein concentration and UK-14304 concentration on functional signalling activity and is described below.

4.3.3.3. Assay time dependence

The time course of agonist promoted [^{35}S]GTPγS binding to the activated G$\alpha_{i1(6xHIS)}$ in the presence of Ni(NTA) is shown in **Figure 4.8**. The amount of [^{35}S]GTPγS bound increased over time and the fold stimulation was approximately equal to 3 after a 60 and 90 min incubation. The basal binding also increased with time which may indicate non-specific protein binding. From this point forward assays were performed at a time point of 90 min.
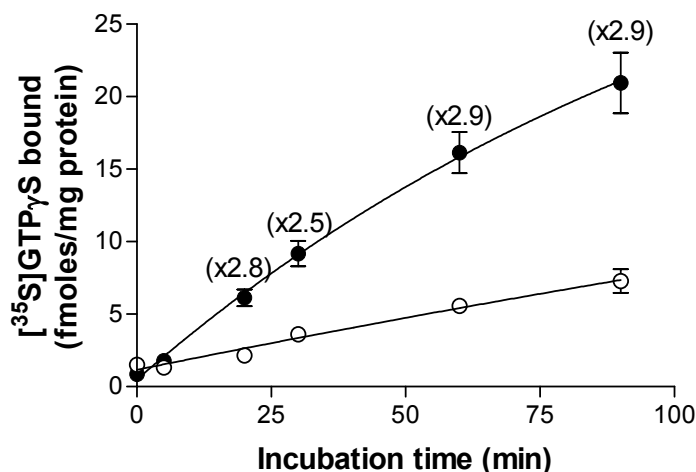

Figure 4.8: The effect of time on α_{2A}AR activated [^{35}S]GTPγS binding in the presence of Ni(NTA) beads

Assays were incubated as described in the materials and methods section ensuring the following: G-proteins (20nM G$\alpha_{i1(6xHIS)}$ and 20 nM $\beta_1\gamma_2$) were combined with 0.2 mg/mL urea treated membranes expressing α_{2A}AR and 5 μL Ni(NTA) agarose beads in an assay volume of 100 μL. Basal [^{35}S]GTPγS binding is represented by unfilled circles (○) and 100 μM UK-14304 induced [^{35}S]GTPγS binding is represented by filled circles (●). The fold stimulation after UK-14304 induced [^{35}S]GTPγS binding is shown above the points. Each data point represents n = 3, mean ± SEM. Where error bars are not visible they are contained within the data point.

Note also that preliminary experiments had shown that 0.2 mg/mL of membrane protein worked as effectively as 0.4 mg/mL (data not shown) and thus 0.2 mg/mL was used from this point forward unless indicated otherwise. Using a particular wild type formyl peptide receptor, Simons *et al.* (2003) determined that the complex assembly half time on beads was 13 min. However, complex assembly time varied

depending on the receptor and specific agonist used, thus these authors chose to use a standardised incubation time of 2 hours to ensure adequate assembly.

4.3.3.4. $G\alpha_{i1(6xHIS)}$ and $\beta_1\gamma_2$ concentration dependence

In order to further characterise the binding of the activated $G\alpha_{i1\ (6xHIS)}$ in the presence of Ni(NTA) beads, subunit dependence curves were performed (see **Figure 4.9**). As expected, the accumulation of receptor activated [^{35}S]GTPγS bound increased proportionately to the amount of $G\alpha_{i1(6xHIS)}$ (**Figure 4.9 A)** or $\beta_1\gamma_2$ (**Figure 4.9 B)** added to the assay. The fold stimulations are shown above the data points and from these levels of stimulation, a concentration of 50 nM for each subunit was used in the next experiment. The half saturation points were 75 nM and 35 nM for $G\alpha_{i1(6xHIS)}$ and $\beta_1\gamma_2$ respectively, compared with 8.1 nM and 3.5 nM, respectively when subunit dependence was determined in the absence of beads. Although different preparations of subunits were prepared in this case (comparing G-protein concentration dependence in the presence or absence of beads), this difference in half saturation point can not be totally attributed to biological variability. Perhaps this means that the presence of beads hinders transductosome assembly to an extent.
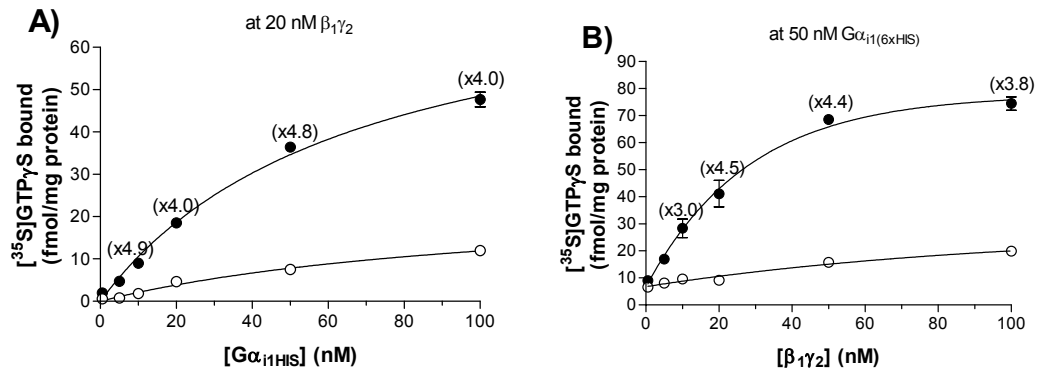
A) at 20 nM $\beta_1\gamma_2$

B) at 50 nM $G\alpha_{i1(6xHIS)}$

Figure 4.9: **The effect of subunit concentration on $\alpha_{2A}AR$ activated [^{35}S]GTPγS binding in the presence of Ni(NTA) beads.**
Assays were incubated as described in the materials and methods section ensuring the following: $G\alpha_{i1(6xHIS)}$ and $\beta_1\gamma_2$ were combined with 0.2 mg/mL urea treated membranes expressing $\alpha_{2A}AR$ and 5 μl Ni(NTA) agarose beads in an assay volume of 100 μL. Basal [^{35}S]GTPγS binding is represented by unfilled circles (o) and 100 μM UK-14304 induced [^{35}S]GTPγS binding is represented by filled circles (•). **A)** 20 nM $\beta_1\gamma_2$ was used and the for $G\alpha_{i1(6xHIS)}$ half saturation point and the B_{max} were determined to be 75 nM and 57 fmol/mg protein respectively upon UK-14304 stimulation. **B)** 50 nM $G\alpha_{i1(6xHIS)}$ was used and for $\beta_1\gamma_2$ the half saturation point and the B_{max} were determined to be 35 nM and 102 fmol/mg protein respectively upon UK-14304 stimulation. The fold stimulation after UK-14304 induced [^{35}S]GTPγS binding is shown in brackets above the points. Each data point represents n = 3, mean $\pm$ SEM. Where error bars are not visible they are contained within the data point.

4.3.3.5. UK-14304 concentration dependence.

The sensitivity of the membrane containing $\alpha_{2A}AR$ preparations to stimulation by UK-14304 when attached to beads via $G\alpha_{i1(6xHIS)}$ linkage, measured by the [^{35}S]GTPγS binding assay is shown in **Figure 4.10**. These experiments were carried out simultaneously to eliminate any biological variability that was previously seen when using different subunits (see previous chapter). The sensitivity of UK-14304 to the transductosome in the presence of beads was similar to the sensitivity observed using a solution-based assay (no beads) suggesting that the agonist binding ability of the preassembled transductosome did not change as a result of immobilisation of $G\alpha_{i1(6xHIS)}$ as reflected in the EC_{50} values determined. As expected, the selective $\alpha_{2A}AR$ antagonist, Rauwolscine blocked the agonist induced [^{35}S]GTPγS binding in the presence of Ni(NTA) beads.
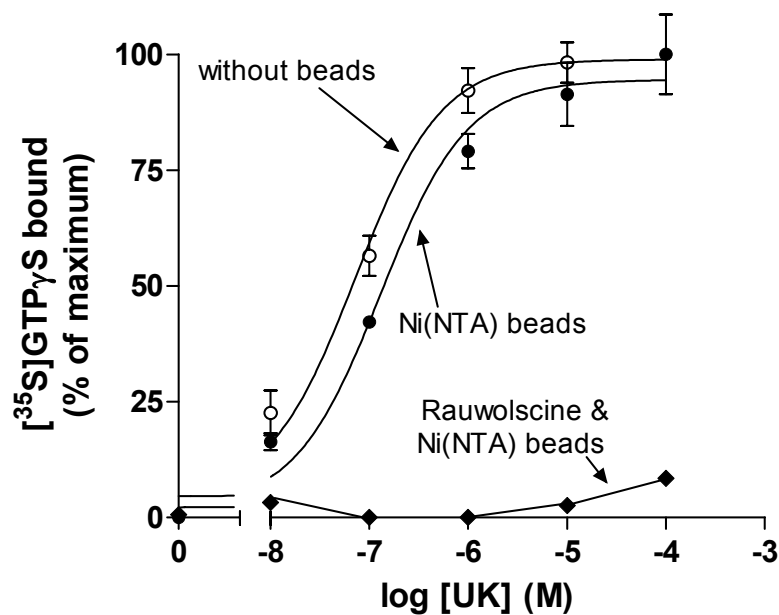
Figure 4.10: **The effect of UK-14304 and Rauwolscine on α_{2A}AR activated [^{35}S]GTPγS binding in the presence and absence of Ni(NTA).**
Assays were incubated as described in the materials and methods section ensuring the following: $G\alpha_{i1(6xHIS)}$ (50 nM) and $\beta_1\gamma_2$ (50 nM) were combined with 0.2 mg/mL urea treated membranes expressing α_{2A}AR in an assay volume of 100 μL. All reactions were initiated by the addition of various concentrations of the adrenaline analogue agonist UK-14304 (concentrations as indicated). UK-14304 stimulated [^{35}S]GTPγS binding in the absence (represented by unfilled circles (○)) or presence of the platform (5 μl Ni(NTA) beads) (represented by filled circles (●)) is shown and normalised. The potent α_{2A}AR antagonist 500 μM Rauwolscine in the presence of varying concentrations of UK-10304 (represented by filled circles (♦)) is shown. The EC_{50}s were determined to be 72 nM (no beads) and 155 nM (beads). Each data point represents n = 3, mean ± SEM. Where error bars are not visible they are contained within the data point.

