THE EFFECT OF RECEPTOR DENSITY ON THE
BINDING AND FUNCTIONAL PROPERTIES OF THE
HUMAN ADENOSINE A₁ RECEPTOR

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The adenosine A<sub>1</sub> receptor is a member of the 7-transmembrane G-protein coupled receptor family. It has an important signalling role in both normal physiology and disease. In addition to containing binding sites for adenosine and G-proteins, the A<sub>1</sub> receptor contains an allosteric binding site which binds synthetic molecules such as the allosteric enhancer PD 81,723. The availability of this compound, together with high affinity antagonists, highly potent agonists with a range of efficacies, the ability to determine ligand binding to the G-protein coupled and uncoupled states of the receptor and to readily measure function in membranes, make the A<sub>1</sub> receptor an ideal candidate for testing the predictions of mathematical models of drug receptor interactions.

The effects of receptor density, guanine nucleotides, and agonist efficacy on the ligand binding properties of the human adenosine A<sub>1</sub> receptor, recombinantly expressed at different densities, in membranes from Chinese hamster ovary cells have been examined. The proportions and ligand affinities of receptors in the G-protein coupled and uncoupled states of the A<sub>1</sub> receptor have been measured. The results suggest that there is a significant amount of receptor-G protein precoupling in the absence of ligand but the overall results are not quantitatively compatible with all the predictions of the ternary complex model of agonism. Radioligand dissociation kinetic studies show the novel phenomenon of agonist-induced agonist dissociation when the receptors are expressed at high but not low expression levels. These results are compatible with the presence of receptor
dimers, or another form of receptor cross-talk that occurs when agonists are bound to receptor G-protein complexes at high expression levels.

The effects of receptor density and agonist efficacy on the functional properties of the adenosine $A_1$ receptor were examined using a $[^{35}S]GTP\gamma S$ binding assay. Marked novel biphasic dose-response curves were observed for high efficacy agonists in membranes from a high expressing $A_1$ receptor cell line. Experiments were performed to investigate the nature of this response, its reversibility, and the effects of an inverse agonist and agonists of different efficacy. A detailed study of the kinetics of association of $[^{35}S]GTP\gamma S$ following agonist exposure suggest that a reversible feedback mechanism is operating, with a short lag-time for its induction.

The mechanism of action of the allosteric enhancer, PD 81,723, on the binding of a series of ligands with a range of intrinsic activities to the coupled and uncoupled states of the adenosine $A_1$ receptor was investigated. The effects of PD 81,723 on the functional properties of these ligands, measured at different receptor expression levels, were also studied. The results of these studies were not compatible with the detailed predictions of the ternary complex model of agonism if PD 81,723 simply enhanced the affinity of the receptor or if it increased agonist efficacy. A quaternary complex model of allosterism and agonism was developed which is compatible with the actions of PD 81,723 in binding and function. These data can be explained by PD 81,723 activating the receptors from the allosteric site and by its ability to act as a co-agonist with agonist binding to the orthosteric site to increase affinity of the $A_1$ receptor for its cognate G protein.
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ABBREVIATIONS

ADA  adenosine deaminase
Ado  adenosine
Bmax  maximum radioligand binding capacity
BSA  bovine serum albumin
BW-A844U  3-(4-amino)phenethyl-1-propyl-8-cyclopentylxanthine
cAMP  adenosine 3',5'-cyclic monophosphate
CHA  N6-cyclohexyl adenosine
CHO  Chinese hamster ovary
DMSO  dimethyl sulfoxide
DPCPX  8-cyclopentyl-1,3-dipropylxanthine
EDTA  ethylenediaminetetraacetic acid
ETCM  extended ternary complex model
frH  the fraction of binding sites of high affinity
GDP  guanosine 5'-diphosphate
GPCR  G-protein coupled receptor
Gpp(NH)p  guanosine 5'-[(β,γ-imido)triphosphate
GR 190178  ((2R,3R,4S,5R)-2-{6-[(3-fluoro-4-hydroxyphenyl)amino]-2-methyl-9H-purin-9-yl-5-(methoxymethyl)tetrahydrofuran-3,4-diol)
GR 161144  (9-[3R,4S-dihydroxy-5-[4-methyl]-1,2,4-oxadiazol-2-yl]-tetrabradfuran-2(R)-yl]-6-[[tetradecyran-4-yl]amino]-9H-purine)
GR 162900  ((2R,3R,4S,3R)-2-[6-[(1-methylethyl)amino]-9H-purin-9-yl]-5-[4H-5-methyl-1,2,4-triazol-2-yl]tetrahydrofuran-3,4-diol)
GTP  guanosine 5'-triphosphate
HE  High expressing CHO adenosine A1 cells
HEPES  N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
K_A  affinity constant of a ligand for a receptor.
K_G  affinity constant for the binding of receptor to G-protein.
$K_{PD}$ affinity constant for the binding of PD 81,723 to the adenosine $A_1$ receptor.

LE Low expressing CHO adenosine $A_1$ cells

NECA 5'-&(N-ethylcarboxamido)-adenosine

PD 2-amino-4,5-dimethylthien-3-yl[3-(trifluoromethyl)phenyl]-methanone, PD 81,723;

$pE_{C50}$ negative log of the concentration of a drug required to produce 50% of its own maximum response.

$pI_{C50}$ negative log of the concentration of a drug required to produce 50% of its own maximum inhibitory effect.

$pK_B$ negative log dissociation constant of an antagonist or inverse agonist for a receptor.

$pK_D$ negative log dissociation constant of a radioligand for a receptor.

$pK_i$ negative log dissociation constant of an unlabelled ligand for a receptor, determined from a radioligand competition binding experiment.

$pK_H$ negative log dissociation constant of a ligand for the high affinity state of a receptor.

$pK_L$ negative log dissociation constant of a ligand for the low affinity state of a receptor.

QCM quaternary complex model

R-PIA R-N^6-(2-phenylisopropyladenosine)

TCM ternary complex model

TM transmembrane spanning domain
Chapter 1: Introduction

1.1 G-protein coupled receptors

1.1.1 General Introduction

The family of seven transmembrane G-protein coupled receptors (7TM GPCRs) is one of the most abundant families of proteins known, with ~1000 members, representing approximately 3% of the mammalian genome (Foord, 2002; Takeda et al., 2002) and 5% of the genome of C. elegans (Bargman, 1998). In addition, 7TM receptors are extremely important in medicine. Approximately 50% of drugs used clinically mediate their effects by either activating or blocking this family of proteins (Drew, 1996).

GPCRs have a characteristic structure and mechanism of action. They can be subdivided into three main families based on their primary amino-acid sequence homology (Humphrey et al., 2000): rhodopsin like (family A), which constitutes the majority of 7TM receptors (~90%) and encompasses ‘classical’ 7TM receptors such as muscarinic and adrenergic receptors; the secretin-like family (family B), which includes receptors for calcitonin, glucagon and vasoactive intestinal peptide; and the metabotropic glutamate and GABA$_B$ receptors (family C). More recently, receptors for fungal pheromones, cAMP, and Wnt signalling factors (frizzled/smoothened receptors) have been identified as separate sub-classes of G-protein coupled receptors (Horn et al., 1998).
1.1.2 General structure of GPCRs

The general structure of 7TM receptors consists of seven transmembrane domains interspersed with three intra- and three extracellular loops, an extracellular N-terminus and an intracellular C-terminus (Fig. 1.1). This basic structure has been predicted from hydropathicity analysis of the primary structures of a large number of 7TM receptors (Baldwin, 1993). For both rhodopsin and the β2 adrenergic receptor it has been demonstrated that the N-terminus is on the extracellular face and the C-terminus is on the intracellular face (Applebury & Hargrave, 1986; Wang et al., 1989). A fourth intracellular loop is formed when one or more cysteine residues in the C-terminal segment are palmitoylated (Bouvier et al., 1995).

The transmembrane domains of GPCRs are of similar lengths across families, which reflects the constancy of the orientations of these domains relative to the width of a phospholipid bilayer. Conserved amino acids in these domains tend to be at the inner faces (Baldwin, 1993). The lengths of all three external loops, and internal loops one and two are relatively conserved and of similar lengths. The N- and C-termini and intracellular loop three are least conserved in sequence homology and overall length, which may in part reflect different modes of ligand recognition (at the N-terminus) and intracellular interactions with other proteins. There is no amino acid sequence homology between the three major GPCR families, whilst approximately 20% amino acid homology can be found between members of the rhodopsin family. Receptor subtypes, which share the same endogenous ligand, can have 50-60% homology, whilst the homology
Figure 1.1. The general structure of a family A, rhodopsin-like G-protein coupled receptor.
The general structure of the human $A_1$ receptor as an example of a rhodopsin like, Family A, seven transmembrane G-protein coupled receptor, illustrating the extracellular N-terminus, the three extracellular and three intracellular loops, and the intracellular C-terminus as described in section 1.1.2.
between species homologues of the same receptor can be 80-99% (Baldwin, 1993).

Most of the structural information on seven transmembrane receptors has come from studies on opsins, light detecting receptors found in certain species of bacteria and in the mammalian and amphibian retina. Bacteriorhodopsin is a light activated proton pump from *Halobacterium Halobium* whose seven-helical structure is known (Henderson et al., 1990; Luecke et al., 1999). This protein, however, does not couple to G-proteins, its sequence shows none of the distinctive patterns of the rhodopsin family and its structure, therefore, is not thought to be relevant to that of the rhodopsin family of GPCRs (Baldwin, 1993).

Rhodopsin itself is unique in that its ligand, 11-cis-retinal, is bound covalently to a conserved lysine in TM7. A photon at the appropriate wavelength causes the ligand to isomerise to the all-trans form which activates the receptor and allows interaction with its cognate G-protein, transducin (Khorana, 1992). In contrast to rhodopsin, other GPCRs are activated by the reversible binding of a diffusible ligand. Despite this difference, and the modest amino-acid homology between rhodopsin and other GPCRs (~20%) the overall structures of the transmembrane domains are thought to be remarkably similar (Ballasteros et al., 2001).

Low temperature electron diffraction studies of two dimensional crystalline receptor arrays have generated low resolution projection density maps showing the arrangement of transmembrane α-helices for frog and bovine rhodopsin (Schertler & Hargrave, 1995; Schertler et al., 1993). The structure was demonstrated to be a bundle of seven transmembrane helices with four α-helices perpendicular to the
membrane and the remaining helices being more tilted (Unger et al., 1997). The probable arrangement of the helices, relative to each other and to the lipid environment of the membrane, is consistent with the relative content of hydrophobic residues in the transmembrane regions (Baldwin, 1993). Thus, TM3 is the least lipophilic and is postulated to be hidden within the alpha-helical bundle, whilst TMs IV and V are most lipophilic and are thought to face towards the lipid bilayer.

The crystal structure of bovine rhodopsin has recently been determined to a resolution of 2.8 Å by X-ray crystallography (Palczewski et al., 2000) and has confirmed the general 7-transmembrane structure postulated from previous studies. Of interest was the unanticipated finding that the C-terminal tail forms an 8th cytoplasmic helix which lies along the surface of the membrane to which it is anchored via palmitoyl groups attached to a conserved pair of cysteine residues. Regions of the receptor involved in G-protein interactions are thought to involve the inward facing surfaces of the cytoplasmic ends of TMs 3, 5, 6 and 7, the second and third intracellular loops, and helix 8. The structure also confirms the bends in a number of helices predicted by the presence of conserved proline residues, in particular the conserved proline in TM6, mutation of which is thought to produce long-range distortion of the third intracellular loop (Ridge et al., 1999).

Most GPCRs contain consensus sequences for N-linked glycosylation in the amino terminus. There are conflicting reports regarding its role in receptor expression and function, and therefore an important role may not be universal among GPCRs. For instance, inhibition of N-glycosylation with tunicamycin has no effect on β2-receptor expression, ligand binding or function in S49 cells, but
decreases the function of prostaglandin E\textsubscript{1} receptors in the same cell line (George et al., 1986). In contrast, tunicamycin treatment of A431 cells has been demonstrated to decrease the expression and G-protein coupling of the β\textsubscript{2}-adrenoceptor (Boege et al., 1988; Cervantes-Olivier et al., 1988). Disruption of potential glycosylation sites by site-directed mutagenesis does not affect expression, ligand binding or function of the muscarinic M\textsubscript{2} receptor (van Koppen & Nathanson, 1990) but decreases the expression of the β\textsubscript{2} receptor (Rands et al., 1990) and the lutropin receptor (Liu et al., 1993).

Two cysteine residues in first and second extracellular loops are highly conserved amongst 7TM receptors and play a major role in receptor structure and stability. Disulphide-bonds between these cysteines have been demonstrated biochemically in rhodopsin (Findlay & Pappin, 1986), muscarinic receptors, (Curtis et al., 1989) and other GPCRs (Ji et al., 1998). In the crystal structure of rhodopsin, the disulphide bond between the two cysteines is thought to pull the second extracellular loop over the opening of the core of the receptor and, together with the N-terminus, contributes to the formation of the β-structure above a central cavity (Palczewski et al., 2000). Site directed mutagenesis of either of these cysteines in rhodopsin profoundly affects the tertiary structure, reducing expression levels, altering glycosylation and decreasing the ability to bind 11-cis-retinal and transducin (Davidson et al., 1994; Karnik et al., 1988). A similar approach dramatically alters the ligand binding ability of the β-adrenergic receptor (Dixon et al., 1987; Dohlman et al., 1990).
1.1.3 Ligand recognition

Despite the relatively low primary amino acid sequence homology between 7TM receptors, the basic three-dimensional architecture has been preserved throughout eukaryotic evolution. However, 7TM receptors have evolved to utilise an array of different modes of ligand recognition involving transmembrane regions, the N-terminal tail and extracellular loops. Thus Ji et al. (1998) have proposed that 7TM receptors can be further subdivided on the basis of how the endogenous ligand binds to its receptor. Ligands can bind exclusively to the transmembrane core (biogenic amines, eicosanoids, nucleosides), to both the core and extracellular loop (small peptides), to the N-terminus and extracellular loops (larger polypeptides) or to the N-terminus alone (glycoproteins).

In general, for biogenic amine receptors, amino acid residues in the transmembrane regions are important for the binding of the endogenous agonist. For rhodopsin itself, the ligand 11-cis-retinal is bound covalently to a lysine in TM7 and sits in a binding pocket formed from amino acids in TM3 and 6 (Palczewski et al., 2000). For the muscarinic M1 receptor the ligand contact points are highly homologous, and acetylcholine-like ligands can be docked along a similar trajectory to that of 11-cis-retinal in rhodopsin (Lu & Hulme, 2000). The positions of these amino acids are well conserved in other 7TM receptors although the side-chains are frequently different, reflecting the different chemical properties of the endogenous ligand (Ji et al., 1998).
1.1.4 Receptor activation

GPCRs are thought to be maintained in a ground state structure by a network of conserved, stabilising intramolecular interactions involving H-bonding networks and Van der Waal's contacts which constrain the TM helices in an inactive conformation. The amino acids involved in maintaining the stabilising interactions are frequently highly conserved. Mutation of these amino acids often results in an increase in agonist affinity, and agonist independent constitutive receptor activation, and suggests a role for these residues in stabilising interactions that constrain the receptor to the inactive ground state (Lefkowitz et al., 1993).

For the muscarinic M1 receptor, a series of highly conserved amino acids in TMs 3, 6 and 7 have been shown to be in close proximity by the creation of high-affinity Zn$^{2+}$ binding sites by histidine substitutions. Substitution of these residues with alanine increased the affinity of acetylcholine, implying the promotion of an activated state (Lu & Hulme, 2000). Replacement of the highly conserved alanine 293 in the C-terminus of the third intracellular loop of the $\alpha_{1B}$ adrenoceptor with any other residue resulted in higher levels of agonist independent receptor activity (Kjelsberg et al., 1992). The invariant arginine in the highly conserved E/D-R-Y motif at the cytoplasmic end of TM3 is postulated to be constrained in a 'polar pocket' formed by conserved neighbouring residues in TM1, 2 and 7. One proposal is that receptor activation leads to protonation of the aspartate or glutamate, causing arginine to move out of the polar pocket and the cytosolic exposure of buried sequences in the second and third intracellular loops. Mutation of the glutamate in rhodopsin (Arnis et al., 1994) or the aspartate in the $\alpha_{1B}$
(Scheer *et al.*, 1996) and \( \beta_2 \) (Rasmussen *et al.*, 1999) adrenoceptors, results in an increase in agonist independent activity.

All GPCRs are thought to share a conserved mechanism of activation. Agonist binding to GPCRs is thought to disrupt the inactive ground state by producing a conformational rearrangement of the transmembrane regions, with the relative movement of TM6 of particular importance. In rhodopsin the use of nitroxide spin labels has demonstrated the outward movement of cytoplasmic region of TM6 following receptor activation (Altenbach *et al.*, 1996; Farahbakhsh *et al.*, 1995; Klein-Seetharaman *et al.*, 1999). The movement of TM6 has also been visualised as a change in fluorescence of environmentally sensitive fluorescent probes introduced into both rhodopsin and the \( \beta_2 \) adrenergic receptor (Dunham & Farrens, 1999; Jensen *et al.*, 2001). Furthermore, the introduction of Zn\(^{2+}\) bridges between TM3 and 6 in rhodopsin, the \( \beta_2 \) adrenergic, and the parathyroid hormone receptors, restricts the relative movements of these transmembrane regions and inhibits receptor activation, suggesting the relative movements of these TM regions to be of particular importance (Sheikh *et al.*, 1999; Sheikh *et al.*, 1996).

The structural rearrangement of the transmembrane helices of GPCRs following agonist activation is postulated to expose regions of the receptor capable of binding and activating G-proteins. From the crystal structure of rhodopsin, these regions are postulated to be the cytoplasmic ends of TMs 3, 5, 6 and 7, the second and third intracellular loops and helix 8 (Palczewski *et al.*, 2000). This supports previous studies that demonstrate that short peptides derived from the C-terminus of the third intracellular loop can mimic G-protein activation by a receptor (Malek & Palm, 1993; Okamoto *et al.*, 1991; Okamoto & Nishimoto, 1992). In addition,
mutation of a four amino acid motif at the cytoplasmic end of TM6 of the muscarinic M2 receptor alters its G-protein coupling selectivity from Goi to Gcq (Kostenis et al., 1997b). A similar result has been observed with hybrid muscarinic M2/M3 receptors where regions of the second and third intracellular loops of the M2 receptor are replaced by the corresponding regions of the M3 receptor (Kostenis et al., 1997a). Furthermore, the third cytoplasmic loop of rhodopsin has been shown to be in close proximity to the N- and C-termini of transducin by covalent cross-linking following receptor activation (Cai et al., 2001; Ito et al., 1994).

1.2 G-proteins

The primary mechanism by which activated GPCRs generate changes in intracellular signalling is via the activation of heterotrimeric guanine-nucleotide binding proteins (G-proteins). Heterotrimeric G-proteins are composed of α-, β- and γ-subunits. To date there are 23 distinct α subunits encoded by 17 different genes which can be subdivided into four sub-families, Goi0, Goζ, Gcq and Go12, according to the homology of the primary amino acid sequence. The α subunits have a conserved primary structure (50-90%) but have diverse profiles of effector coupling. The cellular concentrations of Goi0 considerably exceed those other subtypes and in the brain Goζ may represent 1-2% of the total membrane protein (Hepler & Gilman, 1992). Goα subunits are anchored to the cytosolic face of the plasma membrane by lipid modification, either by N-myristoylation or palmitoylation at the N-terminus (Wedegaertner et al., 1995).
Six β- and twelve γ- subunits have been described. The β- and γ- subunits form a functional βγ heterodimer that is not dissociable except by denaturation. β subunits cannot form dimers with all γ subunits; for example, β1 interacts with either γ1 or γ2, β2 interacts with γ2 but not γ1; and β3 cannot associate with either γ1 or γ2. In addition β subunits are selective in their coupling to α subunits. This selectivity in subunit interactions underlies the fidelity of receptor-G-protein interactions and contributes to the specificity of receptor-effector coupling (Gudermann et al., 1996). βγ subunits are also capable of stimulating diverse effector mechanisms, including activation of inwardly rectifying potassium channels, phospholipase Cβ, as well as adenylyl cyclase and the MAP kinase cascade (Clapham & Neer, 1997). The G-protein signalling pathways will be described in more detail in section 1.4 in the context of the adenosine A1 receptor.

The α subunit cycles between an inactive, GDP bound, conformation and an active GTP form (Fig. 1.2; Gilman, 1987). Activated GPCRs bind to Go-GDP,βγ and induce conformational changes in the G-protein. This results in the exchange of GDP for GTP and the dissociation of the Go-GTP and βγ subunits. Subunit dissociation reveals regions of both the α-GTP and βγ subunits which had been previously hidden at the interface of the heterotrimeric complex, and which are capable of activating downstream effector pathways (Lambright et al., 1996; Wall et al., 1995). The intrinsic GTPase activity of Go hydrolyses GTP to GDP, which is followed by the reassociation of the inactive Go-GDP,βγ complex.
Figure 1.2. Receptor - G Protein interactions

A general model for the interactions between agonist (A), receptor (high agonist affinity state, $R_H$, or low agonist affinity state $R_L$), and G protein ($G\alpha\beta\gamma$) as described in section 1.2.
Gα subunits contain two domains, a domain involved in the binding of guanine nucleotides and hydrolysis of GTP that is structurally similar to the superfamily of small GTP-ase proteins and elongation factors (Kjeldgaard et al., 1996), and a unique helical domain (Coleman et al., 1994; Noel et al., 1993). The helical domain contains flexible 'switch' regions which envelopes bound guanine nucleotide and restricts the movements of nucleotides into and out of the binding pocket (Lambright et al., 1994). Activated receptors catalyse the Mg$^{2+}$ dependent exchange of GDP for GTP by loosening a network of hydrogen bonds and Van der Waals contacts between residues in both the core and helical domains, and the guanine ring of the bound nucleotide (Noel et al., 1993). In general, the intracellular loops of GPCRs are too short to interact directly with the nucleotide binding site of Gα, which is probably located ~30Å from the plasma membrane (Lichtarge et al., 1996). The receptor must therefore work 'at a distance' to change the conformation of the protein by triggering an allosteric transition. Following the binding of GTP, the additional $\gamma$-phosphate of GTP interacts with a small number of amino acids in the switch regions to induce conformational changes that result in the dissociation of both receptor and $\beta\gamma$ from Gα-GTP (Coleman et al., 1994; Noel et al., 1993).