1.1.1. α_{2A}AR signalling activity in the presence of Ni(NTA) beads in 96-well format

Although it was established that the α_{2A}AR-G-protein complex (the transductosome) was functional in the presence of the Ni(NTA) beads, these assays were still being performed in a test tube assay format which was relatively laborious and undesirable in terms of developing a format that increased even moderately assay throughput. Therefore, it was imperative that the throughput of this assay be increased to a 96-well plate format. An assay similar to the one previously constructed and described in chapter 3 using Millipore MultiScreen^{TM} 96-well GF/C plates, was considered. Various 96-well microplate platforms from Whatman and Millipore were evaluated although at the time there was not a 96-well MultiScreen^{TM} plate manufactured with

Whatman #1 inserts. The closest that could be found was a MultiScreen[TM] Durapore® plate. Durapore® filters were tested in the test tube assay and worked as effectively as the Whatman #1 (data not shown) and thus this platform was used. The assays that examined the effects of time and G-protein subunit dependence on the reconstituted α_{2A}AR signalling complex in the presence of Ni(NTA) beads (**Figure 4.8**, **Figure 4.9** and **Figure 4.10**) were repeated using the new 96-well assay format[36]. This data compared well with previous data. Interestingly, the problem with the biological activity of [^{35}S]GTPγS was discovered when a new batch of radioactivity was purchased (see Appendix on page 246).

The receptor promoted [^{35}S]GTPγS binding in the presence of Ni(NTA) beads was further characterised using a competition assay to examine the relative potencies of a variety of adrenergic antagonists (**Figure 4.11**). For many cellular responses, antagonist screening is performed near to, or at the top of, the agonist response curve (eg EC_{80} or above) in order to minimise assay variability (Moore and Rees, 2001). Hence, the agonist concentration of 1 μM UK-14304 was chosen. The pharmacological profile displayed compares well with the rank order potency shown for this receptor class in previous reports (Jasper *et al.*, 1998). The antagonist rank order potency of each of these ligands for [^{35}S]GTPγS incorporation correlated with their ability to displace the α_{2A}AR antagonist [^{3}H]MK-912[37] in a radioligand binding assay (Leifert *et al.* (b) 2005). This indicates that the pharmacological response for α_{2A}AR in terms of rank order potency of different antagonists was unaltered in the presence of Ni(NTA).

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[36] These assays were preformed by Leifert and Burnard.
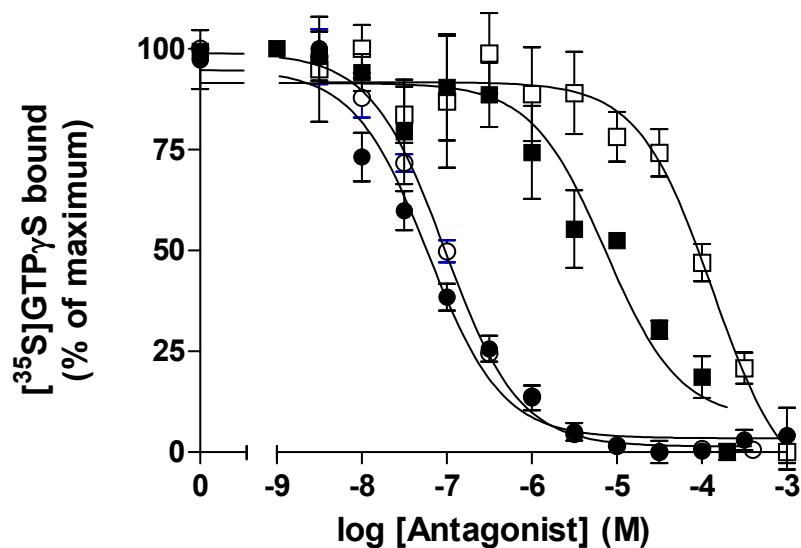[37] as shown by Amanda Aloia and Kelly Bailey (data not shown)

Figure 4.11: **The effect of adrenergic antagonists on α_{2A}AR-activated [^{35}S]GTPγS binding in the presence of Ni(NTA) beads.**
Assays were incubated as described in the materials and methods section ensuring the following: Urea treated membranes expressing α_{2A}AR (0.1 mg/mL) and 10 μL Ni(NTA) agarose beads were combined with 20nM G$\alpha_{i1(6xHIS)}$ and 20 nM $\beta_1\gamma_2$. Agonist, UK-14304 (1μM) was added to start the reaction and the following antagonists were included: Rauwolscine ($\bullet$), Yohimbine (o), Prazosin ($\blacksquare$) and Propranolol ($\square$). The IC$_{50}$ values for each of the antagonists was determined as 0.06 μM (Rauwolscine :selective α_2AR antagonist), 0.09 μM (Yohimbine: selective α_2AR antagonist), 7.4 μM (Prazosin: selective α_1AR antagonist) and 133 μM (Propranolol: βAR antagonist). Each data point represents n = 3, mean $\pm$ SEM. Where error bars are not visible they are contained within the data point.

4.3.3.6. G-protein subunits and the histidine motif

To further rule out the possibility that the attachment was not specific via the nickel and histidine tag on the protein, two separate incubations were performed differing only in the inclusion of Gα_{i1} with or without a histidine tag and then monitoring the UK-14304-stimulated [^{35}S]GTPγS binding. The data in **Figure 4.12** shows that reconstitution with G$\alpha_{i1(6xHIS)}$ and $\beta_1\gamma_2$ (20 nM) (condition **A**) considerably elevated the agonist-promoted binding compared to reconstitution with the subunit lacking the histidine attachment linkage (i.e. Gα_{i1} and $\beta_1\gamma_2$) (condition **B**). Interestingly, there was a degree of stimulation (represented by a fold stimulation of 2.8) when Gα_{i1} minus the tag was used for the reconstitution. Perhaps this condition might be a more appropriate "blank" control. However, this incubation contained a different subunit and thus, as discussed previously, there is variability between each preparation of

subunit. In any case, these results suggest that the receptor activated $G\alpha_{i1(6xHIS)}$ is immobilized on the Ni(NTA)-agarose bead.
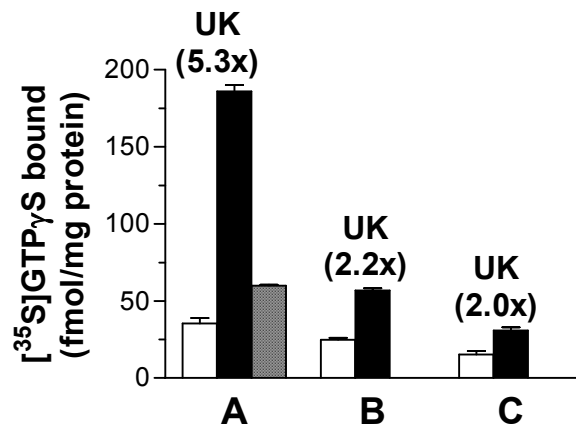

Figure 4.12:   The effect of histidine tagged subunits.
Assays were incubated as described in the materials and methods section ensuring the following: Urea treated membranes expressing $\alpha_{2A}AR$ (0.2 mg/mL) and 5 μL Ni(NTA) agarose beads in an assay volume of 100 μL were combined with: A) 20nM $G\alpha_{i1(6xHIS)}$.and 20 nM $\beta_1\gamma_2$ B) 20 nM $G\alpha_{i1}$ and 20 nM $\beta_1\gamma_2$ or C) 20 nM $G\alpha_{i1}$ and 20 nM $\beta_1\gamma_{2(6xHIS)}$. Basal [^{35}S]GTPγS binding is represented by white bars and 100 μM UK-14304 induced [^{35}S]GTPγS binding is represented by black bars. The antagonist Rauwolscine (500 μM) was included in the reaction that is represented by a checked bar. The fold stimulation after UK-14304 induced [^{35}S]GTPγS binding is shown in brackets above the bars. Each data point represents n = 3, mean ± SEM.

In addition, immobilisation via the histidine tag on the $\beta_1\gamma_{2(6xHIS)}$ subunit was investigated (**Figure 4.12**). This experiment was repeated on several occasions with the same result, of a lack in $\alpha_{2A}AR$-induced [^{35}S]GTPγS binding in the presence of Ni(NTA) when a histidine tagged $\beta_1\gamma_2$ dimer and non–histidine tagged $G\alpha_{i1}$ were used. Although preliminary attempts to follow the $\beta_1\gamma_{2(6xHIS)}$ attachment on the bead using SDS PAGE and silver staining and fluorescein labelled subunits using a method described by (Sarvazyan *et al.*, 1998) were both found to be unsuccessful, a much simpler yet very effective 'filter stack' technique was established by Leifert *et al.* ((b) 2005). This method showed that upon receptor activation the dimer and $G\alpha_{i1}$ dissociated and thus the radiolabelled $G\alpha_{i1}$ was not captured on the Whatman #1 filter paper in these experiments. This activated subunit could be captured only if a GF/C filter was placed underneath the Whatman #1 paper filter.

The overnight storage potential of the transductosome was also investigated (data not shown). However under the conditions utilised (-80°C, -20°C or 4°C) transductosome functionality was not recovered. More recently, solubilised receptors have been shown to withstand freeze-thaw cycles indicating protein stability in micelles (Waller *et al.*, 2003).

Summary of results

In conclusion, two methods of surface attachment were considered. The first involved the preparation of magnetic beads conjugated with the AT_1 cognate ligand Ang II. These ligand-beads were shown to compete with unmodified Ang II in a competition binding dose response curve using membrane homogenates from turkey liver. Next, the $G\alpha_{i1(6xHIS)}$ was captured on Ni(NTA) agarose beads and various modifications directed towards the $[^{35}S]GTP\gamma S$ binding assay were carried out. These modifications enabled the measurement of $\alpha_{2A}AR$ activated $[^{35}S]GTP\gamma S$ binding in the presence of the platform Ni(NTA) to be measured. The specificity of the surface attachment was shown. The concentration dependence of the G-protein subunits, the assay incubation time and sensitivity to the agonist UK-14304 were unhindered in the presence or absence of the Ni(NTA) agarose beads. Furthermore, the single test tube assay was extended to incorporate a 96-well assay format. The rank order potency of selected α_2AR antagonists, an α_1AR antagonist and a β_1AR antagonist was unhindered in the presence of the beads. Moreover, $\alpha_{2A}AR$ activated $[^{35}S]GTP\gamma S$ was only captured when the histidine tag was associated with the $G\alpha_{i1}$ subunit.

4.4. DISCUSSION

GPCR display may be achieved by the direct association of GPCRs with an appropriate surface (Fang *et al.*, 2002c; Sklar *et al.*, 2000) or indirectly through the display of ligands, G-proteins or other binding partners (Waller *et al.*, 2004). Although ligand attachment was considered, it was decided that for the purposes of this system, attachment via the G-proteins would be more appropriate. Attachment to the surface was accomplished by utilising the histidine tagging on the G-protein, which was also used to purify these proteins. The most important points identified pertaining to the construction of a functional cell-free and tethered GPCR assay systems are;

1) G-protein coupling (can this additional level of complexity be incorporated into a cell-free system?)

2) Method of surface attachment (Is hindrance a problem? What about surface affinity?)

3) The associated lipid environment (how important is this?)