The amino acids of Gα involved in the interactions with receptor, βγ, and effectors, have been identified from experiments with peptides, bacterial toxins, antibodies, crosslinking agents and mutagenesis, together with information from the crystal structures of Gα-GDP (Lambright et al., 1994), Gα-GTPγS (Coleman et al., 1994; Noel et al., 1993) and Gα-GDP.βγ (Lambright et al., 1996; Wall et al., 1995). Multiple receptor contact regions have been identified on Gα subunits.
which include the N-terminal 23 residues and the C-terminus (Hamm et al., 1988). The best characterised of these regions is the C-terminus which is thought to interact with regions of the second and third intracellular loop of GPCRs (Kostenis et al., 1997a; Kostenis et al., 1997b). Chimeric Gα subunits have demonstrated that the last three - five amino acids of the C-terminus are critical for the specificity of receptor-G-protein interactions (Conklin et al., 1993; Kostenis et al., 1997a). Furthermore, short peptide sequences from the α-subunit C-terminus are able to disrupt receptor-G-protein coupling (Rasenick et al., 1994).

The Gβ subunit consists of two domains, an N-terminal α helix, and a β-propeller structure made up of seven repeated ‘WD-40’ motifs. The γ subunit is tightly bound to the β subunit through extensive non-covalent interactions with both the coiled region and the propeller region of the β subunit, which explains its resistance to denaturants (Sondek et al., 1996). Prenylation of the C-terminus of the γ-subunit anchors the βγ complex to the plasma membrane (Wedegaertner et al., 1995). In addition to activating downstream effectors, the βγ subunit has also been demonstrated to influence receptor function. βγ subunit have been shown to bind directly to purified β-adrenergic receptors (Heithier et al., 1992) and to rhodopsin (Phillips & Cerione, 1992), and to enhance the binding of Gα to its appropriate receptor (Phillips et al., 1992). The βγ subunit is also able to facilitate receptor phosphorylation by binding to β-adrenergic receptor kinase and bringing it into close proximity to the receptor (Inglese et al., 1995). This latter function has led to the suggestion that the general role of βγ subunits is to promote macromolecular assembly as part of the regulation of intracellular vesicular traffic by heterotrimeric G-proteins (Clapham & Neer, 1993).
The intrinsic GTPase activity of the G-protein α subunits is regulated by a large family of proteins termed regulators of G-protein signalling (RGS). RGS proteins negatively regulate the activity of Gα subunits by increasing the rate of GTP hydrolysis by up to 100 fold and for this reason are also termed GTPase activating proteins (GAPs, Berman & Gilman, 1998). Members of the phospholipase Cβ family of effector enzymes have also been demonstrated to have Gαq-specific GAP activity, suggesting a role in negative feedback regulation of G-protein function (Biddlecome et al., 1996; Chidiac & Ross, 1999).

G-protein activation can be measured directly in a binding assay using the radiolabelled analogue of GTP, [35S]GTPγS. Following receptor activation [35S]GTPγS binds to G-protein α subunits and stimulates subunit dissociation in a similar manner to GTP. However, unlike GTP, [35S]GTPγS is poorly hydrolysable and accumulates in membranes and can be measured using conventional filtration techniques (Hilf et al., 1989; Lazareno, 1997). This method allows the measurement of the number of G-proteins capable of binding guanine nucleotide. The ability of G-proteins to hydrolyse GTP can be measured in a GTPase assay. This method measures the rate or amount of [32P] liberated from [γ-32P]GTP as a result of hydrolysis by the α subunit (Brandt & Ross, 1986; Cassel & Selinger, 1976).

A number of biochemical tools are available for investigating the G-protein α subtypes involved in G protein coupled receptor signalling. Pertussis toxin (or islet activating protein) from Bordetella pertussis, catalyses the ADP-ribosylation of a cysteine residue four amino acids from the C-terminus of G-protein α
subunits of the class Go \( \alpha \) and Go \( \delta \) (Hurley et al., 1984; West et al., 1985). The result of this covalent attachment is to inhibit G-protein interactions with receptors, which further supports the importance of the Go C-terminus in receptor interactions. Cholera toxin from Vibrio cholerae catalyses the ADP-ribosylation of an arginine residue at position 174 of the \( \alpha \) subunit of transducin (Abood et al., 1982) and the corresponding position 201 in bovine Go\( \alpha \) subunits (Robishaw et al., 1986). This residue is positioned in the switch II region of the helical domain and interacts with the \( \gamma \)-phosphate of GTPyS (Lambright et al., 1994). ADP-ribosylation of this arginine residue inhibits the GTPase function of Go\( \alpha \) and disrupts receptor – G-protein interactions by decreasing the affinity of Go\( \alpha \) for G\( \beta \gamma \), although basal GTPase activity of ADP-ribosylated Go\( \alpha \) is unimpaired (Kahn & Gilman, 1984).

1.2.1 Other receptor-protein interactions

Activated G-protein coupled receptors are substrates for several families of kinase proteins, which include second messenger-regulated kinases such as PKA and PKC (Hausdorff et al., 1990), and G-protein coupled receptor kinases (GRKs). The conformational changes which occur as a result of agonist binding and receptor activation expose a number of serine and threonine residues in the C-terminus and the third intracellular loop of GPCRs, which greatly enhances receptor phosphorylation by GRKs. There are currently 6 members of the GRK family (GRK 1-6). The most commonly studied are GRK1, or rhodopsin kinase (Kuhn, 1978), and GRK2, or \( \beta \)-adrenergic receptor kinase, which is now thought to phosphorylate a large number of GPCRs (Krupnick & Benovic, 1998).
Phosphorylated receptors are substrates for a family of cytosolic proteins called arrestins (Krupnick & Benovic, 1998). Whilst GRK catalysed phosphorylation of GPCRs alone may modestly desensitise receptor signalling (Bennett & Sitaramayya, 1988) the binding of arrestins to phosphorylated receptor is the primary mechanism for the rapid uncoupling of receptors from G-proteins (Krupnick & Benovic, 1998). Furthermore, receptor phosphorylation and arrestin binding are critical steps in the initiation of internalisation of muscarinic M<sub>2</sub> (Tsuga et al., 1994) and the β<sub>2</sub>-adrenergic receptor (Ferguson et al., 1995). The internalisation of surface expressed GPCRs following prolonged agonist exposure was originally considered to be part of the process of receptor down regulation. However, internalisation has also been demonstrated to be critical for receptor dephosphorylation and resensitization of the β<sub>2</sub>-adrenergic receptor (Yu et al., 1993; Zhang et al., 1997). More recently it has been discovered that receptor internalisation is a prerequisite for the G-protein coupled receptor mediated activation of mitogenic signalling pathways (Daaka et al., 1998; Luttrell et al., 1999).

Support for the C-terminus of GPCRs as the initial component for ‘scaffolds’ of other proteins comes from the interactions between the β<sub>2</sub> receptor and the Na<sup>+</sup>/H<sup>+</sup>-exchanger regulatory factor (NHERF, Hall et al., 1998b). The NHERF family of peptides contain PDZ domains which recognise the C-terminal motif D-S/T-x-L of both the β<sub>2</sub>-adrenergic receptor and the purinergic P2Y<sub>1</sub> receptor (Hall et al., 1998a). However, the precise biological relevance of the NHERF interaction appears unclear.
The secretagogue, type II receptor family contain a group of 7TM receptors whose expression and pharmacological properties are dependent on interactions with accessory proteins termed receptor activity modifying proteins (RAMPs, Foord & Marshall, 1999). It had been recognised that whilst the operational characterisation of secretagogue receptors had demonstrated the existence of at least five members of the family (calcitonin, amylin, calcitonin-gene-related-peptide (CGRP) -1 and -2, adrenomedullin), only two genes could be cloned (the calcitonin receptor and the calcitonin receptor like receptor CRLR, Flühman et al., 1995). Meanwhile, cRNA from CGRP receptor expressing cells was found to induce novel CGRP responses in Xenopus oocytes (McLatchie et al., 1998). The protein responsible was cloned and termed receptor activity modifying proteins (RAMP1). Two additional RAMPs were cloned (RAMP 2 and 3) and demonstrated to produce adrenomedullin receptors when co-expressed with CRLR (McLatchie et al., 1998). Amylin receptors can be created by the actions of RAMPs with a calcitonin receptor gene product (Chen et al., 1997). The biological functions of RAMPs are integral to the functioning of calcitonin related receptors in that they transport the receptors to the cell surface, determine the receptor pharmacology and influence receptor glycosylation states (McLatchie et al., 1998).

The expression and function of the family III GPCR gamma-aminobutyric acid (GABA\textsubscript{B}) receptor is dependent on the co-expression of two separate 7TM receptors GABA\textsubscript{BR1} and GABA\textsubscript{BR2} (Mohler & Fritschy, 1999). Native GABA\textsubscript{B} receptors regulate potassium and calcium channels via G-protein activation (Bowery, 1993). When expressed alone, GABA\textsubscript{B}R1 fails to bind agonists with
high affinity or couple efficiently to signal transduction pathways (Kaupmann et al., 1997). Fully functional recombinant GABA_B receptors, with similar pharmacological properties to native receptors, are generated only upon co-expression of a second GABA_B receptor, the GABA_B2 receptor (Jones et al., 1998; Kaupmann et al., 1998; White et al., 1998).

The concept that family A receptors can form dimers and higher order multimers, and that these complexes are important for receptor function, is beginning to emerge. Pharmacological evidence for 7TM receptor heterodimers has been demonstrated for the κ and δ opioid receptors which, when coexpressed, demonstrate ligand binding and functional properties that are distinct from those of either receptor (Jordan & Devi, 1999). Evidence for an interaction between family A 7TM GPCRs at the molecular level has been demonstrated using α2-adrenergic and M3-muscarinic receptors in which TM 6 and 7 have been exchanged (Maggio et al., 1993). No radioligand binding could be detected when the chimeric receptors were expressed separately. However, when the constructs were coexpressed in the same cell line, binding of both the muscarinic and adrenergic radioligands could be detected. Based on the relative orientations of the transmembrane regions, it is postulated that dimers occur either as contact dimers or 'domain swapped' dimers, involving TM5 and 6 (Gouldson et al., 2000).

Co-immunoprecipitation studies have provided supportive evidence that differentially epitope tagged receptors may exist as homo- (Cvejic & Devi, 1997) and hetero- (Salim et al., 2002) dimers. In intact cells, resonance energy transfer can be demonstrated between receptors differentially labelled with energy donor
and acceptor molecules (for a review see Salim et al., 2002). Resonance energy transfer occurs across distances that are typically less than 100Å thus suggesting molecular proximity between different receptors. Both co-immunoprecipitation and resonance energy transfer techniques have demonstrated that agonists are able to perturb the extent of receptor proximity. For the δ-opioid receptor, increasing concentrations of the agonist DADLE decreased the level of the dimer in immunoprecipitates, and correspondingly increased the level of monomer (Cvejic & Devi, 1997). In contrast, using fluorescence or bioluminescence energy transfer, the agonist [D-Ala², D-Leu⁵]enkephalin had no effect on the homooligomerisation status of the δ-opioid receptor and actually increased the heterooligomerisation between the δ-opioid and β₂-adrenergic receptors (McVey et al., 2001). Whether the oligomers demonstrated in co-immunoprecipitation and resonance energy experiments actually represents functionally relevant complexes, or simply receptors in close proximity to one another remains to be determined. There is some evidence for the localisation/clustering of GPCRs to lipid rafts, microdomains in the cell membrane that are enriched in cholesterol and sphingolipid (Anderson, 1998).
1.3 Models of receptor-G-protein activation

1.3.1 The Ternary Complex Model.

The simplest mathematical model which describes the interaction of an agonist A, receptor R and G-protein G is the ternary complex model (Fig. 1.3a, De Lean et al., 1980). Within this model the binding of A and G for unoccupied receptor is described by the affinity constants $K_A$ and $K_G$ respectively. Hence, A and G can be considered as ligands for R. The effect of the occupancy of one ligand on the affinity of the second ligand for R is determined by the cooperativity constant $\alpha$. When $\alpha > 1$, the binding of A to R increases the affinity of G for R and A can be considered an agonist. When $\alpha = 1$, the binding of A has no effect on the affinity of R for G and A can be considered an antagonist. If $\alpha < 1$, the binding of A to R decreases the affinity of R for G and $\alpha$ is considered an inverse agonist. $\alpha$ is therefore recognised as molecular efficacy and is independent of $K_A$ and $K_G$.

Ligand binding is assumed to represent the sum of AR and ARG complexes, whilst receptor function can be considered the sum of basal RG and agonist induced ARG complexes.

The binding of an agonist to a receptor is frequently complex. In competition binding experiments, the inhibition of the binding of a radiolabelled antagonist by an agonist is often biphasic or has a Hill slope $< 1$. This type of experimental data can be analysed by a two-site model which allows the quantification of three parameters: $K_H$ and $K_L$, the affinity of the agonist for the high and low affinity states, and $f_{RH}$, the fraction of high affinity binding sites. Both the ratio of
Figure 1.3. The Ternary Complex and Extended Ternary Complex Models of drug receptor interactions.

The ternary complex model (panel a) describes the interaction between a ligand (A), receptor (R), and G-protein (G). A and G can bind both separately and simultaneously to R and can therefore be considered as ligands for R. The binding of A and G to unoccupied R is described by the affinity constants $K_A$ and $K_G$. When R is occupied by one ligand, the affinity of the other ligand is altered in a reciprocal manner by the allosteric cooperativity constant $\alpha$. If $\alpha > 1$, positive cooperativity exists between A and G, and the binding of A will increase the binding of G, and A is considered an agonist. If $\alpha = 1$, the occupancy of R by A does not influence the equilibrium binding properties of G. This is described as neutral cooperativity and A will behave as a simple, competitive antagonist. When $\alpha < 1$, the binding of A to G to R is negatively cooperative. A will decrease the affinity of R for G and behave as an inverse agonist (see section 1.3.2).

The ternary complex model (panel a) is formally identical to the allosteric ternary complex model when G is replaced by an exogenous ligand (X) which can bind to an allosteric site that is distinct from the primary ligand binding site.

The extended ternary complex model (panel b) incorporates a receptor isomerisation step from inactive R to active R* which is governed by the isomerisation constant $J$. In this scheme, an agonist can possess two types of molecular efficacy: the ability to increase receptor isomerisation (described by the allosteric constant $\beta$), and the ability to increase the affinity of R for G ($\alpha$).
a

\[
\begin{align*}
G + R + A & \xleftrightarrow{K_A} G + R.A \\
\alpha K_G & \xleftrightarrow{K_G} \alpha K_A \\
G.R + A & \xleftrightarrow{K_G} G.R.A
\end{align*}
\]

b

\[
\begin{align*}
A + R & \xleftrightarrow{K_A} A.R \\
J & \xleftrightarrow{\beta K_L} \beta J \\
A + R^* & \xleftrightarrow{\beta K_L} AR^* \\
+ G & \xleftrightarrow{K_G} + G \\
A + R^*G & \xleftrightarrow{\alpha \beta K_L} \alpha K_G \\
\end{align*}
\]
the high and low agonist affinities, and the fraction of high affinity binding sites, have been demonstrated to correlate with the efficacy of the competing agonist (Birdsall et al., 1978; Kearn et al., 1999; Kent et al., 1980; Lahti et al., 1992). De Lean and coworkers (1980) were the first to demonstrate that, for the β-adrenergic receptor, many of the features of heterogenous agonist binding and guanine nucleotide sensitivity could be explained by a simple ternary complex model (Fig. 1.3a). The high and low affinity agonist binding sites are believed to represent agonist binding to receptor coupled to, and uncoupled from, G-protein, respectively. Pharmacological agents such as guanine nucleotides and pertussis toxin decrease the affinity of agonists for the high affinity binding site and are postulated to mediate this effect by disrupting receptor-G-protein coupling. The ternary complex model therefore provides a molecular mechanism for high and low agonist affinity states and the modulation of agonist binding by guanine nucleotides.

The ternary complex model has proved useful for describing and analysing drug receptor interactions at other GPCRs (Ehlert, 1986; Wreggett & De Lean, 1984). It is of interest however, that for both the β-adrenergic and dopamine D2-receptors, the fraction of high affinity binding sites is found to be an agonist dependent property (De Lean et al., 1980; Wreggett & De Lean, 1984). Biphasic agonist binding is predicted when the concentration of G-proteins is lower than the concentration of receptor. Under these conditions, there is insufficient G-protein for all receptors to form high affinity RG complexes, and the concentration of G-protein is said to be limiting. \( f_{RH} \) is predicted by the TCM to reflect the relative concentrations of receptor and G-protein and would therefore be expected to be
constant within a particular cell type, and not dependent on the properties of an agonist. This has been highlighted by a number of authors as a deficiency in the ternary complex model (Lee et al., 1986; Neubig, 1994).

1.3.2 Constitutive activity and inverse agonism.

An additional prediction of the ternary complex model is that receptors can couple to G-proteins in the absence of an agonist and that such a complex may be functionally active. Such constitutive activity is predicted to be dependent on the relative levels of receptor and G-protein and the magnitude of the affinity constant for the binding of G-protein to the receptor, $K_g$. The model also predicts that a ligand with a higher affinity for the uncoupled state of a receptor ($\alpha<1$, Fig 1.3a) will give a response in the opposite direction of a conventional agonist, and act as an inverse agonist to decrease basal receptor activity.

Constitutive receptor activity and inverse agonism was first convincingly demonstrated by Costa and Hertz (1989) for the GTPase activity of the δ-opioid receptor endogenously expressed in NG108-15 neuroblastoma-glioma cells. In this study, the inverse agonist response of the opioid ligand ICI 174864 ([N,N'-diallyl-Tyr$^1$,Aib$^{2,3}$]Leu$^5$enkephalin) was competitively antagonised by the silent antagonist MR2266 with a similar affinity as for antagonism of the full agonist DADLE ([D-Ala$^2$,D-Leu$^5$]enkephalin), thus demonstrating that the inhibitory response is mediated by the same receptor as the stimulatory response. Costa and colleagues subsequently demonstrated that this phenomenon could be predicted by the ternary complex model of agonism (Costa et al., 1992). Reports of constitutive activity and inverse agonism associated with a number of different
7TM GPCRs, either endogenously or recombinantly expressed have subsequently been published (for a review see De Ligt et al., 2000).

As predicted by the ternary complex model, constitutive receptor activation can be increased by increasing receptor density (Samama et al., 1993; Smit et al., 1996), which increases the number of active RG complexes. Increasing the expression levels of G-proteins also increases constitutive activity by increasing both the number and proportion of active RG complexes (Burstein et al., 1997).

1.3.2 The Extended Ternary Complex Model.

The realisation that receptor activation involves a conformational change in GPCRs led to the development of mathematical models that incorporate a receptor isomerisation step to describe drug receptor interactions (Leff, 1995; Samama et al., 1993; Weiss et al., 1996). As described in section 1.1.4 above, agonist binding to a receptor leads to the destabilising of amino acid interactions that maintain the receptor in an inactive conformation, and the formation of new interactions which stabilise an active conformation capable of activating G-proteins. Receptors can therefore be considered to exist in an equilibrium between an inactive (R) and active (R*) conformation (Fig. 1.3b) described by the equilibrium constant J, and agonists are believed to increase the proportion of receptors in the R* conformation (Leff, 1995).

Constitutively active receptor systems have been generated by site directed mutagenesis of amino acids thought to be important in maintaining a receptor in an inactive conformation (see, for example, Kjelsberg et al., 1992; Lu & Hulme, 2000). Samama and colleagues (1993) replaced the C-terminal portion of the third
intracellular loop of the $\beta_2$-adrenoceptor with the homologous region of the $\alpha_{1B}$-adrenergic receptor and found an increase in agonist-independent receptor activation of adenylyl cyclase. This mutation resulted in an increase in affinity of up to thirty-fold for agonists binding to the uncoupled state of the receptor. However, this increase in affinity varied between different agonists and was correlated with agonist intrinsic activity. This result suggested that the apparent affinity of a ligand for the uncoupled state of a receptor is not an unconditional constant, but depends on both the affinity and the efficacy of that ligand.

In the study of the mutant $\beta_2$-receptor (Samama et al., 1993) it was concluded that the data could not be explained by the ternary complex model in terms of an increase in the affinity constants $K_A$ or $K_G$. An extended ternary complex model, incorporating an isomerisation step from $R$ to $R^*$ was proposed (Fig 1.3b) and the mutation was postulated to increase agonist affinity by producing an alteration in the $R/R^*$ isomerisation constant. However, the effects of the mutation may not be consistent with a change in isomerisation alone. Basal cAMP production for the mutant receptor was similar to that measured for the wild type receptor in the presence of isoproterenol, and the addition of isoproterenol to the mutant receptor was able to produce a further increase in cAMP production. The authors suggest that the mutation may also be affecting both $K_G$ and $\alpha$. Furthermore, the GTP-shifts for the full agonist isoproterenol and the partial agonist dobutamine are not affected by the mutation which is also not consistent with solely an increase in the isomerisation constant contributing to the observed changes in the binding properties. These observations would suggest that the mutation is having a more
profound effect on the $\beta_2$-adrenergic receptor, influencing both the ability of the receptor to isomerise and to activate G-proteins.