These important points are discussed below. Furthermore, the [^{35}S]GTPγS Ni(NTA) bead assay was used to investigate the events leading up to transductosome assembly, specifically G-protein subunit dissociation and these events are explored in more detail.

4.4.1. Ligand-Receptor capture:

Magnetic Spherotech streptavidin beads conjugated with biotin-Ang II linkers were used to capture AT_1 receptors from turkey liver plasma membrane. Ligand affinity values to plasma membrane homogenates from turkey liver in competition with the Ang II-biotin beads were comparable to those obtained using unmodified Ang II. This suggests that the Ang II-beads can capture AT_1 receptors in turkey liver

membranes without detectable hindrance which may have been caused by the introduction of beads. As explained, this approach of GPCR capture was not continued although these preliminary results looked promising. Primarily this was because of the inherent complications in developing this technique into a functional screening platform. The move away from natural/tissue receptor sources to recombinant expression systems was also a factor. Additionally, the major factor for the decision lay with the fact that although certain ligands have been used for cognate receptor attachment, for example with regard to the β_2AR (Caron *et al.*, 1979; Simons *et al.*, 2004) the technique may not be generic. Some ligands may lose their specific binding activity if they were to undergo chemical modifications to allow for surface attachment.

The rationale behind using ligand-beads to capture GPCRs was based on early work whereby ligand-affinity chromatography had been used to capture solubilised receptors (Caron *et al.*, 1979). Using a similar rationale Simons *et al.* (2004) extended these earlier reports with the solubilised β_2AR containing a green fluorescent protein (GFP) tag. This tag facilitated the visualisation of the solubilised receptor. Obviously for detecting GPCRs within tissue (a possible application of the technique) such molecular enhancements would not be feasible. The present technology is compared to the technology reported by Simons *et al.* (2004) in **Table 4.2**. Another difference between the approaches was the preparation of the ligand-beads; while biotinylation was used in the present study, biotinylated ligands did not capture solubilised receptors in the study reported by Simons *et al.* (2003). Background fluorescence of the GFP fused receptor was also a technical drawback identified by Simons *et al.* (2004).

The major limitation with the ligand-bead approach is that the assay is not a functional signalling assay and can only be used to determine binding at the receptor. Reconstituting the ligand captured receptor with the G-protein subunit combination and detecting the signalling may be possible with this system.

	Simons *et al*. (2004)	This study
Receptor	β_2AR	AT_1
Origin of receptor	Recombinant (mammalian)	Turkey liver plasma membranes
Is receptor solubilised?	Yes	No
Are there G-proteins?	No	Endogenous
Molecular enhancement?	GFP fusion	No
Immobilisation of ligand	DHA beads conjugated on cross linked dextran/agarose beads.	Ang II-biotin immobilised onto streptavidin coated magnetic Spherotech beads.

Table 4.2: **Comparison of ligand-bead capture (Simons *et al.*, 2004).**

4.4.2. G-Protein- receptor capture:

In this study, the [^{35}S]GTPγS Ni(NTA) bead assay was used successfully to demonstrate the functional assembly of a surface associated GPCR transductosome i.e. a cell-free combination of receptor and associated G-proteins capable of exhibiting ligand-induced signalling activity whilst the complex was attached to a surface. This assay required modification and optimisation from the traditional suspension based approach such as determining the appropriate methodology to allow the separation of bound and free radioactivity in the form of [^{35}S]GTPγS. Although GF/C filters are generally used to capture membranes, the present study found that they specifically captured soluble Gα subunits, whether obtained from *Sf*9 cells or obtained from bacteria and not myristoylated (Leifert *et al.* (b) 2005). These properties are useful for suspension assays but result in high background for bead based assays. Thus, the usefulness of Whatman #1 filter paper and then later Durapore® membranes for capturing Ni(NTA) bead associated G$\alpha_{i1(6xHIS)}$ subunits (with attached [^{35}S]GTPγS) released following activation of signalling was

established). These filters showed low levels of non-specific binding. However, they were able to provide a physical barrier to capture the larger Ni(NTA) agarose beads. The validity of the adapted assay system was confirmed by leaving out components such as the G-protein subunits, membranes containing $\alpha_{2A}AR$ and the Ni(NTA beads). When these components were not included signalling was not apparent. Furthermore, $G\alpha_{i1(6xHIS)}$ subunit attachment was shown to be selective to the Ni(NTA) beads as competition with NiCl, imidazole and nickel depleted beads all failed to show captured receptor activated $[^{35}S]GTP\gamma S$ binding.

To further validate and optimise the assay the following conditions were considered: the number of washes required to remove non-specific binding; the amount (volume) of beads for maximal attachment; the assay time and the amount of G-protein subunits. Sensitivity of the reconstituted signalling complex to the agonist UK-14304 was similar in the presence and absence of Ni(NTA) beads using the $[^{35}S]GTP\gamma S$ binding assay. Others have reported that when cell homogenates (without prior purification) containing the GPCR neurokinin-1 receptor tethered in a membrane were used ligand binding activity was fully preserved (Martinez *et al.*, 2003). Moreover, the effect of various adrenergic antagonists on agonist stimulated $[^{35}S]GTP\gamma S$ binding activity of reconstituted $\alpha_{2A}AR$ membrane preparations in the presence of Ni(NTA) beads was examined and the rank order potency of these compounds was maintained. The membrane-associated receptor, from cloned sources, was shown to elicit a functional signalling response measured by the $[^{35}S]GTP\gamma S$ binding assay in the presence of Ni(NTA) beads and the data suggests that the beads were selectively capturing activated $G\alpha_{i1(6xHIS)}$ subunits.

Recently, a technology has been developed whereby the reconstitution of various ligand-receptor-G-protein complexes in detergent on beads can be monitored via flow cytometry (Simons *et al.*, 2003). Their technology is compared to the [^{35}S]GTPγS Ni(NTA) beads assay in **Table 4.3** and the approach adopted by Fang *et al.* (2002).

	Simons *et al.* (2003, 2004) Approach	Fang *et al.* (2002) Approach	This study
Receptor	β$_2$AR and formyl peptide receptor (FPR)	More than 10 receptor-ligand pairs	α$_{2A}$AR
Origin	Recombinant (mammalian)	Purchased commercially	Recombinant (insect)
Is the receptor solubilised?	Yes	No	No
G-proteins present?	Yes- purified reconstituted	Endogenous	Endogenous removed by urea. purified reconstituted
Attachment	Gγ-histidine tag	GAPS-Non-specific attachment to glass via cell membranes containing GPCR	Gα- histidine tag
Detection of activation	Fluorescent ligands/ GFP fusion to receptor (flow cytometry)	Fluorescent ligands and radioactive ligands	Receptor activated [^{35}S]GTPγS binding to Gα$_{i1}$

Table 4.3: Comparison of the technology for surface immobilised GPCR transductosomes (Fang *et al.*, 2002a; Fang *et al.*, 2002c; Fang *et al.*, 2003; Simons *et al.*, 2003; Simons *et al.*, 2004).

The S/B ratio obtained in the present study using the [^{35}S]GTPγS Ni(NTA) assay ranged from 4-6:1 while those of Simons *et al.* (2004) with solubilised receptor reported S/B ratios of around 8:1. In a previous report, Sklar *et al.* (2000) reported very high capture of receptors from crude membrane preparations. Using a fluorescent peptide ligand to the receptor they were able to achieve specific binding displaying an optimal S/B ratio of 30:1 (Sklar *et al.*, 2000). The lack of availability of florescent ligands for all GPCRs may limit the universality of this technology (Fang *et al.*, 2002c; Simons *et al.*, 2003). Additionally, these receptors were captured

directly via a histidine tagged receptor containing endogenous G-protein subunits thereby limiting the control and specificity of G-protein signalling (Sklar *et al.*, 2000). The intrinsic activity of an agonist is influenced by G-protein coupling (Fay *et al.*, 1991; Jasper *et al.*, 1998). Thus, premeditated assembly of a specific signalling complex is probably more commercially attractive than simply inferring GPCRs remain coupled to G-proteins constructed in the assay format. Furthermore, Simons and co-workers (2004) reported that cognate agonist, receptor and G-protein were all necessary to obtain specific assembly as the ternary complex. These researchers developed a receptor fusion protein (i.e. receptor + a GFP tag) that obviated the use of fluoresceinated ligand for the receptors studied (Simons *et al.*, 2003). GFP carries a chromophoric group which emits efficiently in the green spectral region only if the protein is structurally intact (Schmid *et al.*, 1997). Although not identified with the GPCRs used in the proof-of-concept experiments, the addition of this large protein may cause some steric hindrance problems with other GPCR subtypes, and similarly, it is likely that the more molecular enhancements that are made the less physiologically relevant the assay will become. In this regard, it is of some note that the fusion of GFP to a G-protein subunit did not hinder GTP binding in an agonist dependent fashion but did inhibit downstream functional activation of adenylate cyclase (Leaney *et al.*, 2002).

4.4.2.1.   Signalling complex assembly

Cellular modulators of GPCR signal transduction would be expected to be less of a complication when receptor induced signalling activity is performed in a cell-free assay format. In terms of the likely attachment of the membrane-associated GPCR and associated G-proteins used in this study, a number of questions need to be addressed for example; does attachment to the nickel surface occur before or after GPCR-activated [^{35}S]GTPγS binding? Attempts to show independently that the

α_{2A}AR was binding to the $G\alpha_{i1(6xHIS)}$–Ni(NTA) beads using the radiolabelled α_{2A}AR antagonist [^{3}H]MK-912 were unsuccessful due to high background binding which could not be avoided (data not shown). In the presence of GTPγS, the heterotrimer has been shown to dissociate from the isoproterenol-β_2AR complex in the presence of detergent (Simons *et al.*, 2004). In another study, the separation of liganded-FPR from G-protein was induced by GDP in the soluble system (Waller *et al.*, 2004). In addition, the "filter stack" method distinctively demonstrated that there is GPCR – induced subunit dissociation (Leifert *et al.* (b) 2005). In this study a "reconstitution" mix was added to beads and receptor activated [^{35}S]GTPγS binding was shown. Interestingly, if beads were coated with only G-proteins and then the liganded GPCR was added functional signalling was not shown (Leifert *et al.*, (b) 2005). This may indicate that the G-proteins bind to the bead after dissociation from the receptor. Furthermore, Simons *et al.* (2004) showed that the β_1AR-GFP did not bind to the $G\alpha_s$- beads spontaneously or in the presence of antagonist, binding only occurred in the presence of the agonist isoproterenol. In terms of kinetics, in a soluble system, the separation of liganded FPR from the G-protein is faster than the separation of Gα from the obligatory dimer (Simons *et al.*, 2003). However, the affinity of the agonist (partial or full) must also be considered when trying to understand the kinetics of transductosome assembly (Simons *et al.*, 2004).