The simple two-state model and the extended ternary complex model are simplifications of the cubic ternary complex model, which incorporates both receptor-G-protein coupling and receptor isomerisation and allows both $R$ and $R^*$ to bind to $A$ and $G$ (Weiss et al., 1996). These models share similar properties in that they predict that there are two types of molecular efficacy. An agonist may produce a response by increasing the extent of isomerisation from $R$ to $R^*$ ($\beta$ in figure 1.3b), or increase the affinity of $R$ for $G$ ($\alpha$ in Fig. 1.3b). Receptor isomerisation models may be of use in explaining the lack of a correlation between $K_l/K_h$ and agonist efficacy for a number of GPCRs (Fisher et al., 1984; Gardner & Strange, 1998). Receptor isomerisation models have also been used to describe receptor systems in which receptor desensitisation occurs. In this instance $R^*$ is considered to be the inactive, desensitised species (Gero, 1983).

The significance of the formation of $R^*$ and $AR^*$ complexes has been discussed in detail (Colquhoun, 1998; Strange, 1999). When $R^*$ is allowed to accumulate, the apparent affinity of a ligand for the G-protein uncoupled receptor is a macroscopic affinity constant containing elements of both agonist affinity and efficacy for promoting the $R^*$ conformation. Hence affinity and molecular efficacy cannot be considered as separate entities. In instances where $K_l/K_h$ and agonist efficacy correlate, this may indicate that a system consistent with a ternary complex model is operating, and that there is no significant accumulation of $R^*$. 
1.4 Adenosine and its receptors

1.4.1 Physiological role of adenosine

Adenosine is present in all body fluids and elicits an extensive range of physiological functions. These include effects on cardiac rate and contractility, neurotransmitter release, smooth muscle tone, sedation, platelet function, lipolysis, renal function, and the regulation of cells of the immune and inflammatory system (Ramkumar et al., 1988). Adenosine levels increase as a result of ATP metabolism in response to cardiac hypoxia (Fenton & Dobson, 1987), CNS ischaemia (Hagberg et al., 1987) and seizure activity (During & Spencer, 1992) and is considered to have an important role in cytoprotection. For this reason, adenosine has been called a ‘retaliatory metabolite’ (Laghi Pasini et al., 2000). Despite the diverse physiological effects of adenosine, the only current therapy which exploits an adenosine receptor interaction is the use of adenosine itself for the treatment of supraventricular tachycardias (Biaggioni et al., 1986).

The processes of adenosine synthesis and metabolism are clearly important physiologically. However, an understanding of these processes is also essential for in vitro studies when attempting the pharmacological analysis of adenosine receptors. Under normal physiological conditions adenosine is present at concentrations of 10-30 nmoles.g\(^{-1}\) in tissues and 0.03-2.6 μM in body fluids (Arch & Newsholme, 1978). In addition, both intact and homogenised tissue and cellular preparations are capable of releasing large amounts of adenosine which can be present at concentrations which are sufficient to activate adenosine receptors (Prater et al., 1992). For this reason adenosine receptor studies are
frequently conducted in the presence of adenosine deaminase to degrade endogenous adenosine to inosine (Linden, 1989). Receptor activation can then be examined using synthetic analogues of adenosine which are resistant to the actions of this metabolic enzyme.

Under normal conditions, adenosine is produced continuously both intracellularly and extracellularly. The major route of intracellular adenosine synthesis is the dephosphorylation of adenosine monophosphate (AMP) by the action of 5'-nucleotidase (Arch & Newsholme, 1978). 5'-nucleotidase can be inhibited experimentally with the use of α,β-methylene adenosine 5'-diphosphate (Schütz et al., 1981). In certain tissues such as cardiac tissue significant levels of adenosine can be generated by the action of S-adenosylhomocysteine hydrolase on S-adenosylhomocysteine (Schütz et al., 1981). Adenosine can also be produced by the action of alkaline phosphatases which catalyse hydrolysis of 5'-AMP to adenosine (Fernley, 1971) and by purine nucleotide phosphorylase, which catalyses the production of adenosine from adenine (Murray et al., 1970). However, at neutral pH the activities of these enzymes are low and they are unlikely to be important physiologically.

Extracellular adenosine is produced by a family of membrane associated ecto-5'-nucleotidases which generate adenosine by the dephosphorylation of extracellular adenine nucleotides (Zimmermann, 2000). These enzymes are essential for the nerve-activity dependent production of adenosine from released ATP under physiological conditions (Dunwiddie et al., 1997). Extracellular levels of adenosine can also be increased by the transport of intracellular adenosine across the plasma membrane (see below).
Intracellular adenosine is metabolised in two ways. Adenosine kinase acts on adenosine to form AMP, the reverse reaction to that catalysed by 5'-nucleotidase (Arch & Newsholme, 1978). Adenosine is also broken down to inosine by the actions of adenosine deaminase (Arch & Newsholme, 1978). Whilst adenosine is the major physiological stimulus for adenosine receptors, inosine has also been demonstrated to activate adenosine receptors, although at higher concentrations than adenosine, and with lower efficacy (Fredholm et al., 2001b). Adenosine deaminase can be inhibited experimentally by erythro-9-(2-hydroxy-3-nonyl)adenine and by 2'-deoxycoformycin (Plunkett et al., 1979). Adenosine kinase can be inhibited by 5'-iodotubercidin and 5'-amino-5'-deoxyadenosine (Newby et al., 1983).

Adenosine is a relatively hydrophilic molecule and its uptake and release from cells depends upon specific transporter proteins in the plasma membrane. Adenosine transporters are of two types, equilibrative and concentrative (Baldwin et al., 1999). Equilibrative transporters allow passive diffusion of adenosine across cell membranes, are widely distributed and can be inhibited by coronary vasodilators such as dipyridamole (Griffiths et al., 1997a; Griffiths et al., 1997b). The expression of concentrative transporters is limited to specialised cells such as intestinal and renal epithelia, and these channels mediate the active transport of adenosine against its concentration gradient (Ritzel et al., 1997; Ritzel et al., 1998).
1.4.2. Pharmacological classification of adenosine receptor subtypes

Adenosine receptors were historically classified as purinergic P₁ receptors, and distinguished from purinergic P₂ ATP receptors on the basis of a greater potency of adenosine, a selective antagonism by methylxanthines and coupling to adenylyl cyclase (Burnstock, 1978).

Adenosine receptors were originally subdivided on the basis of their ability to inhibit (A₁) or stimulate adenylyl cyclase (A₂) in cell cultures from perinatal mouse brain (van Calker et al., 1979). A₁ receptors were also shown to be pharmacologically distinct from the A₂ receptor on the basis of the rank orders of agonist potencies. At A₁ receptors the agonist potency order is R-N⁶-phenyl isopropyladenosine (R-PIA) > adenosine > 5′-(N-ethylcarboxamido)-adenosine (NECA) whilst at A₂ receptors the agonist potency order is NECA > adenosine > R-PIA (Londos et al., 1980). Adenosine itself has also been demonstrated to have a potency in the nanomolar range for A₁ receptors (Cohen et al., 1996a) and micromolar potency for A₂ receptors (Daly et al., 1981) although variations in potency are not always indicative of receptor heterogeneity. The xanthine antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) is selective for the adenosine A₁ receptor (Klotz et al., 1998).

The A₂ receptor can be further subdivided into A₂A and A₂B receptors. In general, A₂A receptors bind agonists such as NECA and 2[p-(carboxyethyl)phenethylamino]-5′-N-ethylcarboxamido-adenosine (CGS21680) with high affinity, whilst A₂B receptors have low affinity for agonists (Bruns et al., 1986; Jarvis et al., 1989). Recently, competitive antagonists have been described.
with a high affinity and selectivity for the \( A_{2A} \) receptor (Linden et al., 1999) and \( A_{2B} \) receptor (Kim et al., 2000).

The existence of the adenosine \( A_3 \) receptor was not suspected from the pharmacological analysis of natively expressed adenosine receptors. The characterisation of the human \( A_3 \) receptor recombinantly expressed in Chinese hamster ovary cells demonstrated that the pharmacological properties of the \( A_3 \) receptor were distinct from the other members of the adenosine receptor family (Salvatore et al., 1993). The \( A_3 \) receptor binds adenosine agonists with the rank order \( \text{R-PIA} = \text{NECA} \) but does not bind the \( A_1 \) selective antagonist DPCPX with high affinity. One of the most selective compounds for the human \( A_3 \) receptor is the antagonist MRE 3008F20 (5-N-(4-methoxyphenylcarbamoyl)amino-8-propyl-2-(2-furyl)pyrazolo[4,3-e]-1,2,4-triazolo[1,5-c]pyrimidine, Varani et al., 2000).

1.4.3 Cloning and molecular characterisation of adenosine receptor subtypes.

The adenosine receptor family were amongst the first 7-transmembrane receptors to be cloned and all are archetypal rhodopsin like Family A receptors. The overall and transmembrane domain amino acid identity among the four human adenosine receptors is 30 and 45% respectively (Olah & Stiles, 1995). In general, adenosine receptors are relatively small, with short N-termini. Consensus sequences for N-linked glycosylation exist on the extracellular regions of all adenosine receptors. For the \( A_1 \) and \( A_{2A} \) receptors two sites are present on the second extracellular loop, but none on the N-termini, and both receptors have been demonstrated to be glycosylated in native tissue (Barrington et al., 1990; Stiles, 1986). However, the functional significance of glycosylation is unknown. The C-termini of the \( A_1, A_{2B} \)
and A₃, but not the A₂A receptor, contain a conserved cysteine residue that may be palmitoylated (Olah & Stiles, 2000). Other structural features of adenosine receptors that are common to many GPCRs include the highly conserved Asp-Arg-Tyr sequence in the cytoplasmic end of TM3 and conserved cysteine residues in the first and second extracellular loops that are likely to participate in a disulphide bond (Olah & Stiles, 1995).

The adenosine A₁ receptor was originally identified as the orphan receptor, RDC7, which was cloned from a canine thyroid cDNA library using a PCR homology screening strategy (Libert et al., 1989; Libert et al., 1990a). The cloned receptor was demonstrated to bind the radiolabelled agonist [³H]N⁶-cyclohexyladenosine with high affinity and to inhibit forskolin stimulated cAMP in a similar manner to endogenous adenosine A₁ receptors (Libert et al., 1991). The rat (Mahan et al., 1991; Reppert et al., 1991) and bovine (Olah et al., 1992; Tucker et al., 1992) adenosine A₁ receptors were cloned before three groups cloned the human adenosine A₁ receptor (Libert et al., 1991; Salvatore et al., 1992; Townsend-Nicholson & Shine, 1992). The human A₁ receptor consists of 326 amino acids and shares 95% sequence identity with the rat A₁ receptor and 94% identity with the canine and bovine A₁ receptors. In rat tissue, analysis of mRNA by Northern blotting and in situ hybridisation shows the A₁ receptor to be highly expressed in brain, spinal cord, fat and testis (Olah & Stiles, 1995).

The canine orphan receptor RDC8 (Libert et al., 1990b) was identified simultaneously with RDC7 and was demonstrated to have a similar mRNA tissue distribution in brain as the high affinity A₂A binding sites (Schiffmann et al., 1990). Subsequently RDC8 was demonstrated to mediate stimulation of cAMP
when expressed in Xenopus oocytes and to have similar radioligand binding properties to native A2A receptors, namely high affinity binding of[^3H]NECA and[^3H]CGS21690 when expressed in COS-7 cells (Maenhaut et al., 1990). The rat homologue was simultaneously cloned in 1992 by Chern et al. (1992) and Fink et al. (1992) before the cloning of the human A2A receptor by Furlong et al. (1992) from cDNA isolated from a human hippocampal cDNA library.

The human A2A receptor is relatively large, consisting of 409 amino acids, the increase in size a result of an additional 80-90 amino acids in the C-terminal tail.

The functional significance of this relatively large C-terminus is unclear as canine adenosine A2A receptors, truncated at amino acid position 309, bind adenosine ligands (Piersen et al., 1994) and stimulate adenylyl cyclase (Olah & Stiles, 1995) in a similar manner to wild type receptors. A number of serine and threonine residues in the C-terminus may be phosphorylation sites for G-protein coupled receptor kinases and may explain the rapid desensitisation response of the A2A but not the A1 receptor which lacks these residues (Ramkumar et al., 1991). The expression of A2A receptor mRNA by Northern blot analysis, in situ hybridisation, and autoradiography indicate a more restricted expression compared to the A1 receptor, with high levels in striatum where the receptor colocalises with dopamine D2 receptor mRNA (Fink et al., 1992; Olah & Stiles, 1995). In the periphery, high levels of A2A receptor expression are found in spleen, thymus, leukocytes and platelets (Fredholm et al., 2001a).

The rat brain cDNA clone RFL9 was demonstrated to have 45 and 46% homology with rat A1 and A2A receptor respectively and have an mRNA distribution distinct from A1 and A2A receptors (Stehle et al., 1992). When expressed in CHO cells,
RFL9 was demonstrated to stimulate cAMP and to have similar pharmacological properties to the human $A_{2B}$ receptor endogenously expressed in the human fibroblastic cell line VA 13 (Rivkees & Reppert, 1992). The human $A_{2B}$ receptor was cloned from a hippocampal cDNA library (Pierce et al., 1992), and highest expression is found in caecum, colon and bladder (Fredholm et al., 2001a). The $A_{2B}$ receptor is of similar size to the $A_1$ and $A_3$ receptors and shares 73% transmembrane region homology with the $A_{2A}$ receptor (Olah & Stiles, 1995).

The existence of the adenosine $A_3$ receptor was not suspected from the binding and functional properties of adenosine receptors in native tissues. The rat $A_3$ receptor was identified as a novel clone from cDNA libraries from testis (Meyerhof et al., 1991) and striatum (Zhou et al., 1992) before the human receptor was identified from a striatal cDNA library using a probe derived from the rat sequence (Salvatore et al., 1993). The distribution of the $A_3$ receptor is not as widespread as the other receptor subtypes and demonstrates marked species differences, being expressed highly in testis and mast cells of rat, and liver and lung of human, which suggests that the physiological role of the $A_3$ receptor may be different between species (Olah & Stiles, 1995). The $A_3$ receptor consists of 318 amino acids, is similar in size to the $A_1$ receptor, and displays ~49% overall homology with the $A_1$ receptor (Olah & Stiles, 2000). The $A_3$ receptor is unusual in that it contains a consensus site for N-linked glycosylation on both the N-terminus and the second extracellular loop (Olah & Stiles, 1995).
1.4.4 Signalling of adenosine receptors

The signalling of adenosine receptors forms part of the basis of the classification of adenosine receptor subtypes. Thus, A₁ and A₃ receptors preferentially couple to pertussis toxin sensitive Gi proteins and mediate inhibition of adenylyl cyclase, whilst A₂A and A₂B receptors couple to Gs proteins and mediate an increase in intracellular levels of cAMP (Fredholm et al., 2001a).

Whilst the primary signalling pathway of A₂ and A₃ receptors is thought to be the modulation of adenylyl cyclase it is now recognised that the A₁ receptor is able to activate multiple signalling pathways (Fredholm et al., 2001a). Coupling to multiple G-protein subtypes (G-protein promiscuity) may be an important element to this heterogeneity in cell signalling. Thus, the adenosine A₁ receptor stably expressed in CHO cells has been demonstrated to activate Gα₆, Gα₄₁ and Gα₄ in CHO cell membranes, as determined by immunoprecipitation of the Gα subunit (Cordeaux et al., 2000).

1.4.4.1 Adenylyl cyclase

Agonist activation of the adenosine A₁ receptor leads to a decrease in cAMP levels in a number of tissues including brain (van Calker et al., 1979), cardiac myocytes (Martens et al., 1987), adipocytes (Londos et al., 1980) and DDT₁ MF-2 smooth muscle cells (Ramkumar et al., 1991). When expressed in cell lines, the A₁ receptor is also capable of decreasing cAMP levels (Reppert et al., 1991). The inhibitory effect of adenosine A₁ receptor activation on cAMP levels is abolished by pertussis toxin, indicating that this response is mediated by Gα₁₃₀ G-proteins (Akbar et al., 1994; Freund et al., 1994).
The A₁ receptor has been demonstrated to interact directly with G-proteins of the Gα₁<sub>o</sub> class. Agonist affinity chromatography of detergent solubilised bovine brain membranes results in the co-purification of the A₁ receptor and Gα₁₁, Gα₁₂ and Gα₆ (Munshi et al., 1991). When reconstituted into human platelet membranes, the A₁ receptor was able to activate native G-proteins in a pertussis toxin sensitive manner (Munshi & Linden, 1990). Immunoprecipitation experiments demonstrate that the human adenosine A₁ receptor stably expressed in CHO cells can activate Gα<i><sub>q</sub></i>(1.3) (Cordeaux et al., 2000). In CHO cells transfected with Gα subunits which have been rendered resistant to pertussis toxin by a cysteine-glycine mutation in the C-termini, activation of Gα₁₁, Gα₁₂, and Gα₁₃ by the human adenosine A₁ receptor has been demonstrated (Wise et al., 1999).

1.4.4.2 Inositol phosphate and diacylglycerol.

Intracellular concentrations of calcium can be elevated by GPCRs via activation of Gq G-proteins. Gα<sub>q</sub> activates a number of isoforms of phospholipase C which cleaves phosphatidylinositol-4,5-bisphosphate to inositol trisphosphate (IP₃) and diacylglycerol (DAG, Berridge, 1993). IP₃ activates specific channel-containing receptors in the endoplasmic reticulum to release stored calcium into the cytosol (Clapham, 1995) whilst DAG activates protein kinase C (Nishizuka, 1988).

The adenosine A₁ receptor has been demonstrated to mediate inositol phospholipid hydrolysis and the mobilization of intracellular calcium for both recombinant (Akbar et al., 1994; Freund et al., 1994) and endogenously expressed (Dickenson & Hill, 1993; Freund et al., 1994; Gerwins & Fredholm, 1992) receptors. These effects are abolished by pertussis toxin, suggesting a role for
Goq/0 rather than a classical Goq mediated effect (Dickenson & Hill, 1993; Gerwins & Fredholm, 1992). Furthermore, adenosine receptor activation can enhance the inositol phosphate response of agonists of Goq coupled receptors (Gerwins & Fredholm, 1992; Megson et al., 1995). The effects of adenosine receptor activation on inositol phosphate hydrolysis appear in part to be mediated by G-protein βγ subunits as the expression of Gβγ-scavenging proteins such as the C-terminus of βARK1 can partially inhibit the effects of adenosine receptor activation of phospholipase C without affecting the inhibition of cAMP levels (Dickenson & Hill, 1998).

1.4.4.3 Ion channels

In cardiac atrial cells (Kurachi et al., 1986b), ventricular myocytes (Kirsch et al., 1990) and striatal neurons (Trussell & Jackson, 1985) adenosine modulates membrane excitability. This effect is mediated by the activation of a family of inwardly rectifying potassium channels, termed GIRKs (G-protein activated inwardly rectifying K+ channel, Dascal, 1997). There are five members of the GIRK family that can form homo- and heterotrimeric channels with different gating properties. The best studied of these is GIRK1 which is found in cardiac atrial cells and activated by a number of GPCRs including adenosine A1, 5-HT1A, muscarinic M2 and δ-opioid receptors (Dascal et al., 1993; Kurachi et al., 1986b). GIRK channel opening has been reported to be mediated by both Gα (Kirsch et al., 1990) and βγ subunits (Logothetis et al., 1987) with activation being sensitive to pertussis toxin (Kurachi et al., 1986a). GIRKs are the only ion channels demonstrated conclusively to be activated by a direct interaction with G protein βγ.
subunits (Dascal, 1997). Indeed, GIRK was the first βγ effector identified (Logothetis et al., 1987). Recently it has been reported that Gα has a role in regulating the basal activity of GIRK channels (Peleg et al., 2002).

Adenosine A_1 receptors have been reported to inhibit calcium channel opening in neurons (Fredholm & Dunwiddie, 1988; Olsson & Pearson, 1990). This effect may occur directly via action of a G-protein (Wu & Saggau, 1994) or indirectly via K^+ channel mediated membrane hyperpolarisation. A_1 receptors have also been demonstrated to modulate Cl^- conductance in brain (Schubert & Mager, 1989) and kidney cells (Kelley et al., 1990).

1.4.4.4 MAP kinases

The MAP (mitogen activated protein) kinase signalling cascade involves up to six tiers of cytoplasmic kinases which transfer extracellular signals from membrane receptors to the cytoplasm and nucleus by a series of sequential phosphorylation steps (Seger & Krebs, 1995). The family of MAP kinases consists of the extracellular regulated kinases (ERK) and the stress activated protein kinases (SAPK), which include p38 and jun-N-terminal kinase (JNK), and play an essential role in cell differentiation, proliferation, survival and death by regulating gene expression (Gutkind, 1998; Johnson & Lapadat, 2002). Whilst these kinases are considered classically to be mediated by receptor tyrosine kinases, (Seger & Krebs, 1995) there is a growing interest in the ability of 7TM GPCRs to modulate proliferative cell signalling pathways such as MAP kinase (Gutkind, 1998; Inglese et al., 1995).
Adenosine receptors have been demonstrated to modulate the regulation and proliferation of vascular smooth muscle cells (Jonzon et al., 1985) which may suggest a role for the MAP kinase cascade. When transiently expressed in COS-7 cells (Faure et al., 1994) or stably expressed in CHO cells (Schulte & Fredholm, 2000) adenosine A\textsubscript{1} receptors can mediate the activation of ERK1/2 in a pertussis toxin sensitive manner. Indeed, all four adenosine receptor subtypes are capable of stimulating ERK phosphorylation when stably expressed in CHO cells (Schulte & Fredholm, 2000). The effects of adenosine A\textsubscript{1} receptor activation on MAPK activation appear, in part, to be mediated by G-protein \(\beta\gamma\) subunits (Dickenson et al., 1998; Faure et al., 1994) and are independent of protein kinase C activation (Dickenson et al., 1998).

In summary, both endogenous and recombinantly expressed adenosine A\textsubscript{1} receptors are able to activate a diverse range of intracellular signalling pathways. Whilst the A\textsubscript{1} receptor can be demonstrated to interact promiscuously with a number of different G-protein subtypes, the effects of A\textsubscript{1} receptor activation appear to be mediated primarily by \(G\alpha_i\) and/or its associated \(\beta\gamma\) subunits.
1.5 Allosteric modulation of 7TM GPCRs

1.5.1 The Allosteric Ternary Complex Model

Allosteric interactions occur when the binding of a ligand to a site (termed the allosteric site) that is distinct from the primary (orthosteric) binding site modifies the binding of the primary ligand. The simplest description of this interaction is given by the allosteric ternary complex model which is identical to the ternary complex model illustrated in Figure 1.3a, with the exception that G is replaced by an exogenous allosteric ligand X and \( K_X \) is the affinity constant for the binding of X to R. The principal difference between the allosteric ternary complex model and the ternary complex model of agonism is that in the former the concentration of the allosteric ligand is known because it is an exogenously applied ligand, whilst in the latter the concentration of allosteric ligand is unknown because it is a G-protein. This important difference allows the quantitation of the allosteric interaction between two exogenously applied ligands at a receptor (Lazareno & Birdsall, 1995).

Allosteric effects are observed if the ternary complex ARX can form and the binding of one ligand alters the binding of the second ligand. If the affinity of A is increased in the presence of X, \( \alpha > 1 \) and positive cooperativity is demonstrated. If the affinity of A is decreased in the presence of X, \( \alpha < 1 \) and negative cooperativity will be demonstrated. When \( \alpha = 1 \) the interaction is neutrally cooperative. Allosteric interactions are unique for each receptor subtype and each ligand pair and the magnitude of the cooperative interaction is reciprocal in that if
X affects the binding of A then the binding of X is modified with the same magnitude and direction as that of A.

In the special case when $\alpha = 1$ the equilibrium binding of A will not be affected by the presence of X, and the allosteric ligand will appear to be inactive. However, allosteric ligands may also alter the kinetics of ligand dissociation (Lazareno & Birdsall, 1995; Leppik et al., 1998), a property not shared by competitive ligands, which can only bind to the unoccupied primary binding site and consequently decrease the apparent affinity of a ligand. The effect of a compound on the dissociation kinetics of ligand at the primary site therefore provides a test for the presence of an allosteric interaction. X may also bind to the allosteric binding site but have no effect on either the equilibrium or kinetic binding properties of A. The activity of such a ligand can still be detected and quantitated (Leppik et al., 1998). It will behave as a competitive antagonist of the equilibrium and kinetic effects of another allosteric ligand which binds to the same site.

1.5.2 Allosteric modulation of G-protein coupled receptors

Allosteric binding sites have been demonstrated at a number of 7TM GPCRs. By far the best characterised are the muscarinic receptors which have a well defined allosteric binding site on all five receptor subtypes (Lazareno et al., 1998). In general, the behaviour of allosteric ligands at muscarinic receptors is consistent with the scheme in Fig. 1.3a.

An allosteric interaction at muscarinic receptors was first demonstrated for the neuromuscular blocker gallamine at the $M_2$ receptor which displays negative cooperativity for a range of muscarinic ligands (Clarke & Mitchelson, 1976;
Positive cooperativity was demonstrated for the neuromuscular blocker alcuronium, which exhibits a 4-fold positive cooperativity with the antagonist $[^3\text{H}]$NMS (Tucek et al., 1990). This study also showed that the cooperative effects of alcuronium were dependent on both the receptor subtype and the radioligand used. More recently, a second allosteric binding site has been demonstrated on $M_1$ receptors which can also mediate positive cooperativity with acetylcholine (Lazareno et al., 2002; Lazareno et al., 2000).

Amiloride and amiloride analogues have been shown to increase the off-rate of radiolabelled antagonist from both the $\alpha_{1A}$ and $\alpha_{2A}$-adrenergic receptors in a manner consistent with the allosteric ternary complex model (Leppik et al., 1998; Leppik et al., 2000). Amiloride and amiloride analogues have also been shown to enhance the off-rate of radiolabelled antagonist from $D_2$ dopamine receptors (Hoare & Strange, 1996).

### 1.5.3. Allosteric modulation of adenosine $A_1$ receptors

A series of 2-amino-3-benzoylthiophenes have been shown to have allosteric properties at the adenosine $A_1$ receptor (Bruns & Fergus, 1990; Bruns et al., 1990). The best characterized of this series of compounds is 2-amino-4,5-dimethylthien-3-yl [3-(trifluoromethyl) phenyl]-methanone (PD 81,723) which has been shown to slow down agonist dissociation and enhance agonist binding in rat brain (Bruns & Fergus, 1990; Jarvis et al., 1999), guinea pig forebrain (Kollias-Baker et al., 1994) and at the human recombinant $A_1$ receptor expressed in CHO cells (Bhattacharya & Linden, 1995; Cohen, 1995). PD 81,723 also potentiates the functional responses of adenosine receptor agonists in a range of functional
assays from membranes, whole cells and isolated intact tissues (Bruns & Fergus, 1990; Cohen, 1995; Kollias-Baker et al., 1994). More recently a number of analogues of PD 81,723 have been described (Beuters et al., 2002; van der Klein et al., 1999), none of which, however, are more potent than PD 81,723.

In equilibrium studies at the G-protein uncoupled adenosine A₁ receptor (in the presence of GTP), PD 81,723 increased the affinity of the agonists CHA, NECA, PIA and the endogenous agonist adenosine by 10-12 fold (Cohen, 1995). These data were analysed with the allosteric ternary complex model to obtain the affinity and cooperativity values for PD 81,723. Therefore, at the uncoupled A₁ receptor PD 81,723 interacts with adenosine agonists via an allosteric site in a similar manner to allosteric ligands acting at muscarinic receptors.

In equilibrium binding studies at the high affinity, G-protein coupled adenosine A₁ receptor, PD 81,723 does not behave in a manner which is consistent with the simple allosteric ternary complex model. There are no reports of PD 81,723 enhancing the affinity of agonists by more than 2-fold at the G-protein coupled A₁ receptor, which is surprising given the 10-fold increase in affinity at the uncoupled receptor. In addition, there are conflicting reports of the effects of PD 81,723 on the Bmax of radiolabelled agonists. In guinea pig forebrain PD 81,723 increased the Bmax of [³H]CHA three fold (Kollias-Baker et al., 1994) and in CHO cells expressing the recombinant A₁ receptor (Bhattacharya & Linden, 1995) the Bmax of the agonist [¹²⁵I]ABA was increased by the same amount. In contrast PD 81,723 had no effect on the Bmax of [³H]CCPA in rat brain (Kourounakis et al., 2000) or [³H]CHA in CHO A₁ cell membranes (Cohen, 1995). This discrepancy, in part, is due to differences in the ionic composition of the buffers used in the
various binding experiments, as the effects of PD 81,723 on agonist binding to the G-protein coupled state of the A1 receptor is less evident at high concentrations of Mg2+ (Cohen, 1995). Observations that the effects of PD 81,723 are sensitive to the G-protein coupled state of the A1 receptor are supported by results showing that PD 81,723 decreases the potency of guanine nucleotides to inhibit radiolabelled agonist binding (Bhattacharya & Linden, 1995; Kollias-Baker et al., 1994). In addition to potentiating the responses of adenosine agonists in functional assays, PD 81,723 has been shown have agonist properties which are independent of endogenous adenosine (Kollias-Baker et al., 1997; Musser et al., 1999).

Therefore, the effects of PD 81,723 on agonist binding to the coupled A1 receptor and in functional assays indicate a complex mechanism of action which is not predicted by the simple allosteric ternary complex model. At present no molecular scheme has been proposed which can explain all of the actions of PD 81,723.

1.5.4 The potential therapeutic advantages of allosteric modulators of 7TM GPCRs

Many drugs used clinically mediate their effects by either stimulating or blocking 7TM GPCRs at the primary ligand binding site (Drew, 1996). This is a very crude way of regulating the functions of 7TM receptors when compared with the tightly controlled spatial and temporal elements of neurotransmission in vivo. Agonist therapies are often associated with receptor desensitisation and downregulation whilst side effects frequently occur because of a lack of receptor and tissue selectivity.
Allosteric ligands may offer a number of advantages over drugs targeting the primary ligand binding site. Allosteric ligands are generally without effect unless in the presence of the endogenous ligand and will thus mimic the temporal aspects of neurotransmission. In addition, the effect of an allosteric ligand on the binding of an endogenous ligand is saturable, that is once the allosteric site is fully occupied no further effects on agonist binding are observed. This 'ceiling' of activity is defined by the cooperativity constant and suggests that allosteric ligands as therapeutic agents would be less likely to show mechanism-related toxicity (Fig. 1.4).

An example of allosteric ligands in clinical use is the benzodiazepines. These compounds bind to an allosteric binding site on the GABA**A** receptor (a ligand gated chloride ion channel) and stabilise an 'open' form of the GABA**A** receptor to favour the binding of the endogenous agonist (Ehlert, 1986). The allosteric mechanism of action of benzodiazepines provides a rationalisation of their acceptable side effect profile.

The lack of selective ligands, and in particular agonists, for muscarinic receptors is thought to result from the high degree of amino acid sequence homology in the acetylcholine ligand binding domain in all five receptor subtypes (Hulme et al., 1990). However, the allosteric binding site of muscarinic receptors may be less well conserved (Matsui et al., 1995) and thus allow the development of subtype selective allosteric ligands. In addition, the principle of neutral cooperativity allows for the possibility of absolute receptor subtype selectivity, which is independent of the concentration of the allosteric ligand. This can be achieved with an allosteric ligand which has cooperativity (positive or negative) at the
Figure 1.4. The potential therapeutic benefits of an allosteric enhancer.

Panel a. A hypothetical response of a receptor to a nerve impulse (arrow) in normal tissue and a smaller response in diseased tissue where the symptoms result from a local deficit in neurotransmitter release. An allosteric enhancer mediates its pharmacological action only whilst the neurotransmitter is released. Hence, it augments the receptor response in a temporal manner. The maximum amplification of the response is restricted to be equal to or less than the cooperativity factor and the enhancement will be greater in the diseased tissue, i.e. where it is needed. In contrast, conventional agonist replacement therapy is associated with continuous receptor activation and the maximum response may exceed that elicited by the endogenous ligand.

Panel b. In another tissue unrelated to the disease where the neurotransmitter is not released, the allosteric enhancer has no action even if the receptors are present. In contrast, agonist replacement therapy results in continuous receptor activation and may be associated with unwanted additional effects of the drug in this tissue.
Normal Disease

(a) untreated + allosteric enhancer + agonist replacement

(b)

response vs. time
receptor of interest and neutral cooperativity at the remaining receptor subtypes. This has been demonstrated for brucine analogues at muscarinic receptors (Lazarenco et al., 1998).

Allosteric enhancers may be of use in diseases associated with a deficit of endogenous neurotransmitter such as acetylcholine (Alzheimer's disease) and dopamine (Parkinson's disease). A number of diseases are associated with a focal release of adenosine (During & Spencer, 1992; Fenton & Dobson, 1987; Hagberg et al., 1987) in response to an increase in metabolic demand, in an attempt to conserve oxygen and to maintain cellular viability. An adenosine receptor enhancer could increase the cytoprotective effect of adenosine in areas of hypoxia or ischaemia, whilst having little or no effect on adenosine receptor function in tissues that are not being exposed to released adenosine.
1.6 Project Aims

The overall aim of this project is to characterise the binding, pharmacological and functional properties of the human adenosine A1 receptor stably expressed in CHO cells at different levels of expression and to attempt to incorporate the results within mathematical models of receptor binding and function. The thesis is divided into three experimental chapters:

i) The radioligand binding studies evaluated the effects of receptor expression levels on the actions of guanine nucleotides, agonists of varying efficacy, and the kinetics of agonist and antagonist dissociation. The ability of the ternary complex model to predict the experimental observations was evaluated.

ii) Receptor activation, assessed in a [35S]GTPγS assay, was monitored as a function of ligand efficacy and expression level. During the course of these studies a novel inhibitory response following activation of the A1 receptor was observed. This response was characterised in detail and experiments were performed to understand the basis for this response.

iii) Finally, the effect of the allosteric enhancer PD 81,723 on the binding and functional properties of the adenosine A1 receptor expressed at different levels was evaluated. Attempts were made to incorporate these results within an allosteric model of receptor - G-protein interaction.
Chapter 2. Materials and Methods

2.1 Materials

*Tissue culture*: Chinese hamster ovary cells stably expressing high (HE) and low (LE) levels of the adenosine A₁ receptor were a kind gift from Prof. Stephen Hill, Nottingham University; DMEM/HAMS F-12 was from SIGMA. Foetal calf serum was from Global Phamaceuticals, genetecin was from GIBCO. Penicillin/Streptomycin, trypsin/versene and phosphate buffered saline were from media supplies, National Institute for Medical Research, Mill Hill. All tissue culture plastics were from Costar.

*Drugs*: PD 81,723 was a kind gift from Parke-Davis. GR 162900, GR 161149 and GR 190178 (Fig. 2.1) were synthesised by the medicinal chemists of GlaxoWellcome, Stevenage. ADA was from Calbiochem. Adenosine, BSA, DPCPX, CHA, DMSO, GDP, GTP, HEPES, NECA, R-PIA, saponin (catalogue # S1252), Sephadex G50M were from Sigma, Poole, Dorset. EDTA, NaCl, MgCl₂, were from BDH-Merck.

*Radioligands*: [³H]CHA (30 Ci.mmol⁻¹), [³H]DPCPX (110-120 Ci.mmol⁻¹) and [¹⁵S]GTPγS (1250 Ci.mmol⁻¹) were from Dupont-NEN. [³H]NECA and [³H]R-PIA (20-30 Ci.mmol⁻¹) were from Amersham Biosciences.

Readysafe liquid scintillation cocktail and 6ml. Pony vials were from Beckman.

Glass fibre filter paper (GF/B) was from Whatman; 96 well GF/B filter mats and
Figure 2.1. Structures of the compounds used in this study.

The chemical structures of the high efficacy agonist CHA, the lower efficacy agonists GR190178, GR161144 and GR162900, the antagonist/inverse agonist DPCPX and the allosteric enhancer PD 81,723. Adenosine is also included for comparison.
solid scintillant were from Wallac; 5ml polyprep gel filtration columns were from Biorad.

2.2. Experimental Procedures

2.2.1. Tissue culture

CHO cells expressing the human recombinant adenosine A₁ receptor were cultured in DMEM/F12 medium containing 15mM HEPES, pyridoxine and sodium bicarbonate, supplemented with 10% foetal calf serum, 2mM glutamine, 1mg.ml⁻¹ genetecin, and penicillin/streptomycin at 37°C in 5% CO₂. Cells were generally subcultured in 175cm² tissue culture flasks twice weekly at a ratio of 1:10. For membrane preparations, cells were seeded into 850cm² roller bottles in 100ml tissue culture medium (without genetecin) and rotated at 0.2 rpm in normal air at 37°C. Cells were typically confluent in 2 days.

2.2.2 Membrane Preparation

Confluent CHO cells in 850cm² roller bottles were washed with phosphate buffered saline and then incubated in 30 ml homogenisation buffer (20mM HEPES, 10mM EDTA, pH 7.4) whilst being rotated at 2 rpm. After a brief period of time, cells were detached by gently tapping the roller bottles. Cells were disrupted using an Ultraturrax homogeniser (3 x 10 second bursts, full power at 4°C). Membrane and cytosolic fractions were separated by centrifugation at 40,000g for 20 min. The supernatant was discarded and the membrane pellet re-homogenised in buffer (20mM HEPES, 0.1mM EDTA, pH 7.4) as above. The homogenate was then centrifuged at the same speed for 20 min. The pellet was
then resuspended at a concentration of ~1mg protein.ml\(^{-1}\) in binding buffer (20mM HEPES, 100mM NaCl, 10mM MgCl\(_2\), pH 7.4) and stored at -70°C in 1ml aliquots. The membrane protein concentration was determined using the method of Bradford (1976), using bovine serum albumin as a standard reference. The yield of protein was typically 5-10mg per roller bottle.

### 2.2.3 Radioligand binding

CHO cell membranes were incubated with ADA (3U.ml\(^{-1}\)) for 30 min. at 25°C to catabolise the endogenous adenosine. Typically, membranes from low expressing CHO A\(_1\) cells (20-40\(\mu\)g) or high expressing (1-2\(\mu\)g) were added to radioligand, competing ligand and saponin (30\(\mu\)g.ml\(^{-1}\)) in a final volume of 1ml in a buffer of the following composition: 20mM HEPES, 100mM NaCl, 10mM MgCl\(_2\), pH 7.4. Membranes were incubated for 60 min. at 23±1°C. Non-specific binding was determined as the radioligand bound in the presence of 3mM theophylline. Saponin, guanine nucleotides and theophylline were soluble in binding buffer and fresh solutions were made on the day of the experiment. In general, adenosine agonists and antagonists, and PD 81,723 were stored as aliquots in 10mM solutions in DMSO. No loss of activity was detected with storage time. The final concentration of DMSO was typically 1-2%, which had no effect of the binding of either \(^3\)H]DPCPX or \(^3\)H]CHA. All binding experiments were performed in either duplicate or triplicate.
Saponin was included in all saturation and competition binding experiments as previous studies have shown it to be necessary for the complete conversion of the A1 receptor to its low affinity state by guanine nucleotides (Cohen et al., 1996b).

2.2.3.1 Saturation Binding

Following incubation with adenosine deaminase as described above, membranes were incubated with increasing concentrations of either [³H]DPCPX or [³H]CHA in the absence (total binding) or presence (non-specific binding) of 3mM theophylline. The concentrations of radioligand spanned an approximate range of 10-0.1nM, each concentration being 2-fold lower than the preceding concentration. After a 60 min. incubation at room temperature, bound and free radioligand were separated by rapid filtration and washing with ice cold distilled water using a 48 well Brandel cell harvester.

Binding reactions were carried out in 75mm polypropylene tubes (Rohren). Bound radioactivity was determined by punching the samples into liquid scintillation vials and 4ml scintillation fluid was added. The samples were counted on a Beckman liquid scintillation counter once the samples had been left to stand overnight at room temperature to permit the samples to become universally translucent.

2.2.3.2 Competition Binding

Following incubation with adenosine deaminase, membranes were added to increasing concentrations of unlabelled ligand in the presence of [³H]DPCPX (0.4-
0.6nM) or \[^{3}\text{H}]\text{CHA} (1-2\text{nM}) and saponin (30\mu\text{g.ml}^{-1}) in the absence or presence of guanine nucleotides or PD 81,723 (PD). In general, unlabelled ligands spanned the concentration range, 100\mu\text{M}-0.1\text{pM}, using a 0.5 log unit dilution series. In experiments where the effect of increasing concentrations of guanine nucleotides on radiolabelled agonist binding was investigated, the guanine nucleotides were treated as described above for an unlabelled ligand.

Bound and free radioligand were separated by rapid filtration and washing with ice cold distilled water using either a 48 well Brandel cell harvester or a Tomtek cell harvester, using GF/C glass fibre filter paper. Glass fibre filters from the Brandel harvester were processed as described above.

When the Tomtek harvester was used, binding reactions were carried out in 1.2 ml 96 well deep well blocks (Receptor Technologies ltd). The Tomtek cell harvester allows the simultaneous filtration of 96 samples onto an 8x12 GF/B filtermat (Wallac). After filtering, the filtermat was dried at room temperature overnight. Solid scintillant (Meltilex, Wallac) was then melted onto the filtermat, and the radioactivity detected by scintillation spectrometry using a Wallac Trilux Microbeta counter. Ligand affinity data determined using the Tomtek harvester were identical to that generated on the Brandel harvester.

2.2.3.3 Kinetics of radioligand dissociation.

The dissociation of \[^{3}\text{H}]\text{DPCPX} and \[^{3}\text{H}]\text{CHA} from the adenosine A\text{1} receptor was performed using a reverse time course strategy to allow the batchwise processing of samples by simultaneous filtration (Hulme & Birdsall, 1992). After
incubating with ADA, membranes were added to radioligand and incubated for 60 min. at 23±1° in a volume of 50-100µl. Dissociation was initiated by the addition of 2ml of buffer containing either vehicle (1% DMSO), 100µM CHA, 1µM DPCPX or 100µM GTP. Membranes were kept on ice prior to being added to radioligand to ensure membrane, receptor, and G-protein stability when long time courses were being evaluated.

2.2.4 [35S]GTPγS functional assays

2.2.4.1 Measurements of agonist potency and antagonist affinity.

After incubation with ADA, membranes from LE and HE CHO A1 cells (20µg per assay point) were incubated with GDP (10µM), [35S]GTPγS (0.1nM) and increasing concentrations of agonist for 30 min. at 30°C in a final volume of 1ml in a buffer consisting of 20mM HEPES, 100mM NaCl, 10mM MgCl2, pH 7.4. At this time the binding reaction was terminated by rapid filtration and washing with ice cold distilled water. The radioactivity which had been trapped to the filter was determined using liquid scintillation spectrometry as described in section 2.2.3.1.

In experiments where the effects of the antagonist DPCPX on the functional responses to CHA were investigated, membranes were first incubated with GDP, agonist and antagonist for 30 min. at 30°C in a final volume of 1ml. This was to allow both agonist and DPCPX to equilibrate with the receptor. After this time [35S]GTPγS (10µl, 0.1nM final assay concentration) was added and the tubes were vortexed and incubated for a further 30 min. at 30°C. There was no difference
between the agonist control concentration-response curves (in the absence of antagonist) in this experiment, and the agonist concentration-response curves determined as described in the previous paragraph, providing evidence that the agonist-receptor-G-protein-GDP system equilibrated rapidly.

2.2.4.2. Gel filtration of \[^{35}\text{S}]\text{GTP} \gamma \text{S supernatant.}

To detect soluble G-protein G\(\alpha\) subunits, Sephadex G50M (Sigma) which has a molecular weight fractionation range of 1.5-30,000 was used. This gel should exclude globular proteins with a mass of a G-protein G\(\alpha\) subunit (40-44,000 Daltons, Hepler & Gilman, 1992).

Sephadex was prepared by soaking overnight in excess water at room temperature. The Sephadex gel was then washed twice in water to remove fines and then water added to twice the volume of the gel. 4ml of the gel was added to each Polyprep column and 2 x 4ml assay buffer was run through the column.