4.4.3.   Surface attachment:

Immobilisation of proteins on a surface may restrict the movement and influence the dynamics of the interactions (Gales *et al.*, 2005). High-quality array fabrication ensures that protein immobilisation is at the optimal density to the surface for efficient binding while minimising non-specific binding (Venkatasubbarao, 2004). For attachment using a histidine-tagging nickel affinity protocol, the position of the

histidine tag on the protein may potentially influence the extent of protein-bead and protein-protein interactions (Simons *et al.*, 2003). In terms of immobilised GPCRs, Simons *et al.* (2003) reported that the solubilised carboxyl histidine tagged FPR GPCR consistently bound more nickel beads than the same receptor when tagged at the amino terminus. Also with GPCR immobilisation, a C-terminal biotinylation tag has been used effectively to attach cell homogenates containing the neurokinin-1 receptor (Martinez *et al.*, 2003). The alternative immobilisation with the G-protein subunits used in this study, gives a uniform orientation and is a relatively minor modification to the signalling system. An immobilisation approach that incorporated these factors as well as enabled both the agonist binding site and the G-protein activating site to be accessible (i.e. not immobilised) (Karlsson and Lofas, 2002) would be particularly favourable.

4.4.3.1.   Nickel/histidine affinity

There are conflicting reports regarding the affinity of nickel/histidine binding in the literature. Nieba *et al.* (1997) reported a K_d value of 7×10^{-7} M for attaching histidine tagged proteins (isolated from *E.coli*) to Ni(NTA) surfaces for SPR measurements. Meanwhile others reported a range of K_d values from 2×10^{-4} M to 6×10^{-6} M and this value depended on the buffer salt concentration (50-1000 mM) and pH (6-9) (Gershon and Khilko, 1995). However, Kosaza and Gilman (1995) stated a much higher affinity for the nickel/histidine interaction with a value of 10^{-13} M. In the present study, when 20 to 50 nM $G\alpha_{i1(6xHIS)}$ was used in the receptor signalling assay in the presence of Ni(NTA) beads almost all the [^{35}S]GTPγS –bound $G\alpha_{i1(6xHIS)}$ protein was captured therefore less than 0.2 nM [^{35}S]GTPγS was bound. This would equate to a K_d value likely to be less than 0.2×10^{-9} M. Furthermore if the nickel histidine affinity is buffer dependent as inferred by (Gershon and Khilko, 1995) then using a similar buffer to the one used by Kosaza and Gilman (1995) as was used in

this study would imply similar affinity values. Therefore, based on the data (Kozasa and Gilman, 1995) using 5-10 μL of Ni(NTA) beads should be sufficient to capture all the activated $[^{35}S]GTP\gamma S\text{-}G\alpha_{i1(6xHIS)}$ subunits and this was confirmed by the data shown within the results section.

4.4.4. The lipid environment

Although several reports indicate that some solubilised GPCRs retain their binding activity for both ligands and G-proteins (Kobilka, 1995; Sklar *et al.*, 2000), maintaining a lipid environment is absolutely necessary when studying the interactions of integral membrane proteins such as GPCRs and for retaining receptor integration (Fang *et al.*, 2002a). It is important to recognise that the methodologies used by Fang *et al.* (compared to this study in **Table 4.3**) or Sklar and colleagues earlier approaches (Sklar *et al.*, 2000) are not functional assays but rather receptor-ligand binding assays. Thus they can not differentiate between agonist and antagonist, a useful drug discovery advantage. Using the G-protein beads in conjunction with ligand-beads, Waller *et al.* (2004) proposed that fluorescent 'sensing' would allow for the discrimination of ligands on the basis on their antagonistic or agonistic properties. However, this two-tiered approach appears to be unnecessarily excessive. In addition, functional assays using the radionucleotide $[^{35}S]GTP\gamma S$ have shown less non-specific binding compared to ligand binding assays with receptor preparations derived from tissues from natural sources (eg brain homogenates) as used by Shiba *et al.* (2002). In contrast, the lipid environment hinders the freezing potential (and thus storage capacity) and so may hinder the technology transfer applicability. Reconstituted transductosomes using membrane homogenates did not withstand free/thaw cycles whereas solubilised receptors did (Waller *et al.*, 2003). The importance of having the receptor in the endogenous state (or as close as possible to this state), in terms of the lipid environment and to the

phenotypic diversity of the receptor (that is the receptor plus facilitating proteins) for the development of GPCR screening platforms, has been emphasised recently (Gilchrist, 2004; Nature Reviews Drug Discovery GPCR Questionnaire Participants., 2004).

Conclusion

In conclusion, a customizable assay, suitable for compound profiling specifically targeting the α_{2A}AR which could be surface attached, was described. In addition the M$_2$ receptor reconstituted with G$\alpha_{i1(6xHIS)}$ and $\beta_1\gamma_2$ was also shown to function when associated with Ni(NTA) beads (Leifert *et al.*, unpublished data). Using this relatively straight forward attachment method was preferred because additional molecular enhancement of the proteins was not required for proof-of-concept as these subunits were histidine tagged for purification purposes. A radioactive assay ([^{35}S]GTPγS binding) was used to optimise the approach as proof-of-concept. The pharmacological profile of the captured preassembled signalling complex was comparable to those profiles reported for the same receptor using membrane binding data. The level of signalling in the absence of Ni(NTA) beads was comparable to that obtained in the presence of beads. This suggests that this method of surface attachment and assay construct is relatively non-invasive to the signalling complex proving useful as a design prototype for high throughput drug discovery applications.

Chapter 5.
Final Discussion and Some Applications and Opportunities

"Healing is a matter of time, but it is sometimes also a matter of opportunity."

Hippocrates

Cell-free screening would be one way of obtaining high information content for functional outcomes regarding GPCR activity that avoided the necessity of maintaining cell cultures or developing of reporter gene constructs. Indeed, there seems to be a proliferating number of opportunities for such a cell-free GPCR assay platform and such technology may accelerate the comprehensive understanding of GPCR diversity. M Garcia Guzman (Vertex Pharmaceuticals, USA) expressed the following; "Technology innovation is a major driver of competitive advantage in the field of GPCR research" (Gilchrist, 2004). This thesis focused on the strategic development towards a cell-free functional GPCR screening platform using the $\alpha_{2A}AR$ reconstituted signalling complex (transductosome) as the model system. In total, three receptors were investigated for the reasons discussed below. In this final discussion some potential applications and future directions will also be explored.

5.1.1. Summary of thesis

The β_1AR and the AT_1 GPCRs were chosen as the initial targets for this study because of their patho-physiological relevance, specifically in cardiovascular health as well as their reported biochemical characterisation, with both receptor types being key targets for the pharmaceutical industry (Klabunde and Hessler, 2002). As the study progressed it became apparent that the β_1AR could not be easily utilised for proof-of-concept studies for two reasons. Firstly, this receptor signals endogenously through the $G\alpha_s$ signalling system and despite many attempts a palmitoylated $G\alpha_s$ subunit for reconstitution purposes could not be produced. Furthermore, it was not possible to prepare ligand-conjugated streptavidin Spherotech beads to capture the β_1AR because of certain intrinsic difficulties preparing a catecholamine/biotin linker. The problems encountered with the AT_{1A} receptor were related to the restoration of

high affinity binding and cell-free reconstitution, and these problems were discussed in Chapter 2 and Chapter 3, respectively.

In contrast the α_{2A}AR, reconstituted with $G\alpha_{i1(6xHIS)}$ and $\beta_1\gamma_2$ enabled more definitive studies to be carried out with regard to the development of potential, cell-free signalling formats. The baculovirus expression system in *Sf*9 cells was successfully used to express both the receptor and G-proteins as described in Chapter 2. These proteins were functionally reconstituted (Chapter 3) as shown by the agonist induced binding of [^{35}S]GTPγS. This functional assay was optimised for the α_{2A}AR signalling complex and techniques used to increase the fold stimulation over basal binding were investigated. The fold stimulation routinely obtained using this reconstituted signalling system ranged from 4-8 fold with cognate agonist. Later, the single tube [^{35}S]GTPγS binding assay was modified to incorporate a 96-well format which enabled better reproduction of data and allowed for a decrease in assay volume and an accompanying reduction in the usage of expressed protein(s). The use of histidine tagged G-protein subunits ($G\alpha_{i1(6xHIS)}$ and $\beta_1\gamma_{2(6xHIS)}$) was also monitored using the [^{35}S]GTPγS binding assay (Chapter 3). These modified G-protein subunits seemed to work as effectively as unmodified G-proteins in this assay and thus it was likely that the histidine motif did not hinder the functional signalling of the complex. The importance of this result became more apparent in Chapter 4 when the histidine tag was used to immobilise the $G\alpha_{i1(6xHIS)}$ subunit to nickel agarose beads used to capture the α_{2A}AR upon agonist activation. As discussed, Simons *et al.* (2004) used G-protein beads (attached via a histidine tag on the γ subunit) to capture the β_1AR in a detergent solubilised system. Following assay modification, the α_{2A}AR activated [^{35}S]GTPγS binding was shown to be unhindered in the presence of nickel beads. Furthermore, the activated $G\alpha_{i1(6xHIS)}$ associated [^{35}S]GTPγS was specifically captured

in the presence of nickel beads and the rank order potency of α_{2A}AR antagonists as well as the sensitivity of the agonist UK-14304 was maintained in this captured functional signalling system. Establishing and validating this protocol could enable advances to be made in the development of appropriate cell-free, reconstituted assay platforms with respect to GPCR function. Although ligand screening and drug discovery are the most obvious applications of this technology, it is important to recognise and consider future growth areas and market needs.

5.1.2.   Applications: drug screening

Developing drugs is a difficult endeavour plagued by technical pitfalls, clinical failure and competitor hurdles[38] with drug discovery paradigms being rapidly transformed by robotics, high throughput systems, automated assays, and advanced software systems[39]. GPCRs occupy a unique position as the single largest drug class currently on the market (Nambi and Aiyar, 2003). Despite the historical GPCR drug discovery successes, current efforts such as the reliance on higher throughput heterologous, cell-based assays are proving to be less productive. In addition, the wealth of new information regarding novel GPCR behaviours (such as receptor dimerisation) or additional GPCR drug binding sites such as allosteric binding sites, may be troubling industry decision makers (Nature Reviews Drug Discovery GPCR Questionnaire Participants., 2004[40]). Drug hunting practices need to tailor for the richness of the functional effects without being confined within the limits of conventionally used cell-based approaches (Nature Reviews Drug Discovery GPCR Questionnaire Participants., 2004). Innovative technologies will be used to guide the drug discovery process with more confidence and product knowledge.

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[38] http://www.bccresearch.com/biotech/DDR00.html
[39] http://www.bccresearch.com/biotech/DDR00.html-2001
[40] This reference was a whole feature in Nature and represents the contemporary view of 20 experts in the GPCR field. The participants were asked to discuss questions regarding emerging technologies for GPCR research, drug screening and where they anticipate the next wave of opportunity will fall.

The market for technologies utilising membranes for biopharmaceutical discovery, development and commercial production, was estimated at $740 million dollars in 2004, and is expected to rise at an average annual growth rate (AAGR) of 10.7% to $1.23 billion in 2009[41]. In addition, the market for bioanalytical instruments and services for selected products in the drug discovery markets was estimated at approximately $7.7 billion in 2000[42].

Potential drug discovery applications of a universal cell-free functional GPCR assay could be:

1) Compound profiling and selectivity of ligands identified from GPCR-focused libraries and natural sources using known or orphan GPCRs.

2) Screening compounds for allosteric modulation.

3) Screening of compounds that directly act on G-protein signalling.

5.1.2.1.   Compound identification

Advances in genomics, proteomics and bioinformatics will undoubtedly assist in identifying further GPCRs within the human and other genomes and elucidating the mechanisms of signal transduction for potential sites of intervention. There is considerable interest in not only the structure-activity relationships between GPCRs and associated ligands, but also in the methods used to identify therapeutic leads (Sadler, Kristen and Tam, James P., 2004). Due to the limited availability of structural data on GPCRs, the design of ligands for this family still relies heavily on the ligand–based design techniques (Klabunde and Hessler, 2002). Companies have invested heavily in developing GPCR-focused libraries and using these libraries with a higher rate of hits when compared to screening a compound library with random diversity (Gilchrist, 2004). In addition, leads and compound libraries can be generated from natural sources (microbial and plant secondary metabolites) and from

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[41] http://www.bccresearch.com/membrane/C246.html November 2004
[42] (http://www.bccresearch.com/biotech/DDR00.html- 2001

natural products (Wildman, 2003). For example, various molecular constituents of St Johns Wort (natures own Prozac) were systemically screened against a large number of cloned receptors including GPCRs (Butterweck *et al.*, 2002).

5.1.2.2.   Compound profiling and selectivity screening

Saturation binding assays and competition binding assays are conventionally used bioassays for compound screening (Fang *et al.*, 2003). The ability to conduct functional cell-free GPCR assays may extend this profiling to include combined multi-target screening and selectivity profiling. The selectivity of a potential drug compound for a targeted GPCR over other GPCRs is an extremely important factor in drug development (Fang *et al.*, 2003). For example, a particular dopamine receptor antagonist was reported to be a functional antagonist to the CXCR1 chemokine receptor while not being able to displace the radio ligand in a ligand binding assay likely due to the phenomenon of allosteric modulation (discussed below) (Moore and Rees, 2001). Screening compounds against GPCRs from different families and within a family were considered by Fang and colleagues (2003) in the context of a cell-free immobilised GPCR ligand screening platform. In addition, screens could be used to optimise the current drug therapies to exploit the complex pharmacological properties of drugs acting on GPCRs for therapeutic advantage (Brink *et al.*, 2004). Exploring the mechanism by which pharmaceuticals alter signalling, beyond the level of the receptor, to generate a therapeutic effect would be extremely advantageous. Screening protocols investigating the responsiveness of compounds to alter signalling downstream of the receptor targeting asthma and other allergic inflammatory diseases were discussed by Johnson *et al.* (2002).

Functional cell-free tests that allow the discrimination of partial agonists and full agonists are physiologically significant (Simons *et al.*, 2004). However, intrinsic

efficacies of agonists have been difficult to measure (Kenakin, 2002). Jasper *et al.* (1998) reported that the effects of partial agonism using a recombinant $\alpha_{2A}AR$ system needed to be confirmed by co-incubation with full agonists followed by measuring the decrease in agonist induced [^{35}S]GTPγS binding. In addition, Wade *et al.* (1999) reported that although the partial $\alpha_{2A}AR$ agonist *p*-iodoclonidine did not activate the receptor to a great extent, it did result in similar relative stimulation of $G\alpha_{i1}$ (and $G\alpha_s$) in comparison to the full agonist UK-14304. Therefore, G-protein directed, functional signalling assays may also allow for the identification of compounds that would otherwise be classified only with respect to ligand binding abilities.

Additionally, there is the possibility to conduct cell-free functional assays as an adjunct to current ligand screening techniques for de-orphaning receptors. Greater than 100 GPCRs need to be deorphaned (i.e. identification of the cognate ligand) (Gilchrist, 2004). Thus these GPCRs continue to represent a vast untapped market opportunity (Howard *et al.*, 2001). Constitutively active GPCRs that stimulate cellular signalling pathways in the absence of ligand attachment have been used to identify orphan GPCRs (Chalmers and Behan, 2002). This type of screening allows the detection of molecules that block ligand-independent activity (inverse agonists) and such screening could easily be adapted to a cell-free assay format as proposed by this study with advantages of increased information content.

5.1.2.3.   Allosteric compound identification

Recently there has been a marked trend towards the use of functional assays to identify allosteric modulators (or off-target interactions) (Moore and Rees, 2001). These allosteric binding sites represent a potential 'druggable' market and promise to yield therapeutics with potentially greater receptor subtype selectivity, increased

efficacy and an improved safety profile (Christopoulos *et al.*, 2004). Allosteric compounds do not act on the orthosteric site and are thus free to modulate the response independent of any effect on antagonist/agonist affinity. In addition, Hall and Lefkowitz (2002) suggested the disruption of the interaction of GPCRs with scaffold proteins may represent a potential therapeutic target. Binding assays alone cannot be used to detect such modulators as they typically have K_d values which are significantly less than agonist concentration. Thus allosteric modulators are often missed (because receptors are unoccupied) (Litschig *et al.*, 1999). Although, validating putative allosteric modulators using binding assays is important for quantifying the modulators actions, cell-based approaches are far removed from the site of action (Christopoulos *et al.*, 2004). Therefore, a functional cell-free approach may offer significant advantages in this regard.

5.1.2.4. Other targets

While drug discovery efforts have primarily focused on specific receptor ligands for GPCRs, newer drug targets include the G-protein (Holler *et al.*, 1999). Additionally, signalling events such as heterotrimer dissociation may prove to be important targets in the future (Chung and Kermode, 2005). Certain diseases such as bipolar disorder (Manji and Lenox, 2000) are associated with dysfunction in Gα subunits due to alterations introduced by endogenous agents and to somatic or heritable mutations (Landry and Gies, 2002). The heterogeneity of G-proteins may offer great opportunities to develop selective drugs against these proteins and recently the action of lead compounds such as suramin have been studied for the development of discerning G-protein selective drugs (Chung and Kermode, 2005). Short peptides, both naturally occurring and synthetically derived from segments of GPCRs, G-proteins and effectors, have been used extensively to map crucial interaction sites which may antagonise or activate G-proteins (Ja and Roberts, 2005, Gilchrist *et al.*

2002). In addition, large combinatorial peptide libraries are being used to develop G-protein signalling modulators targeting intracellular components (Neubig and Siderovski, 2002; Ja and Roberts, 2005). Some of the known natural and synthetic compounds that interact directly with G-proteins are shown in **Table 5.1**. The part(s) of the activation/deactivation cycle in which these compounds elicit their effect is demonstrated. As the site of interaction of potential lead compounds that modulate at the level of G-protein signalling would be intracellular, it follows that cell-free assays offer the only real way to initially detect such novel compounds.

Activation/stimulation of G-protein
Mastoparan (selectively activates $G\alpha_i$ and $G\alpha_o$ but not $G\alpha_s$ or $G\alpha_t$)
Melittin ($G\alpha_i$, $G\alpha_o$ and $G\alpha_{11}$)
Non-peptide spermine ($G\alpha_o$)
Benzalkonium chloride ($G\alpha_s$)
GEF (guanosine exchange factors; GPCRs are the classical G-protein activators)
Activator of G-protein signalling (AGS)
AlF_4^-
Deactivation/inhibition of G-protein
Melittin ($G\alpha_s$)
Non-peptide spermine ($G\alpha_i$)
Benzalkonium chloride (Mastoparan induced activation of $G\alpha_i$)
GAPs (RGS) by enhancement of hydrolysis
Guanosine dissociation inhibitors (GDIs) which inhibit GDP dissociation

Table 5.1 Some compounds that activate and deactivate G-proteins (De Vries *et al.*, 2000; Landry & Gies, 2002).

5.1.3. Other applications of cell-free GPCR technologies

The design, chemistries and screening parameters of this type of functional cell-free GPCR technology should be applicable to most GPCRs creating opportunities for exploitation in diagnostics, research tools, sensory detection, environmental screening as well as those described for drug screening above. Some of the potential applications for this technology are discussed below.

Personalised medicine (i.e. matching the right drug to the patient) has been rated one of the hottest[43] scientific areas in healthcare biotechnology by key investors (Whelan, 2005). As such technologies that relate to personalised medicine will continue to attract investment money. There is a need to move towards proteomics for a better understanding of cellular events, particularly abnormalities in relation to biomarkers for disease states and cell-signalling dysfunction. In order to improve understanding of normal and disease processes, diagnostic technologies must evolve into automating approaches to analyse protein interactions at the point-of-care. The search for disease specific proteins is a major frontier of biomedical research as many proteins may have medical, diagnostic and commercial potential (Cahill, 2001). As a diagnostics screening technology, assay sensitivity is a major limitation and will need to be increased compared to established diagnostic tests that are conventionally utilised (Westerman *et al.* 2002).

The generation of highly specific research tools as well as identifying new compounds with specific research properties are another potential application for these cell-free immobilised GPCR technologies (Nature Reviews Drug Discovery GPCR Questionnaire Participants., 2004). Emerging information is expected to help elucidate the most basic mechanism by which GPCRs exert their numerous physiological roles, in addition to determining why the perturbation of their function results in many pathological conditions (Marinissen and Gutkind, 2001). Using a surface attached cell-free system, subunit dissociation has been investigated in more detail (Leifert *et al*., (b) 2005). This investigation represented a paramount contribution to the understanding of the events of GPCR activation. Highly specific

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[43] From the article "where's the smart money going in biotech?" key analysts and investors were asked to pick out the science that they think will pay off in 2005 i.e. a hot area of science (Whelan, 2005).

research tools that monitor effects proximal to G-protein signalling events could be used to delineate the physiological and pathological role of GPCRs.

GPCRs are fundamental to the process of sensory perception and it is likely that biomimetic approaches utilising such GPCRs will underpin future biosensor technologies. Sensory GPCRs that detect light (Bieri *et al.*, 1999) and taste (Nofre 2001) are being used to better understand GPCR function. Elucidating the relationship between sensory GPCRs and brain functioning is likely to enrich the current understanding of aspects of sensory perception (Nature Reviews Drug Discovery GPCR Questionnaire Participants., 2004).