1ml aliquots of CHO cell membranes stably expressing a low or high level of the adenosine A\(_1\) receptor were thawed from -70°C, diluted to 200\(\mu\)g.ml\(^{-1}\) membrane protein, homogenised with an Ultraturrax homogeniser (3x5 seconds full power) and incubated with 3U.ml\(^{-1}\) adenosine deaminase for 30 min. at room temperature. The membrane suspension was added to GDP (10\(\mu\)M final concentration), \[^{35}\text{S}]\text{GTP} \gamma \text{S} (0.1nM final concentration) and agonist or vehicle (10% DMSO) and incubated for 30 min. at 30°C. Membranes were either filtered through Whatman GF/B filter paper and washed three times with ice cold distilled water for determination of the total membrane bound \[^{35}\text{S}]\text{GTP} \gamma \text{S}, or placed on ice for 5
min. and then centrifuged at 40,000 g. 0.2ml of the supernatant from the centrifuged samples was added to the Sephadex column and eluted with repeated additions of 0.2 ml ice cold assay buffer (all at 4°C). The total radioligand in each sample (0.2mls of eluate or Whatman GF/B filter disc) was determined by liquid scintillation spectroscopy using 4ml of Readysafe liquid scintillation cocktail.

2.2.4.3. Kinetics of \[^{35}S\]GTP\(\gamma\)S association

The effect of adenosine receptor ligands on the association of \[^{35}S\]GTP\(\gamma\)S to CHO A\(_1\) membranes was determined using a reverse time course strategy to allow the batchwise simultaneous filtration of samples (Hulme & Birdsall, 1992).

Membranes from CHO A\(_1\) HE and LE cells were resuspended to the appropriate protein concentration and incubated with ADA for 30 min. at room temperature, after which the membrane suspensions were placed on ice. Assay tubes were prepared by adding 10\(\mu\)l of GDP and 10\(\mu\)l of either vehicle (1% DMSO final assay concentration), DPCPX (1\(\mu\)M final assay concentration) or CHA (0.1nM - 10 \(\mu\)M final assay concentration). Membranes were added to tubes in a time-staggered manner, and incubated for 30 min. at 30°C. After this time, 10\(\mu\)l of \[^{35}S\]GTP\(\gamma\)S (0.1 or 1nM final assay concentration) was added, the tubes were vortexed, and incubated for the stated period of association time at 30°C. At the end of the experiment, the binding reaction was stopped by the addition of ice cold distilled water followed by rapid filtration and washing as described above. Bound radioactivity was determined by liquid scintillation spectrometry as described above.
2.3 Data analysis

In general, each experiment was performed in duplicate or triplicate and the data expressed as the mean ± s.e. mean for triplicate data points, and the mean ± range/2 for duplicate data points.

Concentration-response curves were analysed individually using non-linear least squares regression using the software package Graphpad Prism. The equations used are described in the following sections.

2.3.1 Analysis of radioligand binding data.

2.3.1.1 Saturation binding

Non-specific binding was a linear function of the free concentration of radioligand and analysed using linear regression. Specific binding was calculated as follows:

\[ \text{specific binding} = \text{total binding} - (M \cdot L + C) \]

where \( L \) = concentration of unbound radioligand and \( M \) and \( C \) are the parameters obtained from the linear regression.

Specific binding was analysed by non-linear regression using the following equation:

\[ \text{specific binding} = \frac{B_{\text{max}} \cdot L}{L + K_D} \]

where \( B_{\text{max}} \) = total concentration of receptors, \( K_D \) = radioligand dissociation constant.
2.3.1.2 Competition binding

Competition binding curves were analysed by non-linear regression using a one site and two site equation and the best fit equation determined by performing an F-test. The two site equation was taken as the better fit when P < 0.05.

1-site

\[
\text{Bound radioactivity} = \text{NSB} + \frac{(\text{Total Binding} - \text{NSB})}{(1 + 10^{(A-\log \text{IC}_{50})})}
\]

2-site

\[
\text{Bound radioactivity} = \text{NSB} + \frac{(\text{Total binding} - \text{NSB}) \cdot (f_{R_H} / (1 + 10^{(A-\log \text{IC}_{50H})})) + (1 - f_{R_H}) / (1 + 10^{(A-\log \text{IC}_{50L})})}{(1 + 10^{(A-\log \text{IC}_{50})})}
\]

where NSB = non-specific binding; A = log (concentration of unlabelled ligand); frH = fraction of high affinity binding sites; IC_{50L} and IC_{50H} = mid-point curve locations for low and high affinity sites respectively. pIC_{50} values were converted to pKi values using the method of Cheng & Prusoff (1973).

In some cases radioligand competition binding data were analysed by fitting to an equation for a single site with a slope factor:

\[
\text{Bound radioactivity} = \text{NSB} + \frac{(\text{Total binding} - \text{NSB})}{(1 + 10^{((A-\log \text{EC}_{50}) \cdot \text{HillSlope})})}
\]

2.3.1.3 Radioligand dissociation

Radioligand dissociation data were analysed by fitting to the following equation:

\[
B_t = (B_0 - B_{\text{min}}) e^{-k_1 t} + B_{\text{min}}
\]
Where $B_t = \text{the binding determined at time } t$, $B_0 = \text{the binding at time } t = 0$, $B_{\text{min}} = \text{the plateau level to which the binding decays}$ and $k = \text{the rate constant}$. The half life is $0.69/k$.

### 2.3.2. Analysis of $[^{35}\text{S}]\text{GTP}y\text{S}$ Functional Data

#### 2.3.2.1 Analysis of agonist concentration-response curves

Agonist concentration-effect curves from $[^{35}\text{S}]\text{GTP}y\text{S}$ binding experiments were fit to a monophasic sigmoid equation of the following form:

$$\text{Response} = \text{Bottom} + \frac{(\text{Top-Bottom})}{(1+10^{(\log \text{EC}_{50}-X)\text{HillSlope}})}$$

or a biphasic equation of the form:

$$\text{Response} = \text{Bottom} + \frac{(\text{max}_1-\text{Bottom})}{(1+10^{(\log \text{EC}_{50,1}-X)})} - \frac{(\text{max}_1-\text{max}_2)}{(1+10^{(\log \text{EC}_{50,2}-X)})}$$

where $X = \log(\text{concentration of agonist})$ and $\text{EC}_{50,1}$ and $\text{EC}_{50,2}$ = $\text{the concentration of drug causing 50% of the maximum stimulatory and inhibitory response}$ respectively; $\text{max}_1 = \text{the maximum stimulatory agonist response}$, $\text{max}_2 = \text{the maximum inhibitory agonist response}$.

#### 2.3.2.2 Analysis of DPCPX antagonist data

Agonist concentration-response curves in the absence and presence of increasing concentrations of DPCPX (10, 30 and 100nM) by non-linear regression as described above. Agonist potency in the absence and presence of increasing concentrations of DPCPX were analysed by the method of Schild (Arunlakshana
(Schild, 1959) for the estimation of the affinity ($K_b$) of DPCPX for the A1 receptor. Each estimate of agonist potency in the presence of antagonist was transformed to log (DR-1) where DR = dose ratio, the EC$_{50}$ in the presence of antagonist divided by the EC$_{50}$ in the absence of antagonist. The log(DR-1) data were then analysed by non-linear regression using an equation for a straight line as follows:

$$\text{log(DR-1)} = m \cdot \text{log[DPCPX]} - \text{log} K_b$$

The intercept on the x-axis gives the $K_b$ when the slope of the line (m) is constrained to unity. This analysis assumes that the agonist concentration-response curves, in the absence and presence of antagonist, reach the same maximum response such that the agonist EC$_{50}$ values represent equi-effective concentrations.

### 2.3.2.3 Association timecourse of $[^3S]GTP\gamma S$

The association of $[^3S]GTP\gamma S$ was analysed by obtaining the best fit from either a straight line or the following equation describing monophasic exponential association:

$$B_t = B_0 \cdot (1 - e^{-kt})$$

Where $B_t =$ the binding determined at time $t$, $B_0$ the binding at time $t=0$, and $k =$ the rate constant. The half life is $0.69/k$.

### 2.4 Simulations

The binding and functional predictions of the ternary complex model and quaternary complex model were simulated using the equations described in
Appendix 1 and 2 respectively. Simulations were performed using an algebraic method to determine the exact solutions for each set of starting parameters, using the spreadsheet functions of an Excel workbook. The simulated data were analysed using non-linear least squares regression analysis as described in Section 2.3, using the appropriate equations for binding or functional data. When binding data were simulated, the concentration of radioligand was given a value 100-fold lower than the $K_D$, such that the Cheng-Prusoff correction was negligible. The simulated IC$_{50}$ data determined in this manner was therefore essentially identical to the theoretical apparent dissociation constants.

This algebraic approach was validated by performing simulations using a numerical method similar to that described previously (De Lean et al., 1980; Lee et al., 1986) using the computer programme SigmaPlot. In contrast to the algebraic approach, the numerical method uses non-linear, least-squares regression to determine the best ‘solution’ for a given set of parameters and starting conditions. Analysis of the simulated data obtained by the numerical method using the methods described in Section 2.3 yielded mid-point curve locations which were essentially identical to those obtained from the algebraic simulation method, and to previously published data from algebraic simulations using the ternary complex model (De Lean et al., 1980; Lee et al., 1986).
Chapter 3. Radioligand binding to adenosine A<sub>1</sub> receptors expressed at high and low densities in Chinese hamster ovary cells

3.1 Introduction

A number of features of the adenosine A<sub>1</sub> receptor make it a unique test system for investigating the predictions of mathematical models of drug receptor interactions, including: (a) the availability of both radiolabelled agonists and antagonists; one of the largest GTP shifts (~1000) documented for 7TM GPCRs (Cohen <i>et al</i>., 1996b), (b) the availability of a range of agonists of different efficacy and (c) the presence of an allosteric binding site which mediates the enhancement of agonist binding (Bruns & Fergus, 1990).

The ternary complex model (TCM) has been commonly used to explain the heterogeneous agonist binding frequently observed at 7TM GPCRs. This model has been used to describe and analyse the agonist binding properties of 7TM GPCRs and predicts that high affinity agonist binding represents the ternary complex, agonist-receptor-G-protein (De Lean <i>et al</i>., 1980). However, there are conflicting reports about the suitability of the TCM to describe agonist binding (see, for example, Lee <i>et al</i>., 1986; Wreggett & De Lean, 1984).

Heterogenous agonist binding to the adenosine A<sub>1</sub> receptor has been frequently observed in both tissues and recombinant receptor expression systems. However, the nature of this heterogeneity has not been explored. In this study the
radioligand binding properties of the adenosine A1 receptor have been investigated and the effects of a number of experimental variables examined (receptor density, presence of guanine nucleotides, agonist efficacy). These results have been compared to the predictions of the TCM when the relevant model parameter is varied.

In kinetic studies investigating the dissociation of a radiolabelled agonist from the adenosine A1 receptor there have been many demonstrations of the presence of long lived, 'locked' agonist-receptor complexes which do not appear to be reversible in the absence of guanine nucleotides (Cohen et al., 1996b; Gerwins et al., 1990; Klotz et al., 1990). The nature of these locked agonist-receptor complexes has not been examined in detail. This study will investigate the kinetics of dissociation of both a radiolabelled agonist and antagonist from the adenosine A1 receptor expressed at different densities in CHO cell membranes.

3.2 Results

3.2.1. [3H]DPCPX and [3H]CHA saturation binding to the adenosine A1 receptor.

The radiolabelled antagonist [3H]DPCPX bound specifically and saturably to CHO cell membranes expressing either a high (HE) or low (LE) density of the adenosine A1 receptor. [3H]DPCPX bound to LE A1 membranes with a negative log dissociation constant (pKd) of 8.82±0.07 and a maximum binding capacity (Bmax) of 0.78±0.18 pmol.mg⁻¹ protein (n=5, Fig. 3.1a). In HE A1 cell membranes [3H]DPCPX bound with a pKd of 8.67±0.07 (n=5; Fig. 3.1b) and a
Figure 3.1. Saturation binding of the radiolabelled antagonist \( ^{3}\text{H}]\text{DPCPX} \) and the radiolabelled agonist \( ^{3}\text{H}]\text{CHA} \) to CHO adenosine \( A_{1} \) low and high expressing membranes.

Following incubation with 3U.ml\(^{-1}\) adenosine deaminase, LE (a) or HE (b) adenosine \( A_{1} \) CHO membranes were incubated with increasing concentrations of radioligand in the presence of 30\( \mu \text{g.ml}^{-1} \) saponin for 1 hour at room temperature. Data are the mean ± s.e. mean of triplicate points and representative of 5 experiments. Specific binding was calculated by subtracting non-specific binding (defined in the presence of 3mM theophylline) from total binding. The line represents the best fit of the data to a single site hyperbolic equation as described in Chapter 2.
Bmax of 18.7±4.5 pmol.mg⁻¹ protein. The pK_D values for [³H]DPCPX binding to LE and HE membranes were not significantly different (p>0.05). If it is assumed that [³H]DPCPX binds to the total population of adenosine A₁ receptors expressed in CHO cell membranes, there is approximately a 24 fold higher expression of A₁ receptors in high expressing compared to low expressing membranes.

The radiolabelled agonist [³H]CHA bound specifically and saturably to both HE and LE membranes. In LE membranes, [³H]CHA bound to A₁ receptors with a pK_D of 9.03±0.04 (n=5, Fig. 3.1a). The Bmax for [³H]CHA binding to HE membranes was 0.60±0.01 pmol.mg⁻¹ protein, which was approximately 80% of the total receptor population labelled by [³H]DPCPX. In HE membranes, [³H]CHA bound to A₁ receptors with a pK_D of 9.01±0.09 (n=5, Fig.3.1b) which was not significantly different from the value determined in LE A₁ membranes (p>0.05). The Bmax of [³H]CHA binding to HE A₁ receptors was 7.7±1.8 pmol.mg⁻¹ protein, which was approximately 12 fold higher than the binding of [³H]CHA to LE A₁ membranes and approximately 42% of the total binding sites labelled with [³H]DPCPX. Therefore, in both LE and HE A₁ membranes, [³H]CHA labelled fewer binding sites than [³H]DPCPX. However, the proportion of high affinity agonist binding sites (compared with antagonist binding sites) in LE A₁ membranes was approximately twice that of HE A₁ membranes.

3.2.2. The effect of CHA on the binding of [³H]DPCPX to the adenosine A₁ receptor.

The inability of the agonist CHA to bind with high affinity to the total population of [³H]DPCPX-labelled A₁ receptors was also observed in CHA competition
experiments (Fig. 3.2). In both LE and HE A<sub>1</sub> membranes CHA competed with [<sup>3</sup>H]DPCPX in a manner which could be described satisfactorily by an equation for two independent binding sites. In LE A<sub>1</sub> membranes CHA inhibited 84±1% of [<sup>3</sup>H]DPCPX binding with high affinity (pK<sub>H</sub> 8.68±0.04, n=5; Fig. 3.2a). The remaining [<sup>3</sup>H]DPCPX binding was inhibited with lower affinity (pK<sub>L</sub> 5.74±0.05). In HE A<sub>1</sub> membranes, CHA inhibition of [<sup>3</sup>H]DPCPX binding gave pK<sub>H</sub> and pK<sub>L</sub> values of 8.72±0.07 and 5.74±0.03 respectively (n=5, Fig. 3.2b). In contrast to LE A<sub>1</sub> membranes and consistent with saturation binding studies, the high affinity component of CHA binding was 46±1% of the specific [<sup>3</sup>H]DPCPX binding. The high efficacy A<sub>1</sub> agonist, R-PIA, also demonstrated heterogeneous agonist binding in both cell lines. R-PIA inhibited 88% and 46% of specific [<sup>3</sup>H]DPCPX with high affinity in LE and HE A<sub>1</sub> membranes respectively (n=1).

In the presence of 100μM guanosine 5'-diphosphate (GDP), the inhibition of [<sup>3</sup>H]DPCPX binding by CHA was monophasic and of low affinity in both LE and HE A<sub>1</sub> membranes. The pK<sub>L</sub> values of 5.81±0.04 and 5.77±0.03 at LE and HE A<sub>1</sub> membranes respectively, were not significantly different from each other and from the values obtained from competition binding experiments in the absence of GDP. A calculation of the ratio of K<sub>L</sub> values, obtained from either a two-site fit of heterogeneous agonist binding or from agonist binding in the presence of guanine nucleotide, to the K<sub>H</sub> values, yields the ‘GTP shift’, an index of agonist efficacy. In both LE and HE A<sub>1</sub> membranes CHA demonstrated GTP shifts in the order of 900-2,200, which is consistent with an agonist of high efficacy.
Figure 3.2. The effect of the adenosine receptor agonist CHA on the binding of the radiolabelled antagonist $[^3]$H]DPCPX in the absence and presence of GDP.

After incubation with 3U.ml$^{-1}$ ADA, LE (a) or HE (b) membranes were incubated with $[^3]$H]DPCPX (0.5nM) and increasing concentrations of CHA and the absence or presence of GDP (100μM) in the presence of 30μg.ml$^{-1}$ saponin. In the absence of GDP inhibition curves were best fit by a two-site model whilst, in the presence of GDP, inhibition curves were best fit by a one-site model. Data are the mean ± s.e. mean of triplicate points and representative of 17 (control) or 5 (GDP) experiments.
3.2.3 Simulation of the effect of changing receptor density on the agonist binding properties of the adenosine A\(_1\) receptor using the Ternary Complex Model.

The effects of increasing receptor density on the behaviour and predictions of the ternary complex model (TCM) were examined (Fig. 3.3). The simulated inhibition curves were analysed in the same manner as the experimental data, by fitting to either a one or two site model to provide estimates of \(pK_H\), \(pK_L\) and \(f_{R_H}\). For simplicity the concentration of total G-protein (\(G_T\)) was set to 1 and the relative concentration of \(R_T\) was varied from 0.1 to 20.

Simulations were performed at \(K_G\) values of, 0.1, 1 and 10. The affinity constant \(K_G\) describes the position of the equilibrium between G-protein coupled and uncoupled receptor and therefore the extent of receptor-G-protein 'pre-coupling', in the absence of agonist. When \([G_T]\) is fixed at a nominal value of 1, \(K_G\) values of 0.1, 1 and 10 represent RG 'pre-coupling' of 10, 50 and 90\% respectively (expressed as a percentage of \(R_T\)). Thus, if \(K_G.G_T << 0.1\), negligible receptor G-protein pre-coupling exists, whilst for \(K_G.[G_T]\) values of \(\geq 0.1\) there is significant pre-coupling.

The main effect of increasing \(R_T:G_T\) is to decrease the fraction of high affinity binding sites (\(f_{R_H}\)). When \(R_T:G_T < 1\), agonist inhibition curves are monophasic and of high affinity. As \(R_T\) increases, the inhibitory curves became biphasic and the proportion of high affinity sites progressively smaller. The TCM therefore predicts that the fraction of high affinity sites is limited by, and equivalent to, the
Figure 3.3. Simulation of competition binding using the Ternary Complex Model: Changing $R_T$.

The simulations were performed as described in Chapter 2 using the equations derived in Appendix 1. The following model parameters were used:

Agonist (A) affinity: $1 \times 10^6 \text{M}^{-1}$

agonist cooperativity constant ($\alpha$): 1000

$G_T$: 1

$K_G$: 0.1 (panel a&b), 1 (panel c & d), 10 (panel e & f)

The radioligand was assumed to be a silent antagonist and present at a concentration (relative to its affinity) at which the Cheng-Prusoff correction was negligible. The model parameters used for the radioligand were as follows:

Radioligand affinity: 1

Radioligand concentration: 0.01

cooperativity constant: 1

The simulated data were analysed by non-linear least squares regression, as described in Chapter 2, for estimation of $K_L$, $K_H$ and $f_{RH}$. The best fit from either a one site, or two, non-interacting, site model was used. When $R_T=0.1$, the simulated data were best fit to a one-site binding model. All other data sets were best fit to a two, non-interacting, site model.

The predicted relationship between $\log K_H$, $\log K_L$ and $f_{RH}$ is presented in panels b, d & f.
relative concentration of $G_T$. Furthermore, the effect of changing $R_T:G_T$ on $f_{H}$ was essentially independent of $K_G$ (Fig. 3.3 b, d & f).

The TCM predicts that the fraction of high affinity agonist binding sites is extremely sensitive to receptor density. A decrease in $f_{H}$ from 83% to 50% is achieved with less than a 2 fold increase in receptor density (Fig. 3.3 b, d & f). It was observed experimentally that a 20 fold increase in receptor density leads to a decrease in $f_{H}$ from 84% to 46%, which was markedly different from the predictions of the TCM. Whilst the differences in $f_{H}$ between the LE and HE cell lines are consistent with changes in the $R_T:G_T$ ratio, the data also suggests that both $R_T$ and $G_T$ may be different between the two cell lines.

Increases in the value of $K_G$ result in an increase in the apparent affinity of the agonist for the coupled state of the receptor ($1/K_H$, Fig. 3.3 a, c & e). Interestingly, at a $K_G$ value of 0.1, differences in the apparent agonist affinity for both the coupled and uncoupled states of the receptor were observed as $R_T:G_T$ increased (Fig 3.3b). These differences in the estimates of $pK_H$ and $pK_L$ at different values $R_T:G_T$ were approximately 0.15 and 0.3 log units, respectively, when $f_{H}$ values were in the range 0.8 to 0.5 (Fig 3.3b). However, at a $K_G$ value of 1, smaller differences in the estimated values of $pK_H$ and $pK_L$ were observed (Fig. 3.3d), and at a $K_G$ value of 10 the values of $pK_H$ and $pK_L$ were essentially independent of the $R_T:G_T$ ratio (Fig. 3.3f). The constancy of the apparent affinity estimates of CHA for the coupled and uncoupled $A_1$ receptor determined experimentally in high and low expressing cell lines would therefore suggest that $K_G$ is in the range of 1-10 in this experimental system.
3.2.4. The binding properties of lower efficacy agonists at the adenosine A\textsubscript{1} receptor.