A receptor-biosensor may have applications with environmental monitoring and screening contaminated surface water. A large group of environmental pollutants are able to mimic or antagonise the effects of endogenous hormones (Rodriguez-Mozaz *et al.*, 2004) and due to the expanding number of new pollutants causing alarming health and environmental consequences, significant control is required (Rodriguez-Mozaz *et al.*, 2004). GPCR receptor biosensors may well have potential application in this area.

5.1.4. Future directions

There were several opportunities identified to improve and extend the technology presented here in this thesis; two of which are presented below and some others are included in the Appendix (page 248).

5.1.4.1. Molecular enhancements

Molecular biology is a tool which will enable every facet of designing and investigating cell-free assays approaches to be improved. Structural enhancements to

GPCRs/ G-protein subunits or effectors will be imperative in the design of novel cell-free assay technologies. Some examples of potential molecular enhancements may include a poly-histidine tag to enhance surface affinity (this is currently being investigated at CSIRO and is based on work published by (Lata and Piehler, 2005; Ratnala *et al.*, 2004)), molecular protein fusions (Milligan *et al.*, 2004) or designing chimeric (Milligan and Rees, 1999) and/or promiscuous G-protein subunits (Hazari *et al.*, 2004; Milligan *et al.*, 1996). This type of protein engineering was reviewed in more detail (Leifert *et al.*, (a) 2005).

5.1.4.2.   High throughput detection

Monitoring the activation of GPCRs is a fundamentally important consideration in increasing the high throughput potential of the assay and ultimate fabrication of GPCR biosensor. Although radioactive assays are a tried and true methodology to measure functional GPCR activity, associated problems and limitations with this method of detection has lead to alternate measurement technologies for GPCR-activated signalling. Such trends are contrary to much of what has been presented in this thesis. However it was important to use an established signalling technique when introducing other variables into the assay such as cell-free functional reconstitution and surface array. Moreover, measuring GPCR activation at the GTP binding level has become quite stagnant in its potential. In contrast, protein/ protein interactions between G-protein subunits and subunits and receptor can be measured by techniques such as fluorescence resonance entry transfer (FRET) and bioluminescence resonance energy transfer (BRET). These techniques are based on the transfer of energy between a donor and an acceptor and the strict dependence on molecular proximity between donor and acceptor molecules for energy transfer (Bouvier, 2002). Such techniques have been used to monitor GPCR activation intracellularly (Janetopoulos and Devreotes, 2002; Janetopoulos *et al.*, 2001; Pfleger and Eidne,

2005). In addition to the plethora of reported and commercially available techniques, novel ways to detect G-protein interactions in a cell-free approach (that may be extended to GPCR activation) using a time resolved fluorescence technique are currently being investigated. Moreover, the potential to linking the assay to an artificially designed secondary messenger system to maintain the phenotype integrity of the GPCR-activation (Gilchrist, 2004) may be another avenue worth pursuing (Allen *et al.*, 2002).

5.1.5. Conclusion

GPCRs are intimately linked to a number of disease conditions and disease processes. The pharmaceutical industry relies heavily on high throughput screening for the identification of potential therapeutics (Dove, 1999). It is important to recognise that the technology in the present study is a model system from which could develop several level approaches to cell-free, surface arrayed assay formats. Several applications and opportunities were showcased to demonstrate where such a cell-free functional assay potentially could be positioned in the marketplace. As well as the need in drug screening, other useful applications for a cell-free GPCR biosensor have been identified. Such a functional assay can offer ligand screening that can be measured at a very early stage in signal generation.

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Chapter 6.
Appendices

"Everything's always ok in the end. If it's not ok, then it's not the end."

Unknown

6.1.   BACULOVIRUS CLONES

Recombinant baculovirus	Donated by
$\beta_1 AR$	Dr Elliot Ross (University of Texas)
$\alpha_{2A} AR$	Professor Rick Neubig (University of Michigan
AT_{1A} receptor	As above
M_2 receptor	As above
$G\alpha_{i1(6xHIS)}$ $G\alpha_{i1}$;	As above
β_1;	As above
γ_2; $\gamma_{2(6xHIS)}$	As Above
$G\alpha_s$	As Above
β_4	Dr James Garrison (University of Virginia)
$G\alpha_s$	Dr Andrejs Krumins/ Professor Alfred Gilman (University of Texas)
$G\alpha_s$ Short	As above
$G\alpha_q$	As above

Table 6.1:   **Donation of recombinant baculovirus used in this project.**

6.2.   RAT HEART MEMBRANES

6.2.1.1.   Membranes containing β_1AR from rat heart

Rat cardiac membranes expressing β_1AR were prepared. The method of membrane preparation closely followed the method by (McMurchie *et al.*, 1987).Ventricular tissue from 3-4 Sprague Dawley rats was removed and placed in ice cold STEM buffer (250 mM sucrose, 20 mM Tris, 1 mM EDTA, 1 mM $MgCl_2$, pH 7.4). The tissue was first roughly chopped and then homogenised using a polytron tissue homogeniser (Kinematica, GmbH, Switzerland) at setting 4 for 3 bursts each of 30 sec. This homogenate was filtered through 4 layers of wide-weave cheese cloth and centrifuged at 500 g using a JA-20 rotor in Beckman J2-21 centrifuge at 4°C for 15 min. The supernatant was saved and the pellet was resuspended in STEM buffer to a final concentration of approximately 7 mg/mL protein and re-centrifuged. The supernatant from this and the first centrifugation were combined and centrifuged at 46,000 x g for 30 min and the pellet resuspended in STEM buffer and the centrifugation was repeated. This time the pellet was resuspended in 20 mM Tris, 2 mM $MgCl_2$, pH 7.4 at a concentration of between 4 and 5 mg protein/mL and aliquoted and snap frozen in liquid N_2. Membranes were stored at -80°C until required. Protein concentration was determined by Lowry (LOWRY *et al.*, 1951) in the range of 8-10 mg/mL per preparation.

6.2.1.2.   Results

The [^{125}I]ICYP binding assay was assessed with natural source of β_1AR found in rat ventricles. Rat ventricular membranes were prepared in the presence of protease inhibitors as per the method outlined by (McMurchie *et al.*, 1987). Freshly thawed membranes were examined for β_1AR binding using the radioligand [^{125}I]ICYP and this binding was compared to data in **Figure 2.1**. Total, non-specific (10 μM

Propranolol) and specific $[^{125}I]ICYP$ binding increased over time as can be seen in

Figure 6.1. In contrast to binding observed to turkey erythrocyte membranes, non-

specific binding was high and ranged from 40-60%. The total binding was also

considerably lower than the total binding obtained using turkey membranes

suggesting that the latter membranes are an enriched natural source of β_1AR as

previously reported (Shorr *et al.*, 1982). Thus further enrichment of this receptor

subtype from the rat heart tissue would be necessary to utilise this source of β_1AR.

Receptor damage due to the isolation procedure is unlikely as this method of

isolation has been used previously. Albeit exhibiting β_1AR ligand binding, using rat

ventricular membranes as a source of receptor was not considered further.
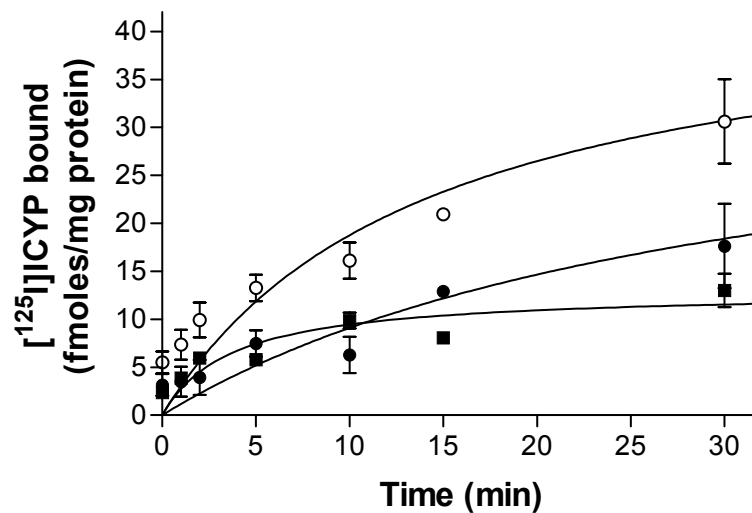
Figure 6.1: $[^{125}I]ICYP$ **binding to rat ventricular membranes over time.**
Rat ventricular membranes (0.5 mg/mL protein per assay) were diluted in TEAM buffer then combined with 300 pM $[^{125}I]ICYP$. The assays were incubated at 37^oC for the specified time. Reactions were terminated by filtering the contents of the entire assay over a GF/C filter and washed with 3 x 4 mL TMN buffer. Non-specific binding (■) was determined in the presence of 10 μM Propranolol. Total binding is represented by (o) and specific binding is represented by (●). Specific binding is defined as total binding minus non-specific binding and data represents (n = 3, mean ± SEM).

6.3. BACULOVIRUS EXPRESSION SYSTEM

The growth conditions of the *Sf*9 insect cells were investigated. To prevent and limit bacterial/fungal contamination, suspension cultures were kept in autoclaved Schott bottles. The use of these bottles also aided in handling of the cells, negating the use of more complicated dilution steps. Optimal cell growth was achieved at 28°C (non-humidified) with agitation at 140 oscillations/min in an orbital shaker. A more consistent atmospheric exposure for the cells was obtained by maximising cell culture volume to one quater that of the total bottle. This was important because oxygen has been shown to be a crucial rate-limiting step as consumption increases drastically after viral infection (Massotte, 2003). Adequate oxygenation was achieved by loosening the cap. Initially stocks were kept at -80°C, however, after 3 months these cells could not be rejuvenated thus it was imperative that stocks were maintained in liquid nitrogen. Uninfected insect cells had a doubling time of approximately 24 hours in the logarithmic phase and then growth was slowed after the culture reached a density of 4×10^6 cells/ml (see **Figure 6.2** filled black circles).