It has previously been reported that agonists of varying efficacy at G-protein coupled receptors have different \( K_I/K_H \) ratios and described different proportions of high affinity binding sites and that these parameters are correlated with agonist efficacy (Birdsall \textit{et al.}, 1978; Kent \textit{et al.}, 1980). The adenosine A\textsubscript{1} agonists GR190178, GR161144 and GR162900 have a lower efficacy than full agonists such as CHA and NECA as they are unable to stimulate maximum responses in a number of functional measurements of adenosine A\textsubscript{1} receptor activation (Sheehan \textit{et al.}, in press). The \( K_I/K_H \) ratios and fr\textsubscript{H} values for CHA, GR190178, GR161144 and GR162900 were determined by incubating LE and HE adenosine A\textsubscript{1} membranes with \(^3\text{H} \)DPCPX and increasing concentrations of each of the agonists in the absence of guanine nucleotides. The data were analysed by fitting to both a one-site and two-site binding model and the model of best fit determined by an F-test. For the two-site fit, the agonist IC\textsubscript{50} for the low affinity binding site was constrained to the IC\textsubscript{50} determined from agonist inhibition of \(^3\text{H} \)DPCPX binding in the presence of 100\( \mu \)M GDP which was performed on the same day. In a separate series of experiments, the \( K_H \) values were determined by incubating increasing concentrations of these compounds with HE membranes and \(^3\text{H} \)CHA. The fr\textsubscript{H}, pK\textsubscript{H} and pK\textsubscript{L} values were then compared to the predictions of the TCM when the cooperativity term \( \alpha \) was varied.

GR190178 (the most efficacious of the three agonists) produced biphasic inhibition of \(^3\text{H} \)DPCPX binding in the absence of GDP with fr\textsubscript{H} values of 77\( \pm \)4\% and 42\( \pm \)5\% in both LE and HE A\textsubscript{1} membranes respectively (n=3; Fig. 3.4
Figure 3.4. The effect of agonists of different efficacy on radioligand binding to adenosine A₁ receptors.

Following a 30 min. incubation with 3U.ml⁻¹ ADA, low expression (A,C,E & G) and high expression (B,D,F & G) membranes were incubated with either the radiolabelled antagonist [³H]DPCPX (~0.5nM) in the absence or presence of GDP (100µM), or radiolabelled agonist [³H]CHA (~1.5nM, high expression membranes only), for 1 hour at room temperature. Data are the mean ± s.e.mean of triplicate data points. Curves are the best fits from a comparison of one and two site models.
Table 3.1. Binding parameters for the inhibition of \[^{3}\text{H}]\text{DPCPX} binding by agonists of varying efficacy at adenosine A\textsubscript{1} LE and HE membranes.

<table>
<thead>
<tr>
<th></th>
<th>(pK_H)</th>
<th>(f_H)</th>
<th>(pK_L)</th>
<th>(pK_i^a)</th>
<th>(K_i/K_H)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Low expression</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CHA</td>
<td>8.80±0.04</td>
<td>0.82±0.01</td>
<td>5.73±0.03</td>
<td></td>
<td>1180±117</td>
</tr>
<tr>
<td>GR190178</td>
<td>8.44±0.02</td>
<td>0.77±0.04</td>
<td>6.19±0.07</td>
<td></td>
<td>210±95</td>
</tr>
<tr>
<td>GR161144</td>
<td>8.21±0.01</td>
<td>0.68±0.02</td>
<td>7.25±0.16</td>
<td>7.73(b)</td>
<td>10±4</td>
</tr>
<tr>
<td>GR162900</td>
<td></td>
<td></td>
<td></td>
<td>7.82±0.04</td>
<td></td>
</tr>
</tbody>
</table>

|            |               |              |          |             |             |
| **High expression** |               |              |          |             |             |
| CHA        | 8.68±0.20     | 0.44±0.04    | 5.71±0.08|             | 1140±530    |
| GR190178   | 7.97±0.04     | 0.42±0.05    | 6.15±0.01|             | 70±10       |
| GR161144   | 8.05          | 0.26         | 7.10     | 7.43±0.17\(c\) | 9           |
| GR162900   |               |              |          | 7.46±0.10   |             |

Following pre-treatment with 3U.ml\(^{-1}\) ADA, CHO A\textsubscript{1} LE and HE membranes were incubated with \[^{3}\text{H}]\text{DPCPX} (~0.5nM) and increasing concentrations of agonist in the presence of 30\(\mu\)g.ml\(^{-1}\) saponin for 60 min. at room temperature. Binding parameters were obtained by obtaining the best fit from either a one- or two-site binding model. Affinity constants were calculated from \(IC_{50}\) values using the Cheng-Prusoff equation as described in Chapter 2. Data are the mean ± s.e.mean of 3 experiments.

- \(a\): \(pK_i\) values when data were best for to a one site model.
- \(b\): Data were best fit to a one site model in one out of three experiments.
- \(c\): Data were best fit to a one site model in two out of three experiments.
Table 3.2. Ligand binding parameters for the G-protein coupled and uncoupled states of the adenosine A₁ receptor in LE and HE membranes.

<table>
<thead>
<tr>
<th></th>
<th>[³H]CHA</th>
<th>[³H]DPCPX + 100μM GDP</th>
<th>K_L/K_H</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pK_H</td>
<td>pK_L</td>
<td></td>
</tr>
<tr>
<td>Low expression</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CHA</td>
<td>N.D.</td>
<td>5.59±0.08</td>
<td></td>
</tr>
<tr>
<td>GR190178</td>
<td>N.D.</td>
<td>6.19±0.06</td>
<td></td>
</tr>
<tr>
<td>GR161144</td>
<td>N.D.</td>
<td>7.08±0.03</td>
<td></td>
</tr>
<tr>
<td>GR162900</td>
<td>N.D.</td>
<td>7.41±0.12</td>
<td></td>
</tr>
<tr>
<td>DPCPX</td>
<td>N.D.</td>
<td>N.D.</td>
<td></td>
</tr>
<tr>
<td>High expression</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CHA</td>
<td>8.96±0.03</td>
<td>5.61±0.06</td>
<td>2270±520</td>
</tr>
<tr>
<td>GR190178</td>
<td>8.48±0.01</td>
<td>6.34±0.15</td>
<td>100±30</td>
</tr>
<tr>
<td>GR161144</td>
<td>8.28±0.05</td>
<td>7.13±0.05</td>
<td>11±1.4</td>
</tr>
<tr>
<td>GR162900</td>
<td>8.24±0.08</td>
<td>7.59±0.13</td>
<td>4±2</td>
</tr>
<tr>
<td>DPCPX</td>
<td>8.90±0.03</td>
<td>9.01±0.02</td>
<td>0.80±0.03</td>
</tr>
</tbody>
</table>

Following pre-treatment with 3U.ml⁻¹ ADA, CHO A₁ membranes were incubated with [³H]CHA (1-2nM; G-protein coupled receptor) or [³H]DPCPX (~0.5nM) plus 100μM GDP (G-protein uncoupled receptor) with increasing concentrations of agonist in the presence of 30μg.ml⁻¹ saponin for 60 min. at room temperature.

Inhibition curves were analysed by fitting to a one-site model. Data are the mean ± s.e.mean of 3 experiments, with the exception of CHA (n=2) which is expressed as the mean ± range/2. Affinity constants were calculated from IC₅₀ values using the Cheng-Prusoff equation as described in Chapter 2. N.D.: binding parameters not determined.
Table 3.1) which was not significantly different from CHA (p<0.05, one-way analysis of variance (LE) or paired t test (HE)). The intermediate efficacy agonist GR161144 inhibited $[^3H]DPCPX$ binding in a biphasic manner in LE $A_1$ membranes in two out of three experiments, and in HE $A_1$ membranes in one out of three experiments with $f_{H}$ values of 68±2% and 26% respectively, which were lower than the $f_{H}$ values determined for CHA. In LE membranes the $f_{H}$ value for GR161144 was significantly lower than for CHA (P<0.05, one-way analysis of variance). In HE membranes the $f_{H}$ value for GR161144 could not be statistically compared with CHA as this represented a single value. In all three experiments the lowest efficacy agonist, GR162900, inhibited $[^3H]DPCPX$ in a manner best described by a one site equation in both LE and HE $A_1$ membranes. The $f_{H}$ for these agonists therefore appears to correlate with agonist intrinsic activity. The difference in apparent affinities of GR162900 determined using $[^3H]CHA$ and $[^3H]DPCPX$ in the presence of GDP (described below) suggests that this agonist is clearly able to bind to G-protein coupled and uncoupled receptors with different affinities. The failure to estimate $f_{H}$ values for this compound in $[^3H]DPCPX$ inhibition experiments in the absence of GDP is probably due to its small value and to scatter in the experimental data.

Agonist $K_{H}$ and $K_{L}$ values for the inhibition of $[^3H]DPCPX$ in the absence of GDP were similar in LE and HE membranes and are shown in Table 3.1. Where the agonist inhibition curves were best fit to a two-site model, the $K_{L}/K_{H}$ values decreased with agonist intrinsic activity, with CHA having the largest $K_{L}/K_{H}$ value of 1100, and GR161144 the smallest $K_{L}/K_{H}$ of 10. As the inhibition curve to GR162900 was best fit to a one-site model, a $K_{L}/K_{H}$ value could not be
determined. The apparent affinity values for CHA, GR190178, GR161144, and GR162900 determined by the inhibition of \(^{3}H\)DPCPX in the absence of GDP (pK\(_l\)) were also similar to the pK\(_l\) values determined by the inhibition of \(^{3}H\)DPCPX in the presence of GDP (Fig. 3.4, Table 3.2).

Low efficacy agonists therefore generate a smaller fraction (number) of high affinity binding sites compared to full agonists. Using a radiolabelled antagonist in the absence of guanine nucleotides, it therefore becomes progressively harder to measure the affinity for the coupled state of the receptor for agonists with diminishing efficacy. In order to make a better estimate of agonist affinity for the coupled receptor, the effect of the partial agonists on the binding of \(^{3}H\)CHA to A\(_1\) HE membranes was investigated (Fig. 3.4). The binding of \(^{3}H\)CHA to the adenosine A\(_1\) receptor can be completely abolished by GDP confirming that \(^{3}H\)CHA selectively binds to the G-protein coupled state of the adenosine A\(_1\) receptor (data not shown).

The pK\(_H\) values for CHA, GR190178, GR161144, and GR162900 in HE membranes determined by the inhibition of \(^{3}H\)CHA were similar to those obtained from the inhibition of \(^{3}H\)DPCPX in the absence of GDP (compare Tables 3.1 & 3.2). The ratio K\(_l\)/K\(_H\) (calculated using K\(_l\) values determined from the inhibition of \(^{3}H\)DPCPX in the presence of GDP) was highest for the full agonist CHA (2270±520) and lowest for the antagonist DPCPX (0.80±0.03; Table 3.2). The rank order of K\(_l\)/K\(_H\) for the ligands tested was CHA >GR190178 >GR161144 >GR162900 >DPCPX which was the same as the rank order of intrinsic activity (Sheehan et al., in press). Therefore, for the adenosine A\(_1\)
receptor, the GDP shift of an agonist (relative to a standard) is a reflection of the relative efficacy for that agonist.

3.2.5. Simulation of the effect of efficacy changes on the predictions of the Ternary Complex Model.

In the ternary complex model the efficacy of an agonist is determined by its cooperativity constant $\alpha$, which is a property of the agonist, receptor and G-protein. The affinity of the receptor for the G-protein, $K_G$, also contributes to the efficacy of an agonist. However, $K_G$ is dependent only on the receptor and G-protein and is independent of the applied agonist. Both $\alpha$ and $K_G$ therefore contribute to the ability of an agonist to stimulate G-protein binding to the receptor, and both of these parameters need to be considered in the TCM simulations of agonist efficacy. The effect of agonist efficacy on radioligand binding was therefore simulated by changing $\alpha$ from 1-100,000 at different values of $K_G$. Values of $R_{T:G_T}$ of 1.2 and 2 (Fig. 3.5 a & b respectively) were chosen as they simulated the values of $f_{H}$ observed in LE and HE membranes. Estimates of $f_{H}$, $K_H$ and $K_L$ were obtained by obtaining the best fit from analysis with either a one- or two-site binding model after constraining $K_L$ to the log $IC_{50}$ determined when $\alpha=1$.

In all simulations, increases in the value of the cooperativity term $\alpha$ result in increases in the value of $K_L/K_H$ at all values of $K_G$ and $R_{T:G_T}$. This supports the postulate that $K_L/K_H$ is an appropriate index of agonist efficacy, and is similar to that found for the adenosine A1 receptor and other G-protein coupled receptors. When $\alpha$ is given a value of one it is predicted that ligand binding will not perturb
**Fig. 3.5a. Simulation of competition binding using the Ternary Complex Model: Changing the cooperativity constant $\alpha$ when $R_T:G_T = 1.2$.**

The simulations were performed as described in Chapter 2 using the equations derived in Appendix 1. The following model parameters were used:

**Agonist (A) affinity:** $1 \times 10^6 \text{M}^{-1}$

**$R_T$:** 1.2

**$G_T$:** 1

$K_G$: 0.1 (panel a&b), 1 (panel c & d), 10 (panel e & f)

The radioligand was assumed to be a silent antagonist and present at a concentration (relative to its affinity) at which the Cheng-Prusoff correction was negligible. The model parameters used for the radioligand were as follows:

**Radioligand affinity:** 1

**Radioligand concentration:** 0.01

**cooperativity constant:** 1

The simulated data were analysed by non-linear least squares regression, as described in Chapter 2, for estimation of $K_L$, $K_H$ and $f_{IH}$. The best fit from either a one site, or two, non-interacting, site model was used. When $\alpha$ was given a value of 1, the data were best fit to a one site model and therefore no value for $f_{IH}$ was determined. To maintain consistency with the analysis of the experimental data, when the two-site binding model was used to analyse the simulated data, the value for $K_L$ was held constant at the value determined when $\alpha = 1$.

The predicted relationship between $\log K_L/K_H$ and $f_{IH}$ is presented in panels b, d & f, together with the experimentally determined values for CHA, GR 190178 and GR 161144.

The $R_T:G_T$ ratio was chosen as this best described the proportions of high affinity binding sites observed for CHA in low expressing membranes.
Figure 3.5b. Simulation of competition binding using the Ternary Complex Model: Changing the cooperativity constant $\alpha$ when $R_T:G_T = 2$

The simulations were performed as described in Chapter 2 using the equations derived in Appendix 1. The following model parameters were used:

Agonist (A) affinity: $1 \times 10^6 \text{M}^{-1}$

$R_T$: 2;

$G_T$: 1

$K_G$: 0.1 (panel a&b), 1 (panel c & d), 10 (panel e & f)

The radioligand was assumed to be a silent antagonist and present at a concentration (relative to its affinity) at which the Cheng-Prusoff correction was negligible. The model parameters used for the radioligand were as follows:

Radioligand affinity: 1

Radioligand concentration: 0.01

cooperativity constant: 1

The simulated data were analysed by non-linear least squares regression, as described in Chapter 2, for estimation of $K_L$, $K_H$ and $f_{rH}$. The best fit from either a one site, or two, non-interacting, site model was used. When $\alpha$ was given a value of 1, the data were best fit to a one site model and therefore no value for $f_{rH}$ was determined. To maintain consistency with the analysis of the experimental data, when the two-site binding model was used to analyse the simulated data, the value for $K_L$ was held constant at the value determined when $\alpha = 1$.

The predicted relationship between $\log K_L/K_H$ and $f_{rH}$ is presented in panels b, d & f, together with the experimentally determined values for CHA, GR 190178 and GR 161144.

The $R_T:G_T$ ratio of 2 was chosen as this best described the proportions of high affinity binding sites observed for CHA in high expressing membranes.
Fractional Radioligand Occupancy

Fractional Radioligand Occupancy

Fractional Radioligand Occupancy

fr_H

fr_H

fr_H

\[ \text{Fractional Radioligand Occupancy} \]

\[ \frac{W_{obs}}{W_{max}} \]

\[ \text{fr}_H \]

\[ \frac{W_{obs}}{W_{max}} \]

\[ \text{fr}_H \]

\[ \frac{W_{obs}}{W_{max}} \]

\( \text{(measured)} \)

\( \text{(predicted)} \)
receptor G-protein coupling. This condition is a requirement for neutral antagonism and inhibition curves are predicted to be monophasic and of low affinity.

In general the TCM predicts that there is very little change in $fr_H$ when $\alpha$ is varied for simulations of LE membranes when $R_T:G_T=1.2$ and $[G_T].K_G=10$ (fig. 3.5a panel e), and for simulations of HE membranes when $R_T:G_T=2$ and $[G_T].K_G=1$ or 10 (Fig. 3.5b panels c & e). However, for values of $[G_T].K_G = 0.1$ or 1 when $R_T:G_T=1.2$, and for values of $[G_T].K_G$ of 0.1 when $R_T:G_T=2$, changes in $fr_H$ are predicted to occur for $K_i/K_h$ values of 10 and 100.

The experimental observation that the rank order of agonist $K_i/K_h$ ratios is the same as the rank order of intrinsic activities is therefore supported by the predictions of the TCM. However, the observation that agonists of different efficacies can describe different proportions of high affinity binding sites is only predicted by the TCM when $[G_T].K_G < 1$.

3.2.6 The effect of GDP on the ligand binding properties of the adenosine $A_1$ receptor.

The effect of increasing concentrations of the guanine nucleotide GDP (1-100μM) on the inhibition of $[^3H]DPCPX$ by the agonist CHA was investigated. LE and HE membranes were incubated with $[^3H]DPCPX$ (0.4-0.6nM) and increasing concentrations of CHA in the presence of saponin (30μg.ml⁻¹) and different concentrations of GDP for 1 hour at room temperature. Similar experiments were performed using the guanine nucleotide GTP, which produced quantitatively similar results with the exception that it was approximately 10 fold more potent
than GDP (data not shown). The GDP data were analysed as it was viewed that at low concentrations, GDP would be more stable than GTP, especially in the presence of agonist.

Increasing concentrations of GDP progressively reduced the affinity of CHA for the high affinity, G-protein coupled state of the receptor (Fig. 3.6 and Table 3.3). In LE membranes GDP decreased the pK_{H} from 8.63 ± 0.06 (control) to 7.05 ± 0.09 (10 μM GDP) whilst in HE membranes the pK_{H} was decreased from 8.68 ± 0.09 (control) to 7.43 ± 0.12 (10μM GDP). In the presence of 100μM GDP, CHA inhibition curves were monophasic and of low affinity, which indicated that the A_{1} receptor had been essentially fully uncoupled from G-proteins by the presence of guanine nucleotide. In both LE and HE membranes, GDP had no effect on the affinity of CHA for the uncoupled state of the A_{1} receptor. This suggests that the low affinity agonist binding component in control membranes represents a state of the A_{1} receptor which is very similar or identical to the state found in GDP treated membranes.

The fraction of high affinity binding sites was also sensitive to GDP. Increasing concentrations of GDP produced a decrease in f_{H} in both LE and HE membranes (Fig. 3.6 and Table 3.3). In LE membranes GDP decreased f_{H} from 83% (control) to 41% (10μM GDP) whilst in HE membranes, f_{H} was decreased from 47% (control) to 28% (10μM GDP). It can be assumed that no high affinity agonist binding sites remain in the presence of 100μM GDP because inhibition curves are monophasic and of low affinity.
Figure 3.6. The effect of GDP on the inhibition of $[^3\text{H}]$DPCPX binding to adenosine $A_1$ receptors by the adenosine $A_1$ agonist CHA.

Following a 30 min. incubation with 3U.ml$^{-1}$ ADA, LE (a) and HE (b) membranes were incubated with $[^3\text{H}]$DPCPX (0.5nM), GDP (0-100μM) and increasing concentrations of CHA in the presence of 30mg.ml$^{-1}$ saponin. Inhibition curves were analysed by obtaining the best fit from either a one- or two-site model as described in Chapter 2. Data are the mean ± s.e.mean of triplicate data points and representative of 5 experiments. The relationship between log [GDP] and the experimentally determined values for $f_{rH}$, log $K_H$ and log $K_L$ are illustrated in panels c and d for LE and HE membranes respectively.
Table 3.3. The effect of GDP on CHA inhibition of $[^3\text{H}]\text{DPCPX}$ binding to LE and HE membranes.

<table>
<thead>
<tr>
<th>[GDP]</th>
<th>$f_r_H$</th>
<th>$pK_H$</th>
<th>$pK_L$</th>
<th>$f_r_H$</th>
<th>$pK_H$</th>
<th>$pK_L$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>83±2</td>
<td>8.63±0.06</td>
<td>5.82±0.05</td>
<td>47±1</td>
<td>8.68±0.09</td>
<td>5.67±0.02</td>
</tr>
<tr>
<td>1 μM</td>
<td>74±2</td>
<td>7.54±0.13</td>
<td>5.86±0.09</td>
<td>35±2</td>
<td>8.05±0.04</td>
<td>5.79±0.02</td>
</tr>
<tr>
<td>3 μM</td>
<td>55±3</td>
<td>7.50±0.05</td>
<td>5.95±0.08</td>
<td>32±4</td>
<td>7.70±0.13</td>
<td>5.73±0.03</td>
</tr>
<tr>
<td>10 μM</td>
<td>41±4</td>
<td>7.05±0.09</td>
<td>5.93±0.08</td>
<td>28±2</td>
<td>7.43±0.12</td>
<td>5.75±0.05</td>
</tr>
<tr>
<td>100 μM</td>
<td></td>
<td>5.81±0.04*</td>
<td></td>
<td></td>
<td></td>
<td>5.77±0.03*</td>
</tr>
</tbody>
</table>

Following pre-treatment with 3U.ml$^{-1}$ ADA, CHO A$_1$ membranes were incubated with $[^3\text{H}]\text{DPCPX}$ (0.4-0.6nM) and increasing concentrations of agonist in the presence of 30μg.ml$^{-1}$ saponin and the stated concentration of GDP for 60 min. at room temperature.