Recombinant baculovirus expressing the $\beta_1 AR$ was donated from Dr Elliot Ross (Southwestern Medical Centre, University of Texas). This virus was amplified as described in the materials and methods section and then used to infect cells in the log phase of growth at a MOI of 2 (see **Figure 6.2** un-filled circles). Cell viability was decreased from 90% to less than 50% indicating successful infection and normal cell growth was inhibited.
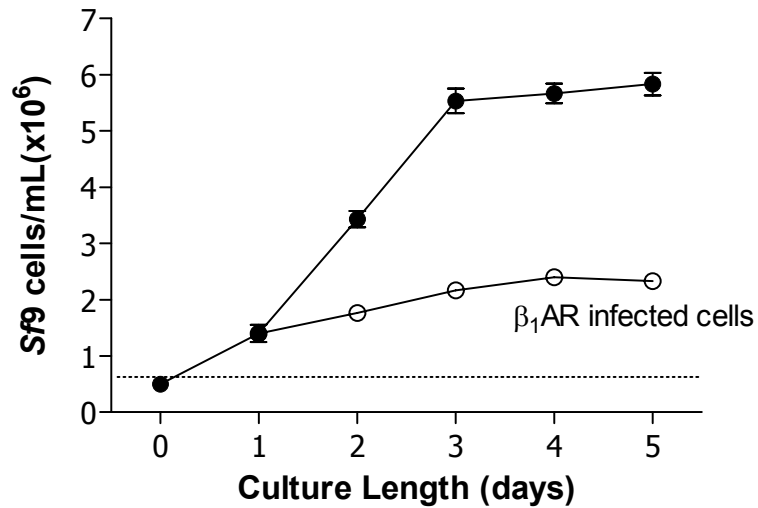
Figure 6.2: *Sf*9 **cell growth and baculovirus infection.**
Spodoptera frugiperda (*Sf*9) insect cells were grown in serum free SF900 media at 28°C with agitation at 140 oscillations/min in an orbital shaker. Cell counts were taken on a daily basis and standard haemocytometer techniques were used to determine cell growth. Trypan blue staining was performed to determine cell viability. Cell viability was greater than 90% for uninfected cells represented by filled black circles (●) and less than 50% for cells that were infected with the baculovirus expressing β1 adrenergic receptor at a multiplicity of infection (MOI) of 2 represented by (○). (n=3, mean ± SEM)

6.4. UREA TREATMENT OF MEMBRANES

6.4.1.1. Urea treatment of membranes expressing $\alpha_{2A}AR$

Binding of the radioligand [^{3}H]MK-912 to urea and non urea treated membranes was compared to ensure that urea treatment did not interfere with ligand binding (**Figure 6.3**). In all cases the treatment of *Sf*9 membranes with high concentrations of the chaotropic agent urea, did not inhibit antagonist binding compared to binding with non-urea treated membranes expressing the β_1AR (Chapter 2 **Figure 2.7**). Furthermore, urea treatment significantly increased [^{3}H]MK-912 binding at most protein concentrations tested and this may indicate that urea treatment enables a semi-purification of the membrane thus removing non-specific binding sites
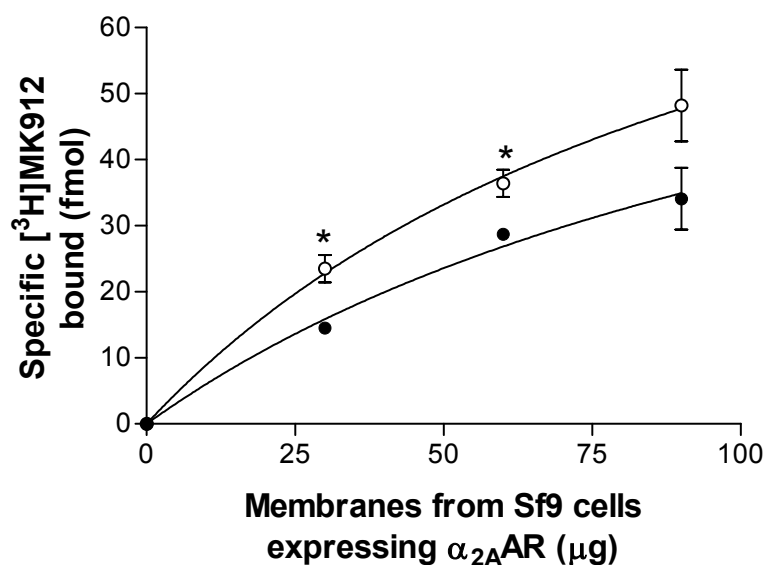

Figure 6.3: The effect of urea pre-treatment on the specific binding of [^{3}H]MK-912 to *Sf*9 cell membranes expressing α_{2A} adrenergic receptors.
Assays were incubated as described in the materials and methods section ensuring the following; urea treated (represented by unfilled circles (o)) and non urea treated membranes (represented by filled circles ($\bullet$)) expressing $\alpha_{2A}AR$ were combined with increasing concentration of the $\alpha_{2A}AR$ antagonist [^{3}H]MK-912 and incubated for 90 min at 30°C. Non-specific binding was determined by adding 100 μM Yohimbine to the incubation mix. Each data point represents n = 3 samples, mean $\pm$ SEM. Where error bars are not visible they are contained within the data point.*$p<0.05$.

6.4.1.2. Urea treatment of CHO cell membranes expressing AT_{1A}

Specific [^{125}I]Ang II binding to both untreated and urea-treated membranes increased linearly with an increase in the amount of membrane protein (see **Figure 6.4**); a

result similar to that described Chapter 2 **Figure 2.11** for untreated membranes. Urea

treated membranes exhibited a 4 fold increase in [^{125}I]Ang II binding compared to

non-urea treated membranes. Lim and Neubig (2001) reported that the urea treatment

of mammalian membranes removed around 75% of proteins from the preparation
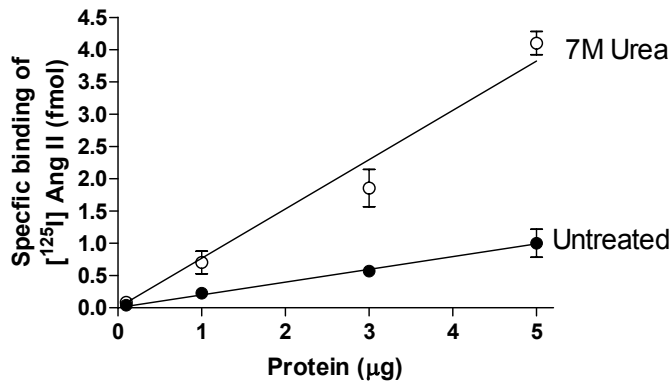

Figure 6.4: The effect of urea treatment on the specific [^{125}I]Ang II binding to increasing amounts of CHO cell membranes expressing the AT$_{1A}$ receptor.
CHO cells from passage 35-39 stably expressing the AT$_{1A}$ were harvested and membranes were prepared with or without treatment with 7M urea. CHO cell membranes (amounts as indicated) stably expressing AT$_{1A}$ were incubated in binding buffer (plus protease inhibitors and 0.1% (w/v) BSA) with 1 nM [^{125}I]Ang II for 60 min at 26°C. Non-specific binding was determined in the presence of 10 μM Losartan. Specific binding to urea-treated membranes is represented by open circles (o) and specific binding to untreated membranes is represented by filled circles (●). Specific [^{125}I]Ang II binding is shown and data represents n = 2, error bars represent the range of duplicates.

6.5. CHO CELL GROWTH

Mammalian cell culture was established in this laboratory so monitoring was essential in the first instance. CHO cells are a fairly robust cell line and there were not many problems experienced with their growth. If subculturing was performed when the cells were over grown the initiation of cell necrosis was observed. This was prevented by subculturing when cell growth, was sparse (approximately 60-70% confluent). CHO cell growth was observed and preliminary observations showed that cells grew optimally when they were split between 1:20 and 1:30 every 3 days for large T175 flasks (**Figure 6.5**).
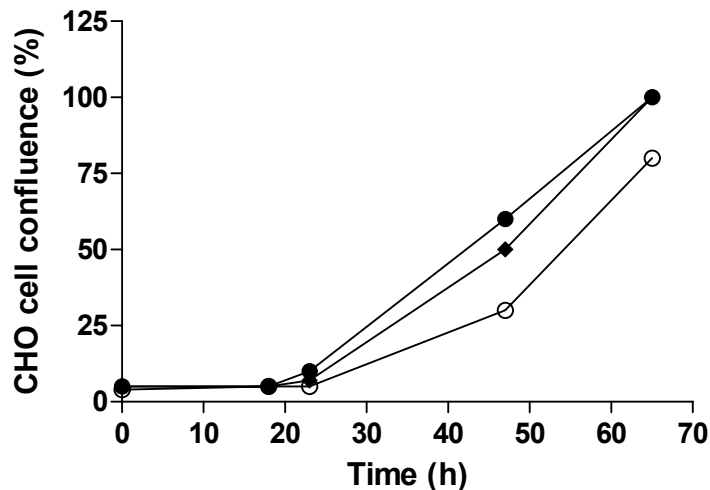

Figure 6.5: **CHO cell expressing the Angiotensin II receptor (AT$_1$) growth to confluence**. CHO cell from passage 30 stably expressing AT$_1$ were grown in complete α-MEM media at 37°C with 5% CO_2. Three different splits were performed: a 1:20 split was performed ($\bullet$), a 1:25 split ($\blacklozenge$) and a 1:30 split (o). Confluence was observed at various time points indicated using an inverted microscope. (n = 2, error bars where visible represent the range of duplicates).

6.6.   THE EFFECT OF $\beta_1\gamma_{2(6xHIS)}$ ON [^{125}I]ANG II BINDING

The effect of the $\beta_1\gamma_2$ dimer on [^{125}I]Ang II binding was examined to elucidate if the observed inhibition of binding is specific to the Gα subunit. Previously, the non histidine tagged dimer was added to non-urea treated membranes in combination with Gα subunit (data not shown) and to the non urea treated membranes alone (data not shown). In all cases, there was a reduction in total [^{125}I]Ang II binding to the CHO cell membranes. With the aim of keeping constituents the same, $\beta_1\gamma_{2(6xHIS)}$ was added to the urea treated system as this subunit was produced concurrently with the dialysed Gα_q (used for the experiments in Chapter 2 **Figure 2.15**). The concentration chosen for this comparison was 150 nM and 1.3 μl volume was required to achieve this concentration (since the histidine tagged subunit was considerably concentrated). Heat denatured protein and buffer E were added as additional controls (**Figure 6.6**).

The addition of $\beta_1\gamma_{2(6xHIS)}$ significantly ($p<0.05$) decreased specific [^{125}I]Ang II binding to urea treated membranes by 18% compared to the specific binding minus added G-proteins. It is likely that the active protein is not responsible for causing this reduction as there was a significant ($p<0.001$) reduction (24%) in the specific binding observed when the heat denatured dimer was added. Further investigation in the contribution of buffer E to the specific binding showed that the addition of 1.3 μl significantly ($p<0.0001$) reduced specific binding to membranes without buffer E. This reduction was significantly lower than the reduction after $\beta_1\gamma_{2(6xHIS)}$ addition ($p<0.0001$) and denatured dimer addition ($p<0.01$). The opposite phenomena was observed previously see **Figure 2.15**. In the case of Gα_q addition, the reduction (in specific binding to membranes minus G-proteins or buffer) is significantly lower

than the reduction when the same volume of buffer E is added. So the only difference

between these two purified G-protein preparations (apart from the biological

difference which can be discounted because there was no significant difference when

the proteins were denatured) is that one contains a histidine tag. Therefore during the

purification process the histidine tagged subunit (or dimer in this case) will stay on

the column a little longer and thus get an extra wash perhaps exchanging/ removing

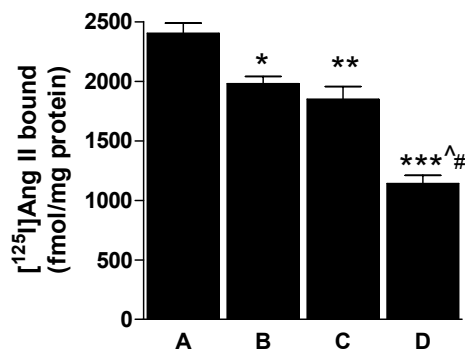the cholate or alternative inhibitory constituents.