Inhibition curves were analysed by fitting to a one- and two-site model and obtaining the best fit from an F-test as described in Chapter 2. Affinity constants were calculated from IC$_{50}$ values using the Cheng-Prusoff equation as described in Chapter 2. Data are the mean ± s.e.mean of 5-6 experiments.

* In the presence of 100μM GDP, inhibition curves were best fit to a one-site binding model.
Table 3.4. The effect of GDP on the binding of $[^3\text{H}]$DPCPX to adenosine $A_1$ receptors in LE and HE membranes.

<table>
<thead>
<tr>
<th>GDP concentration ($\mu$M)</th>
<th>Low expression</th>
<th>High expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>0.3*</td>
<td>136±10</td>
<td>111±6</td>
</tr>
<tr>
<td>1</td>
<td>142±12</td>
<td>129±8</td>
</tr>
<tr>
<td>3</td>
<td>156±11</td>
<td>129±10</td>
</tr>
<tr>
<td>10</td>
<td>152±15</td>
<td>128±11</td>
</tr>
<tr>
<td>100</td>
<td>163±17</td>
<td>125±9</td>
</tr>
</tbody>
</table>

Following pre-treatment with 3U.ml$^{-1}$ ADA, CHO $A_1$ membranes were incubated with $[^3\text{H}]$DPCPX (0.4-0.6nM) in the presence of 30$\mu$g.ml$^{-1}$ saponin and the stated concentration of GDP for 60 min. at room temperature. Non-specific binding was determined by the presence of 3mM theophylline.

Data are expressed as the percentage of the specific $[^3\text{H}]$DPCPX binding in the absence of GDP and are the mean ± s.e.mean from 3(*) or 6 experiments.
The binding of $[^3\text{H}]$DPCPX to the $A_1$ receptor was also sensitive to the presence of GDP. GDP (0.3-100\mu M) increased the binding of $[^3\text{H}]$DPCPX by 60% and 30% in LE and HE membranes respectively (Table 3.4). The increase in $[^3\text{H}]$DPCPX binding appeared to be maximal at concentrations of GDP $\geq$ 1\mu M and is consistent with previous reports of the effects of guanine nucleotides on the binding of $[^3\text{H}]$DPCPX to the adenosine $A_1$ receptor (Cohen et al., 1996b; Klotz et al., 1990). This effect of GDP is unlikely to be due to an increase in the affinity of $[^3\text{H}]$DPCPX given that it has been shown in section 3.2.4 that DPCPX had similar affinities for the coupled and uncoupled states of the $A_1$ receptor (Table 3.2). The change appears to be due to an increase in the apparent Bmax (Cohen et al., 1996b, and references therein).

3.2.7. Simulating the effect of GDP using the Ternary Complex Model

The process by which guanine nucleotides decrease agonist binding is believed to be an indirect effect following binding to the G-protein $\alpha$ subunit (Gilman, 1987). A negative cooperative interaction between the binding of guanine nucleotides and receptor results in a decrease in affinity of the receptor for the G-protein. Whilst the TCM does not consider the role of guanine nucleotides in receptor G-protein interactions, the effects of guanine nucleotides on agonist binding can be simulated by varying $K_G$, the affinity constant for the binding of receptor to G-protein in the absence of agonist.

Decreasing the value of $K_G$ caused a decrease in the apparent agonist affinity for the G-protein coupled receptor, with no change to the apparent affinity for the low affinity binding site (Fig. 3.7). This was observed at $R_T$:$G_T$ ratios of 1.2 (Fig. 3.7 a
Figure 3.7. Simulation of competition binding using the Ternary Complex Model: Changing the affinity constant $K_G$.

The simulations were performed as described in Chapter 2 using the equations derived in Appendix 1. The following model parameters were used:

Agonist (A) affinity: $1 \times 10^6 \text{M}^{-1}$

$G_T$: 1

$R_T:G_T$: 1.2 (panel a & b), 2 (panel c & d)

$\alpha$: 1000

The radioligand was assumed to be a silent antagonist and present at a concentration (relative to its affinity) at which the Cheng-Prusoff correction was negligible. The model parameters used for the radioligand were as follows:

Radioligand affinity: 1

Radioligand concentration: 0.01

cooperativity constant: 1

The simulated data were analysed by non-linear least squares regression, as described in Chapter 2, for estimation of $K_L$, $K_H$ and $f_{RH}$. The data were analysed by obtaining the best fit from either a one site, or two independent site model. In all cases for the model parameters used, the simulated data were best fit to a two-site model.

The predicted relationship between $\log K_L$, $\log K_H$ and $f_{RH}$ is presented in panels b & d.

The $R_T:G_T$ ratios of 1.2 and 2 were chosen as this best described the proportions of high affinity binding sites observed for CHA in low and high expressing membranes.
& b) and 2 (Fig. 3.7 c & d), which were chosen to mimic the agonist binding observed experimentally for LE and HE, respectively, in the absence of guanine nucleotide.

Decreasing the value of $K_G$ also led to a marked decrease in $f_{R,H}$ (Fig. 3.7). This effect was most pronounced with a value of $R_T:G_T$ of 1.2 and at $K_L/K_H$ ratios of $< 10$, which occurred at $[G_T].K_G$ values $1x10^{-2} - 1x10^{-4}$ (Fig. 3.7 a & b). In general, the predicted decreases in $f_{R,H}$, which occurred as $K_G$ was decreased, were observed at $K_L:K_H$ values which were lower than those determined experimentally.

The ternary complex model can therefore predict the qualitative effects of GDP on $K_H$ and $K_L$, and on the fraction of high affinity binding sites, in both LE and HE membranes if it is assumed that GDP decreases the affinity constant $K_G$. However, the effects of GDP on $f_{R,H}$ determined experimentally, appear to occur at values of $K_L/K_H$ which are greater than those predicted by the model.

3.2.8. The kinetics of radiolabelled antagonist and agonist dissociation from the adenosine A<sub>1</sub> receptor.

The kinetics of radioligand dissociation provide a further pharmacological property of the adenosine A<sub>1</sub> receptor for comparison between LE and HE membranes. The first objective of these studies was to compare the dissociation of $[^3H]DPCPX$ and $[^3H]CHA$ from LE and HE membranes in order to examine the effect of receptor density on radioligand dissociation. Following pre-labelling with a radioligand, dissociation can be achieved either by diluting the membrane preparation such that the concentration of radioligand decreases significantly below its $K_D$, or by the addition of an excess concentration of a unlabelled
competitive ligand. Using the first method, a limited amount of re-association of the radioligand to the receptor occurs. However, in the presence of a high concentration of cold competitor no radioligand rebinding occurs. In addition, the dissociation of a radioligand is predicted to be independent of the pharmacological properties of the cold competitor, although there are only isolated literature reports comparing the relative abilities of unlabelled agonists and antagonists to initiate radioligand dissociation (Hirschberg & Schimerlik, 1994). The second objective of these studies was therefore to compare the kinetics of radioligand dissociation when the dissociation is initiated either by dilution, or the presence of an excess concentration of unlabelled agonist or antagonist.

Radiolabelled agonist dissociation from the adenosine A₁ receptor frequently displays the phenomenon of ‘agonist locking’, that is the presence of irreversible (or pseudoirreversible) agonist-receptor complexes which fail to dissociate over a period of hours (Cohen et al., 1996b; Kourounakis et al., 2000; Parkinson & Fredholm, 1992; Stiles, 1985). These complexes have been shown to involve receptor G-protein coupling, because agonists fully and very rapidly dissociate from the complex upon the addition of guanine nucleotides (Cohen et al., 1996b; Leid et al., 1988; Stiles, 1985). Few studies, however, have explored the molecular mechanisms of ‘locked’ agonist-receptor G-protein complexes and a further aim of this study was to investigate whether receptor density can influence the formation of these complexes.

The dissociation kinetics of \[^{3}H\]DPCPX and \[^{3}H\]CHA in LE and HE membranes were examined by incubating membranes with radioligand in a small volume (50-70μl) for 1 hour and then initiating radioligand dissociation in one of three ways:
addition of a 20-40 fold excess of buffer alone (dilution), or addition of a 20-40 fold excess of buffer containing 100μM CHA (dilution plus agonist) or 10μM DPCPX (dilution plus antagonist).

3.2.8.1. $[^3H]$DPCPX dissociation

Receptors were pre-labelled with 2-10nM $[^3H]$DPCPX. Irrespective of the method of initiating radioligand dissociation the binding of $[^3H]$DPCPX to the A<sub>1</sub> receptor decreased rapidly with a $t_{1/2}$ of approximately 2 minutes (Fig 3.8 a & b, Table 3.5). In both LE and HE membranes the dissociation of $[^3H]$DPCPX was well described by a monophasic exponential decay curve with dissociation rate constants ($k_{off}$) that were independent of the method of dissociation (Table 3.5a). There appeared to be a small increase in the values of $k_{off}$ in HE compared to LE membranes. The binding of $[^3H]$DPCPX was fully reversible and reached non-specific binding levels in the dilution plus cold agonist or antagonist experiments. Following dilution alone, the decrease in binding of $[^3H]$DPCPX reached 80-90% of total binding, which was consistent with the expected re-equilibration levels of the lower concentration of radioligand (0.05-0.5nM) after dilution. The dissociation rate constants for $[^3H]$DPCPX were also unaffected by the presence of 100μM GTP in the diluent (Fig. 3.8 a & b, Table 3.5a: n = 1), indicating that the binding of $[^3H]$DPCPX to the A<sub>1</sub> receptor is insensitive to receptor-G-protein interactions.
Fig. 3.8. The dissociation of $[^3H]DPCPX$ from the adenosine A$_1$ receptor in low expressing and high expressing CHO membranes. 
Low expressing (panel a) and high expressing (panel b) adenosine A$_1$ CHO membranes were incubated for 1 hour with 4nM $[^3H]DPCPX$, after which time dissociation was initiated by a 40 fold dilution with buffer containing the reagent as indicated. Data are single points with the exception of total and non-specific binding which are the mean ± s.e.mean of triplicate points. Dissociation with GTP was performed once. All other time courses are representative of three experiments. The lines represent the computer generated best fits of the data to an equation for monoexponential decay as described in Chapter 2.
% dissociation of specific \(^{3}H\) DPCPX binding

% dissociation of specific \(^{3}H\) DPCPX binding

- Dilution alone
- Dilution + 100μM CHA
- Total Binding
- Non-specific binding
- Non-specific binding + 10μM DPCPX

Time (min)
Fig. 3.9. The dissociation of $[^3H]CHA$ from the adenosine A$_1$ receptor in low expressing and high expressing CHO membranes. Low expressing (panel a) and high expressing (panel b) adenosine A$_1$ CHO membranes were incubated for 1 hour with 5nM $[^3H]CHA$, after which time dissociation was initiated by a 40 fold dilution with buffer containing the reagent as indicated. Data are the mean ± range/2 of duplicate points with the exception of dilution + 100μM GTP which are single points and total and non-specific binding which are the mean ± s.e.mean of 6 replicates. Dissociation with GTP was performed once. All other time courses are representative of three experiments. The lines are the computer generated best fits of the data to an equation for monophasic exponential decay as described in Chapter 2.
Dilution alone
- Dilution + 100μM CHA
- Dilution + 10μM DPCPX
- Dilution + 100μM GTP
- Total Binding
- Non-specific binding
**Legend to Table 3.5:**

Binding parameters were determined by non-linear regression analysis of radioligand dissociation data using an equation describing monophasic exponential decay as described in Chapter 2.

a: extent of radioligand dissociation determined from the plateau of the exponential decay and expressed as a percentage of the specific radioligand binding.

b: Data could not be fit to an equation describing monophasic exponential decay. The extent of radioligand dissociation was quantified by expressing the residual binding 1 hour after the initiation of dissociation, as a percentage of the specific radioligand binding.

Data are the mean ± s.e.mean of n=3 experiments with [³H]DPCPX or mean ± range/2 of n=2 experiments with [³H]CHA. Data for dissociation + GTP is from a single experiment.
Table 3.5. Summary of kinetic parameters for the dissociation of $[^3H]$DPCPX (A) and $[^3H]$CHA (B) from the adenosine A<sub>1</sub> LE and HE membranes.

A

<table>
<thead>
<tr>
<th>Method dissociation</th>
<th>Low expression</th>
<th>High expression</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$k_{off}$ (min&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>% dissociation&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>dilution</td>
<td>0.30±0.02</td>
<td>84±7</td>
</tr>
<tr>
<td>dilution + 100μM CHA</td>
<td>0.32±0.02</td>
<td>100±3</td>
</tr>
<tr>
<td>dilution + 10μM DPCPX</td>
<td>0.28±0.01</td>
<td>97±3</td>
</tr>
<tr>
<td>dilution + 100μM GTP</td>
<td>0.36</td>
<td>92</td>
</tr>
</tbody>
</table>

B

<table>
<thead>
<tr>
<th>Method dissociation</th>
<th>Low expression</th>
<th>High expression</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$k_{off}$ (min&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>% dissociation&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>dilution</td>
<td>20±3</td>
<td>28±6</td>
</tr>
<tr>
<td>dilution + 100μM CHA</td>
<td>28±1</td>
<td>0.06±0</td>
</tr>
<tr>
<td>dilution + 10μM DPCPX</td>
<td>29±4</td>
<td>24±1</td>
</tr>
<tr>
<td>dilution + 100μM GTP</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>
3.2.8.2. $[^3]H$CHA dissociation

In contrast to results for $[^3]H$DPCPX dissociation (3.2.8.1) the dissociation of $[^3]H$CHA was complex. In both LE and HE membranes, both the 'dilution alone' and 'dilution plus antagonist' protocols failed to produce greater than 30% dissociation of specific $[^3]H$CHA binding at times of greater than 60 min. (Fig. 3.9, Table 3.5). The data were not well described by expressions for either monophasic or biphasic exponential decay, and for this reason the percentage dissociation after 1 hour was calculated. In LE membranes the effect of CHA in the diluent produced a similar level of dissociation of $[^3]H$CHA as dilution alone and dilution + antagonist (Fig. 3.9a, Table 3.5a). In contrast, in HE membranes the presence of CHA in the diluent produced 63±1% (n=2) dissociation of the specific $[^3]H$CHA binding. The data were described by a single component exponential decay curve with a $k_{off}$ value of 0.059±0.001 min$^{-1}$ (Fig. 3.9b).

In both LE and HE membranes, specific $[^3]H$CHA rapidly dissociated upon the addition of 100µM GTP (Fig. 3.9 a & b). The dissociation rate constant was too fast to be measured accurately. After 1 min., 80-90% of specific $[^3]H$CHA binding had dissociated and after 1 hour no specific binding was observed.

3.3. Discussion

The binding of CHA to the adenosine A$\text{}_{1}$ receptor

The aim of these studies was to compare and contrast the radioligand binding properties of the adenosine A$\text{}_{1}$ receptor expressed in CHO cell membranes with a
20-fold difference in receptor density and then to critically evaluate the ability of the ternary complex model to describe this data.

The binding of CHA to the adenosine A_1 receptor was heterogeneous, with high and low affinity binding sites of identical affinity in LE and HE membranes. The only difference in the equilibrium binding properties of CHA, between LE and HE membranes, was in the fraction of high affinity CHA binding sites. Estimation of $f_{R_H}$, determined either by a comparison of the Bmax values of $[^3H]DPCPX$ and $[^3H]CHA$, or by the two-site analysis of CHA-inhibition of $[^3H]DPCPX$ binding, indicated values of 0.8-0.85 and 0.42-0.46 for LE and HE membranes respectively. Heterogeneous agonist binding has been widely observed for G-protein coupled receptors in both native tissue systems (Birdsall et al., 1978; Kent et al., 1980) and recombinant receptor expression systems (Vogel et al., 1995) and is believed to reflect the G-protein coupled (high agonist affinity) and uncoupled (low agonist affinity) states of the receptor. Heterogeneous agonist binding has also been reported for the adenosine A_1 receptor. In CHO cells expressing 1.2 pmol.mg^-1 receptor CHA inhibited 80% of $[^3H]DPCPX$ binding with high affinity (Cohen et al., 1996b), whilst in guinea pig forebrain where the receptor expression was 3.3 pmol.mg^-1, the agonist R-PIA inhibited 38% of antagonist binding with high affinity (Kollias-Baker et al., 1994).

The biphasic inhibition of the binding of a radiolabelled antagonist by an agonist is a prediction of the ternary complex model when $R_T \geq G_T$ (Fig. 3.3). When $R_T \ll G_T$ agonist binding can be described by a one-site binding model of high affinity. Mechanistically, heterogeneous agonist binding occurs when there is insufficient free G-protein for the total receptor population to form high affinity
ARG complexes. The existence of low affinity agonist binding sites can therefore be considered a consequence of a high $R_T:G_T$ ratio.

The TCM predicts that the fraction of high affinity agonist binding sites will be sensitive to changes in $R_T$ over a certain range of $R_T:G_T$ (Fig. 3). Hence $f_{H}$ decreases from 0.8 - 0.1 when $R_T:G_T$ increases by six-seven fold at a $K_q$ value of 1. Furthermore, the $f_{H}$ values of the range 0.85-0.5, which are observed experimentally in LE and HE membranes, are predicted by the TCM to occur over the $R_T:G_T$ range of 1.2 -2 (Fig. 3.3). If it is assumed that the total cellular G-protein content is similar in LE and HE membranes, the relative $R_T:G_T$ ratios should reflect the relative $B_{max}$ values, which have been determined experimentally to be of the order of twenty-fold different. This is in marked contrast to the predictions of the model. As the relative receptor expression levels in LE and HE membranes have been determined experimentally, whilst the relative levels of G-protein are unknown, the simplest explanation for this discrepancy is that the relative amount of G-protein available to form high affinity ARG complexes is at least ten-fold different between the two cell lines. The relative lack of sensitivity of $f_{H}$ to changes in receptor expression levels has also been shown for muscarinic $M_2$ receptors in CHO cells, where $f_{H}$ increased from 9% to 56% following a 30-fold reduction in receptor number by treating with the slowly dissociating antagonist quinuclidinyl benzilate (Vogel et al., 1995).

Studies which have measured the total cellular content of G-protein have found $G_T$ to be in considerable excess of $R_T$ (Mueller et al., 1991; Neubig et al., 1985; Ransnas & Insel, 1988a), an observation which is discrepant with the requirements for heterogenous agonist binding. This has led Neubig (1994) to propose the
compartmentation of receptor and G-protein such that the total cellular concentrations of receptor and G-protein do not reflect the ratio of R:G at the plasma membrane. A biochemical explanation for compartmentation may come from observations that GPCRs, G-proteins and effector molecules can be organised in specialised membrane domains such as caveolae and lipid rafts (Anderson, 1998).

The apparent high and low affinity agonist dissociation constants for CHA, measured directly in $[^3H]$CHA saturation binding experiments, or by analysis of $[^3H]$DPCPX competition curves, were essentially identical in LE and HE membranes. This result seems entirely intuitive and is predicted in the TCM simulations when $K_G > 1$ (Fig. 3.3f). However, when $K_G < 1$ the independence of $K_H$ and $K_L$ from $R_T$ begins to break down. This prediction would suggest that in LE and HE membranes the value of $K_G$ is required to be greater or equal to 1 to explain the experimental data.

The prediction of the TCM that, when $K_G < 1$, agonist affinity constants may vary over certain ranges of $R_T:G_T$ ratios, is a somewhat surprising finding given that a central tenet of the pharmacological classification of receptors is that ligand affinity is independent of receptor density. The findings with the adenosine A$_1$ receptor however, are consistent with the literature in that the proportions of sites, but not affinities, change with receptor expression. Interestingly, radioligand binding to cardiac membranes from transgenic mice which overexpress the $\beta_2$ adrenergic receptor have demonstrated an eight-fold increase in affinity for the agonist isoprenaline and a loss of guanine nucleotide sensitivity (Gurdal et al., 113
In this paper the increase in affinity was explained using a model of receptor dimerisation.

An important consideration when interpreting the predictions of the ternary complex model, is that when $G_T = R_T$ and $K_{G_t}G_T \leq 0.1$, the predicted data is poorly described by a two-site, non-independent binding model (Lee et al., 1986). This is illustrated by the poor description of the data by a two-site model when $R_T:G_T=1.2$ and $[G_T].K_{G} = 0.1$ (Fig. 3.3 panels a & c). This distortion is a consequence of the depletion of free G-protein following agonist binding to a receptor and forming ARG complexes, and is most extreme when $G_T = R_T$ (data not shown). Analysis of the predicted data when $G_T = R_T$ and $[G_T].K_{G} = 0.1$ with a two-site model of non-interconverting binding sites can generate estimates of $K_L$ and $K_H$ which can deviate from the appropriate parameters of the TCM by 10 - 30 fold (Lee et al., 1986). The good agreement of $K_L$ values determined from the analysis of agonist inhibition curves in the absence and presence of GDP, together with the similar estimates of $K_H$ for CHA in HE and LE membranes, suggests that both $R_T:G_T$ and $[G_T].K_{G}$ are $\geq 1$.

In summary, CHA labelled a heterogeneous population of binding sites in CHO cell membranes stably expressing the human recombinant adenosine $A_1$ receptor which indicates that in both cell lines $R_T:G_T > 1$. The affinity of CHA for the low and high affinity binding sites were similar in the two cell lines and represent the G-protein coupled and uncoupled states of the $A_1$ receptor. The similarity in the affinity values for CHA in low and high expressing $A_1$ membranes suggests that, according to the TCM there should be over 50% receptor G-protein pre-coupling in these membranes. In addition, the concentration of G-protein able to interact
with receptor appears to increase as the concentration of receptor increases, but it is less than the total cellular G-protein concentration.