Figure 6.6: The effect of the addition $\beta_1\gamma_{2(6xHIS)}$ on the specific [^{125}I]Ang II binding to CHO cell membranes expressing the AT$_{1A}$ receptor.
CHO cells from passage 40-42 stably expressing the AT$_{1A}$ were harvested and membranes were treated with 7M urea. CHO cell membranes (0.03 mg/mL) stably expressing AT$_{1A}$ were incubated in binding buffer (plus protease inhibitors and 0.1% BSA) with 1 nM [^{125}I]Ang II for 60 min at 26°C. Non-specific binding was determined in the presence of 10 μM Losartan and was less than 5% of total binding. The incubation also included the addition of A) no G-protein; B) 150 nM $\beta_1\gamma_{2(6xHIS)}$ (1.3 μL); C) 150 nM heat denatured $\beta_1\gamma_{2(6xHIS)}$ (1.3 μL); D) 1.3 μL buffer E. The reaction was terminated by filtering the total assay volume (100 μl) using 0.1% BSA pre-soaked GF/C filters and washing with 3x 4 mL volumes of TMN buffer with 0.1% BSA. Specific [^{125}I]Ang II binding is shown and data in A (n = 9, mean ± SEM, error bars). Data in B, C and D (n = 3, mean ± SEM, error bars). *$p<0.05$, **$p<0.01$, *$p<0.001$ (comparison to A); ^$p< 0.01$ (comparison to C); #$p<0.001$ (comparison to B).

6.7.   CENTRICON G-PROTEINS

The centricon $G\alpha_q$–prepared was added to the incubation in two concentrations 150

nM and 50 nM and to achieve these concentrations the following volumes of stock

subunit were added respectively, 7.4 µL and 2.4 µL (see **Figure 6.7**). The centricon

technique was more effective in buffer exchange than dialysis because in each case

(**Figure 6.7 C** and **E**), the addition of an equal volume of buffer E (as the amount of

subunit added) inhibited the specific binding to a greater extent. This effect was

significant (p<0.001) when the addition of 50 nM $G\alpha_q$ was compared to the addition

of the same volume of buffer E and it was observed that adding an equal volume of

buffer E was not as inhibitory as adding the un-centricon $G\alpha_q$ sample.
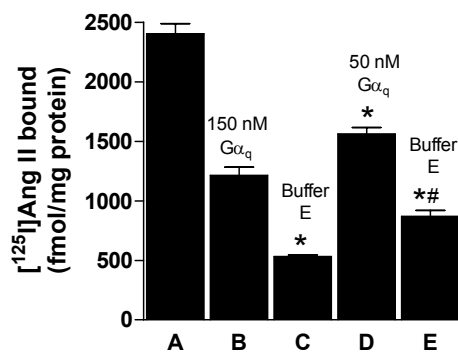

Figure 6.7:   **The effect of Centicon-prepared $G\alpha_q$ on the specific [^{125}I]Ang II binding to CHO cell membranes expressing AT$_{1A}$ receptor.**
CHO cells from passage 40-42 stably expressing the AT$_{1A}$ were harvested and membranes were treated with 7M urea. CHO cell membranes (0.03 mg/mL) stably expressing AT$_{1A}$ were incubated in binding buffer (plus protease inhibitors and 0.1% (w/v) BSA) with 1 nM [^{125}I]Ang II for 60 min at 26°C. Non-specific binding was determined in the presence of 10 µM Losartan and was less than 9% of total binding. The incubation also included the addition of A) no G-protein; B) 150 nM $G\alpha_q$ (7.4 µL); C) 7.4 µL buffer E; D) 50 nM $G\alpha_q$ (2.4 µL); E) 2.4 µL buffer E. The reaction was terminated by filtering the total assay volume (100 µl) using 0.1% BSA pre-soaked GF/C filters and washing with 3x 4 mL volumes of TMN buffer with 0.1% BSA. Specific [^{125}I]Ang II binding is shown and data in A (n = 9, mean ± SEM,). Data in C, D and E (n = 3, mean ± SEM,). Data in B (n = 2, error bars represent the range of duplicates). *p<0.0001 (comparison to A); $^\#$p<0.001 (comparison to D).

6.8. PROBLEMS ASSOCIATED WITH THE [^{35}S]GTPγS ASSAY

It was evident that agonist induced [^{35}S]GTPγS varied quite regularly and thus this was investigated. As each assay was controlled internally and the quantification of [^{35}S]GTPγS was not a direct assay development factor, this investigation was performed after much of the experimental section was completed. However, this data is included here for thesis completeness. The problem encountered was likely a technical issue relating to the stability of the concentrated stock of the isotope. Typically, the stock was thawed 3 separate times and each time it was diluted to a working stock of 40 nM and aliquoted. For each assay a new aliquot was used. The degree of agonist stimulated [^{35}S]GTPγS binding compared using a working stock that was prepared from the once thawed concentrated stock and twice thawed concentrated stock (**Figure 6.8**). Interesting, total binding using each of these stocks was not different which indicates that radioactivity and isotope conjugation is not disturbed by the freeze-thaw process. It is likely that GTPγS binding structures may have been inhibited by the freeze thaw. Perkin Elmer's technical did not think that this could be a problem. Discovery of this phenomenon may shed some light on the maximum binding inconsistencies seen earlier in this thesis.

Another problem associated with [^{35}S]GTPγS is that on occasions when cooled and the re frozen oxidation upon exposure to air occurred (Ferrer *et al.*, 2003). Reduced this problem after acknowledgement and stabilised the radioisotope for long time storage by the addition of reducing agents
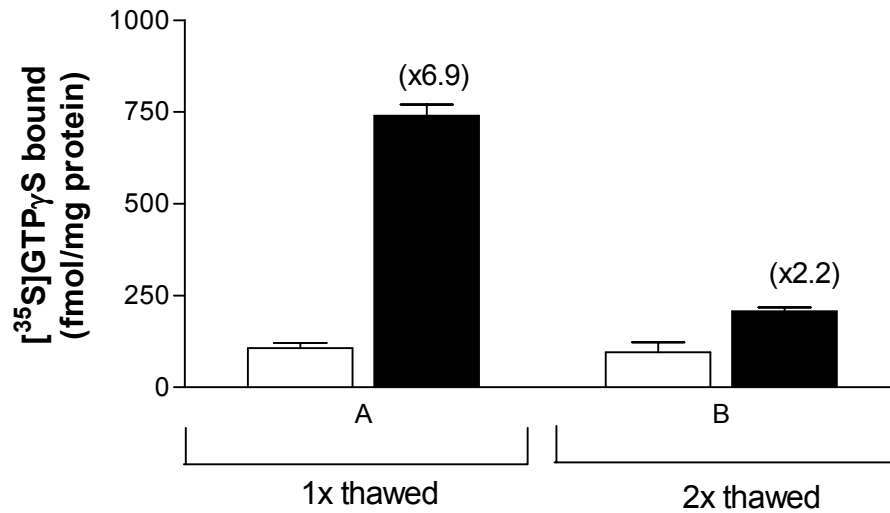
Figure 6.8: **Effect of using twice thawed stocks of [^{35}S]GTPγS.**
All incubations contained 5 μM GDP, 10 μM AMP-PNP and 0.2 nM [^{35}S]GTPγS that was diluted to 40 nM from stock that was once only thawed (first panel) and twice thawed (second panel). The concentrations of the proteins were the same in each assay i.e. urea treated *Sf*9 membranes over expressing α_{2A}AR (0.1 mg/mL), 20 nM G$\alpha_{i1(6xHIS)}$ and 20 nM $\beta_4\gamma_2$. However, three different reconstitution mixes were compared: Reconstitution 1 and 2 used the same preparation of $\beta_4\gamma_2$ and reconstitution 2 and 3 used the same α_{2A}AR and G$\alpha_{i1(6xHIS)}$. The reaction was incubated for 90 min at 26°C and initiated either by the addition of buffer (Basal [^{35}S]GTPγS binding) represented by the white bars or 10 μM UK-14304 (agonist induced [^{35}S]GTPγS binding) represented by black bars. Reactions (100 μl) were filtered over GF/C filter and washed with 3 x 4 mL with ice-cold TMN buffer. (n = 4, mean ± SEM).

6.9. MORE FUTURE DIRECTIONS

6.9.1.1. Customised lipid exchange

On-site lipid reconstitution enables the rapid lipid reconstitution of a receptor attached to a surface (Karlsson and Lofas, 2002). Modifying the lipid environment to mimic diet induced changes might be an interesting extension of this approach such that the effects of particular drugs on receptors in different lipid environments could be monitored. An example of a diet that modifies the lipid environment is one in which high consumption of omega 3 polyunsaturated fatty acids achieves more polyunsaturated n-3 fatty acids into excitable cell membranes (eg heart cells) (Leifert *et al.*, 1999) and the synergistic/additive effects of ingested fish oil with antiarrhythmic class of drugs that directly antagonise the cardiac βAR were investigated in a cell-based assay (Bucco 2000). Therefore, using such a cell-free approach may dramatically reduce costs and time and enable further investigation into such interactions giving yet another example of future application of the technology.

6.9.1.2. Receptor-shape mimetics

Receptor-shape mimetic technology avoids the expression of the entire protein and has been used in rational drug design although its use with GPCRs has been limited because of the debate over whether a generic ligand binding site exists (Sadler, Kristen and Tam, James P., 2004). Perhaps a similar approach could be incorporated into a functional assay whereby the pharmacologically important sections of the GPCR are coupled to the G-proteins (based on data from structure activity relationships). The benefit of this would be a specifically directed functional assay.

6.9.1.3. The immune system

Another biotechnology sector that is delivering compelling science certain to transform medicine in the next decade is the immune system (Whelan, 2005). The sentinels of the immune system, dendritic cells are regulated by both GPCR signalling and toll-like receptors (Shi *et al.*, 2004). Toll-like receptors (proteins that recognise the molecular structure of pathogens) were shown to alter GPCR signalling most likely by altering the expression of RGS proteins in dendritic cells (Shi *et al.*, 2004). Linking GPCRs with toll-like receptors in an assay may be used to screen therapeutics that direct the body's immune response to pathogens, allergens and cancer cells.