*The binding of partial agonists to the adenosine A1 receptor*

This study has demonstrated that a series of adenosine A1 receptor agonists with different reported intrinsic activities (Sheehan et al., in press) demonstrate differences in proportions of high affinity binding and $K_L/K_H$ values (‘GDP shift’). The values of $f_{T,1}$ and $K_L/K_H$ obtained from the inhibition of $[^3H]DPCPX$ in the absence of GDP appeared to be related to the reported intrinsic activities of these compounds. However, for agonists of low efficacy it becomes progressively harder to accurately estimate the values of $f_{T,1}$ and $K_L/K_H$. This was particularly evident for GR 162900, the agonist of lowest efficacy, which demonstrated monophasic inhibition of $[^3H]DPCPX$ binding. Further estimates of $K_H$ and $K_L$ were obtained from inhibition of $[^3H]CHA$, and $[^3H]DPCPX$ in the presence of GDP, respectively. This approach provided a spectrum of ‘GDP shifts’ which ranged from 2000 for CHA, to <1 for the antagonist DPCPX, with intermediate values for the three partial agonists.

The range of GDP shifts for the adenosine A1 receptor described in this study is one of the largest reported for any G-protein coupled receptor. In rat brain homogenate Lorenzen et al. (1996) demonstrated a $K_L/K_H$ range of 19 for the partial agonist cladribine, to 85 for the high efficacy agonist CCPA, estimated from biphasic $[^3H]DPCPX$ inhibition curves. Kouranakis et al (2000) demonstrated a $K_L/K_H$ ratio of 40 for the high efficacy agonist CPA, which was also determined from biphasic inhibition of $[^3H]DPCPX$ in rat membranes.
However, complete receptor G-protein uncoupling could only be achieved by the inclusion of 1M NaCl, 1mM GTP and the G-protein inhibitor suramin and this marked difference in the binding conditions make comparisons between binding sites and theoretical models difficult. In contrast, in this thesis the full conversion to a monophasic, low affinity, G-protein uncoupled state of the A1 receptor has been observed by the simple addition of 100μM GDP.

In addition to the adenosine A1 receptor, the link between functional activity and the ratio of affinities for the G-protein coupled and uncoupled receptor states has been observed for a number of 7TM receptors, including muscarinic (Birdsall et al., 1978), β-adrenergic (Kent et al., 1980), cannabinoid (Kearn et al., 1999) and dopamine receptors (Lahti et al., 1992), and is entirely consistent with the theory and predictions of the ternary complex model.

An understanding of the ability of the GDP-shift to predict relative agonist efficacy comes from examination of the equations of the TCM (Appendix 1). An agonist can bind to the uncoupled state of a receptor with an affinity constant $K_a$ which is unique for each agonist-receptor pair. At the coupled state of a receptor the apparent affinity constant for an agonist is given by $\frac{K_a(1 + \alpha K_G [G])}{1 + K[G]}$ and is therefore dependent on $\alpha$, [G] and $K_G$ as well as $K_A$. The ratio $K_l/K_H$ is given by $\frac{(1 + \alpha K_G [G])}{(1 + K_G [G])}$ which is a function of $\alpha$ and is independent of the agonist affinity constant. The GDP-shift of a test agonist, expressed relative to that of a standard agonist (relative GDP-shift) is therefore $\frac{(1 + \alpha_{test} K_G [G])}{(1 + \alpha_{standard} K_G [G])}$. If it is assumed that
the terms \([G]\) and \(K_G\) are tissue dependent and agonist independent, then the rank order of GDP-shifts will be identical to the relative values of \(\alpha\), the molecular determinant of agonist efficacy in the ternary complex model. The GDP shift is therefore a good index of relative agonist efficacies.

Agonist sensitivity to guanine nucleotides can vary between subtypes of the same receptor, for example cortical (mainly \(M_1\), Hulme & Birdsall, 1992) and cardiac (\(M_2\), Berrie et al., 1979) muscarinic receptors and this may reflect the preference of a receptor for a particular G-protein subtype. There are also reports that agonist binding to 7TM GPCRs is completely insensitive to guanine nucleotides (see, for example, Tietje et al., 1990; Zhang & Pratt, 1996). The reasons for this remain unclear, although it may be due to the formation of other high affinity agonist-receptor-protein complexes such as agonist-receptor-arrestin, which has been demonstrated for both the \(\beta_2\)-adrenergic and the \(M_2\) muscarinic receptors (Gurevich et al., 1997). However, whilst the magnitude of the GDP shift may vary among GPCRs, the utility of the GDP shift in ranking the efficacies of agonists remains.

In this study the partial agonist GR161144 generated significantly fewer high affinity binding sites than the full agonist CHA. Agonist dependent differences in \(f_{H}\) have also been demonstrated for adenosine receptors in rat brain membranes, where the high efficacy agonist CCPA has a \(f_{H}\) of 45% whilst the partial agonist cladribine, with intrinsic activity of 0.2, has a \(f_{H}\) of 27% (Lorenzen et al., 1996). Agonist intrinsic activity has been shown to correlate with \(f_{H}\) at other GPCRs, including \(\beta\)-adrenergic (Kent et al., 1980), muscarinic (Birdsall et al., 1978) and cannabinoid (Kearn et al., 1999) receptors. In contrast, a negative correlation
between \( K_L/K_H \) and \( f_{H} \) has been demonstrated for the dopamine \( D_2 \) receptor (Sibley & Creese, 1982; Wreggett & De Lean, 1984). Therefore, experimental observations clearly demonstrate that different agonists can form different fractions of high affinity binding sites.

As discussed above, however, the predictions of the TCM suggest that the ability to observe low affinity binding sites arises essentially as a consequence of the ratio of \( R_T \) and \( G_T \). When \( R_T \) is in excess of \( G_T \) there are not enough G-proteins to generate 100% high affinity ARG complexes and agonist inhibition curves will be heterogeneous. The ratio of \( R_T \) to \( G_T \) therefore constrains, and in general is proportional to, the fraction of high affinity agonist binding sites.

However, as demonstrated in Fig. 3.5 a and b, there are specific sets of conditions under which \( f_{H} \) can vary as a consequence of agonist efficacy, namely when \( R_T= G_T \) and/or \([G_T]_{K_G} < 1 \). As discussed in the previous section, this change in the fraction of high affinity binding sites arises out of the poor description of the data by a two independent-site binding model under these conditions. Therefore, there are particular ranges of \( f_{H} \) which are permitted within the predictions of the TCM, which vary at different values of \( K_L/K_H \) (Lee et al., 1986). In general, the \( f_{H} \) values generated for CHA, GR190178 and GR 161144 in both LE and HE membranes lie close to, but outside of, these permitted values. This observation is consistent with data from \( \beta \)-adrenergic (Kent et al., 1980), \( D_2 \) dopamine (Sibley & Creese, 1982) and muscarinic (Wong et al., 1986) receptors.

Whilst the TCM predicts that \( K_L/K_H \) will vary with agonists of different efficacy, it is therefore unable to predict agonist dependent differences in \( f_{H} \) which lie
outside the permitted values which arise when \( R_t = G_t \) and/or \([G_t].K_G < 1\). In order to accommodate this discrepancy within the framework of the TCM, it is therefore necessary to postulate that \( R_t:G_t \) is not a fixed property of the cellular system, but is dependent on a property of the agonist. This was implied, but not explicitly discussed, in the analysis of agonist binding to the \( \beta \)-adrenergic receptor using the TCM (De Lean \textit{et al.}, 1980).

The comparison of the proportions of high affinity binding sites for CHA in high and low expression systems presented in Figures 3.1-3.3 has led to the consideration that \( G_t \) is not a fixed parameter, but may vary with receptor density (see discussion above). The requirement for \( G_t:R_t \) to also vary as receptors are occupied by agonists of different efficacy is therefore an extension of this argument. A mechanistic explanation for this may come from biochemical and immunocytochemical studies which demonstrate that receptors are expressed as both diffuse and clustered populations (Daly \textit{et al.}, 1998). In addition, in DDT1MF-2 cells agonist activation of the adenosine \( A_1 \) receptor promotes receptor clustering (Ciruela \textit{et al.}, 1997). In contrast, in unstimulated rat cardiac ventricular myocytes 70\% of adenosine \( A_1 \) receptors are associated with caveolae membrane microdomains and agonist activation results in the rapid translocation of the \( A_1 \) receptor to non-caveolae plasma membrane (Lasley \textit{et al.}, 2000). However, these observations were made in intact cells and are likely to be processes which require energy and the presence of cytoskeletal proteins which are probably not present in membranes.

If agonists are able to alter the patterns of membrane distribution of 7TM GPCRs then the possibility exists for the apparent \( R_t:G_t \) ratio to be dependent on the
agonist used. In order to address this, immunocytochemistry experiments should be performed using antibodies to the A1 receptor, its cognate G-protein and possibly lipid-raft markers such as anti-caveolin antibodies. The effects of agonists of different efficacy on the distribution patterns of receptor and G-protein could then be evaluated.

*The effect of GDP on agonist binding to the adenosine A1 receptor*

A common feature of 7TM GPCRs is the ability of guanine nucleotides to modulate the binding of agonists. This effect is thought to result from the uncoupling of high affinity ARG complexes to generate low affinity AR complexes (Gilman, 1987). Many studies use a high concentration of guanine nucleotide to either inhibit the binding of a radiolabelled agonist or to achieve complete conversion to a low affinity state in radiolabelled antagonist-unlabelled agonist inhibition studies. From both of these approaches however, it can not be determined whether the effect of the nucleotide is to decrease the affinity or the maximum binding capacity of an agonist. Whilst guanine nucleotides are assumed to destabilise, or uncouple, RG complexes, the mechanistic interpretation of this process remains unclear. In these experiments the effects of graded concentrations of GDP on the affinities and proportions of CHA binding sites at the A1 receptor were investigated. The results were not consistent with a simple change in the affinity of receptor for G-protein, but were accompanied by a decrease in $f_{R\text{H}}$ which is not predicted by the ternary complex model.

The TCM predicts that decreasing $K_G$ will result in a decrease in both $K_H$ and $f_{R\text{H}}$. The effect of changing $K_G$ on $f_{R\text{H}}$ is most pronounced at a $R_T:G_T$ ratio of 1.2. This
would suggest that the predicted decreases in frH are a result of the predicted data being poorly described by a two independent site binding model when the conditions are \( R_T:G_T \approx 1 \) and \([G_T].K_G < 1\) (see previous sections). In general, however, the changes in frH observed experimentally with increasing concentrations of GDP, occur at \( K_L/K_H \) values which are greater than those required to satisfy the predictions of the TCM. The effects of GDP on frH observed experimentally cannot therefore be explained by the TCM in terms of only a decrease in \( K_G \).

In order to explain the effects of GDP on frH within the framework of the TCM it is therefore required to postulate that guanine nucleotides increase the ratio \( R_T:G_T \). This has been discussed in the previous sections concerning the effects of low efficacy agonists. However, an alternative explanation for the inability of the model to predict the effects of GDP on frH may be the lack of specific terms for G-protein subunits and guanine nucleotides in the model. Whilst extended versions of the TCM have been developed to accommodate these additional terms (Onaran et al., 1993; Waelbroeck, 2001), the ability of these models to predict the effects of guanine nucleotides on heterogeneous binding has not been evaluated.

Few studies have investigated the effect of graded guanine nucleotide on heterogeneous agonist inhibition of radiolabelled antagonist at the adenosine A₁ receptor. In bovine brain membranes, Lorenzen et al. (1993) showed that increasing concentrations of GDP (1-100μM) decreased the fraction of high affinity binding sites from 80 to 40% whilst the affinity of R-PIA for the G-protein coupled state of the receptor decreased by only three-fold. However, full conversion to a homogeneous, low affinity population of receptors was not
achieved in this study, and the residual GDP resistant, high affinity binding may therefore mask further decreases in agonist affinity. Mahle et al. (1992) have reported that graded increases in the concentration of the metabolically stable guanine nucleotide analogue guanosine 5'-[β,γ-imido]triphosphate (Gpp(NH)p) decreased $B_{\text{max}}$, with little effect on $K_D$, in radiolabelled agonist saturation binding experiments in rat hippocampal membranes. However, in this study approximately 50% of the binding was resistant to Gpp(NH)p. This large amount of residual high affinity binding makes it very difficult to estimate the agonist affinity for the guanine nucleotide sensitive RG complexes.

Kent et al. (1980) examined the effect of increasing concentrations of Gpp(NH)p on the inhibition of the radiolabelled antagonist [3H]dihydroalprenolol by the agonist isoprenalol at the β-adrenergic receptor. In this study, Gpp(NH)p produced a marked decrease in $f_{\text{RH}}$ with little change in $K_L/K_H$. In contrast, at the $\alpha_2$-adrenergic receptor, an intermediate concentration of Gpp(NH)p had no effect on $f_{\text{RH}}$ but produced a 4-fold increase in $K_H$ (Hoffman et al., 1982). In a study of β-adrenoceptors, De Lean and colleagues (1980) examined the effect of graded Gpp(NH)p on the inhibition of radiolabelled antagonist binding by the agonist hydroxybenzylisoproterenol. The data could only be described by the TCM if Gpp(NH)p was allowed to decrease both the ratio $G_T:R_T$ and the agonist efficacy term $\alpha$. Wreggett and De Lean (1984) have also shown that the effect of guanine nucleotides on agonist binding to the D2-dopamine receptor can be adequately fit by the TCM only if it is assumed that the concentration of G-protein is reduced. The effects of GDP on the binding properties of CHA at the adenosine A1 receptor
are therefore generally consistent with the literature reports for other G-protein coupled receptors.

It has therefore been shown that guanine nucleotides can decrease both the agonist affinity for, and the number of, high affinity binding sites. Both of these observations can be qualitatively described by the TCM when it is assumed that the effect of GDP is to decrease the value of $K_q$. However, the predicted effects of GDP on $f_{RH}$ occur at values of $K_I/K_H$ which are lower than those values determined experimentally. This failure of the TCM to satisfactorily describe the effects of guanine nucleotides on agonist binding probably results from lack of the explicit inclusion of nucleotides within the scheme.

The effect of GDP the binding of $[^3\text{H}]$DPCPX to the adenosine A$_1$ receptor

GDP increased the binding of 0.5nM $[^3\text{H}]$DPCPX in LE and HE membranes by 60 and 30% respectively. The magnitude of the increase in $[^3\text{H}]$DPCPX binding to LE membranes was similar to that found previously in this cell line by Cohen et al. (1996b). Guanine nucleotides have frequently been reported to increase the binding of radiolabelled antagonists to the adenosine A$_1$ receptor by increasing the Bmax, with no change in antagonist affinity (Green, 1984; Schiemann et al., 1990; Stiles, 1988). In addition, guanine nucleotides have no effect on the kinetics of radioligand antagonist dissociation to the A$_1$ receptor (Klotz et al., 1990; Parkinson & Fredholm, 1992; Prater et al., 1992), but increase the extent, but not the rate, of radiolabelled antagonist association (Cohen et al., 1996b; Prater et al., 1992).
It has been proposed that guanine nucleotides increase the binding of antagonists in an indirect manner, by decreasing the affinity of endogenous adenosine for the $A_1$ receptor (Prater et al., 1992; Schiemann et al., 1990). However, in saturation binding studies this should not be the case as high concentrations of radiolabelled antagonist should fully compete with endogenous adenosine and label the entire population of receptors. In this situation it would be expected that guanine nucleotides would increase the apparent affinity of the antagonist for the receptor, with no change in $B_{max}$, which is generally not observed experimentally.

An alternative explanation for the effects of GDP on $[^3H]DPCPX$ binding is the presence of 'locked' endogenous adenosine-receptor-G-protein complexes. In washed membranes, in the presence of ADA and the membrane permeabilising agent saponin, the free concentration of adenosine is likely to be very low. However, during the preparation of the membranes, endogenous adenosine is able to bind to $A_1$ receptors and form long lived, 'locked' agonist receptor complexes, as demonstrated in the $[^3H]CHA$ dissociation experiments described in section 3.2.8.2. Endogenous adenosine which is tightly bound to $A_1$ receptors will be insensitive to membrane washing and the presence of ADA. However, it has also been demonstrated that these locked, slowly reversible adenosine receptor complexes are fully dissociated in the presence of GDP. The increase in antagonist binding in the presence of GDP can therefore be explained by the generation of additional binding sites for the radioligand by the uncoupling of receptors tightly bound to endogenous adenosine. The enhanced increase in the binding of $[^3H]DPCPX$ in the presence of GDP in LE membranes probably
reflects the greater proportion of high affinity binding sites in these membranes (see section 3.2.2) available for the binding of endogenous adenosine.

\[^3H\]DPCPX/\[^3H\]CHA dissociation kinetics

The kinetics of \[^3H\]DPCPX dissociation from the A\textsubscript{1} receptor were consistent with a simple, reversible bimolecular interaction between the radioligand and the receptor. This result is similar to previous reports of \[^3H\]DPCPX dissociation from both recombinant (Cohen \textit{et al.}, 1996b) and endogenous A\textsubscript{1} receptors (Gerwins \textit{et al.}, 1990; Klotz \textit{et al.}, 1990). In addition, the absence of any effect of GDP on \[^3H\]DPCPX dissociation indicates that the dissociation of the radiolabelled antagonist is not sensitive to the G-protein coupled state of the A\textsubscript{1} receptor. Klotz \textit{et al} (1990) have also shown the dissociation of \[^3H\]DPCPX from adenosine A\textsubscript{1} receptors in rat brain to be insensitive to guanine nucleotide and Mg\textsuperscript{2+}. It has also been found that the dissociation of the A\textsubscript{1} receptor antagonist \[^125I\]BW-A844U from bovine brain membranes is not affected by either guanine nucleotide or Mg\textsuperscript{2+} (Prater \textit{et al.}, 1992; Schiemann \textit{et al.}, 1990).

In contrast to the simple behaviour of \[^3H\]DPCPX, the dissociation of \[^3H\]CHA was complex. In this study, 70% of specific agonist binding was found to be in the form of ‘locked’, GTP sensitive, pseudoirreversible ARG complexes, which did not dissociate within the time period of the experiment. The formation of pseudoirreversible agonist-receptor complexes has been observed for a number of receptors including muscarinic (Hirschberg & Schimerlik, 1994), \(\beta\)-adrenergic (Severne \textit{et al.}, 1986), somatostatin (Koenig \textit{et al.}, 1997) and calcitonin (Hilton \textit{et al.}, 2000) receptors. In most studies the radiolabelled agonist dissociates in the
presence of guanine nucleotide, implicating the influence of a G-protein in the locked agonist receptor complex. At the calcitonin receptor however, locked agonist-receptor complexes are insensitive to the guanine nucleotide analogue GTPγS (Hilton et al., 2000).

Pseudoirreversible agonist binding to the adenosine A₁ receptor has been frequently reported in a number of species including pig (Leid et al., 1988), rat (Schwabe & Trost, 1980) and human (Waldhoer et al., 1999). In these studies 50-80% of specific radiolabelled agonist binding dissociated following the addition of a large excess of unlabelled agonist. Interestingly Cohen (1996b) showed that 35-50% of specific radiolabelled agonist binding was dissociated following the addition of a high concentration of the antagonist theophylline, whilst Stiles (1988) observed no dissociation of agonist from solubilised rat A₁ receptor following dilution alone. These differences in radiolabelled agonist dissociation may be due to species variation, or the experimental conditions used. However, the most obvious differences in methodology were in the methods used for initiating radioligand dissociation. For this reason, the effects of dilution alone, or dilution in the presence of a high concentration of unlabelled agonist or antagonist were compared in LE and HE membranes. It was demonstrated that agonists, but not antagonists, facilitate the dissociation of a radiolabelled agonist from the adenosine A₁ receptor. This effect is dependent on receptor expression level because it was only observed in HE, but not LE membranes.

Despite the frequent reports of agonist locking at the A₁ receptor and other 7TM GPCRs, few studies have addressed the molecular mechanisms of this phenomenon. For an unlabelled ligand to facilitate the dissociation of a
radioligand from a receptor, information from the unlabeled ligand occupied receptor must be transferred to the radioligand occupied receptor. This transfer of information may occur directly through a receptor-receptor interaction, or indirectly via a G-protein. The former would require the formation of adenosine A₁ receptor dimers, whilst the latter would require the depletion of functionally relevant G-proteins following the addition of excess agonist.

For the porcine muscarinic M₂ receptor Hirschberg and Schimerlik (1994) demonstrated agonist locking in high expressing membranes following dilution, which could be unlocked when dissociation was initiated either by agonist or antagonist. A model was proposed which allowed the formation of asymmetric, non-covalent receptor dimers. Each dimer binds a single molecule of radiolabelled agonist (at low concentrations) in a pseudoirreversible manner. Upon binding of an unlabelled ligand (agonist or antagonist) the dimer dissociates into monomeric receptors which freely dissociate from the radioligand.

The data from the A₁ receptor in this study differ from the porcine M₂ muscarinic receptor in that antagonist insensitive, radiolabelled agonist locking is observed in high and low expressing membranes. Furthermore, locked agonist receptor complexes can be unlocked by agonists in high, but not low, expressing membranes. These observations may be consistent with the presence of adenosine A₁ receptor homodimers in HE membranes only. However, if locked agonist-A₁ receptor complexes reflect the presence of receptor dimers, the dimerisation process would appear to be independent of receptor expression as similar levels of locking are observed in both LE and HE membranes.
Whilst the model of Hirschberg and Schimerlick may explain certain aspects of the $A_1$ receptor the mechanism of agonist locking remains unclear. A number of questions should be addressed through further experimentation. Firstly is there a time dependence for formation of locked complexes and is locking still observed with shorter pre-incubation periods? In this study a 1 hour incubation prior to dissociation was used. Secondly, can the dissociation of locked agonist receptor complexes by cold agonist be considered a ‘functional’ response to agonist? What is the concentration range of unlabelled agonist which induces unlocking, what are the effects of partial agonists, and can this response be blocked by an antagonist? Finally, can other 7TM GPCRs facilitate agonist unlocking through a cross talk mechanism either at the level of receptor or G-protein. It would be expected that a model describing these experimental observations will be able to link receptor density, agonist locking and agonist induced agonist dissociation.
Chapter 4 Pharmacological characterisation of $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding responses in CHO A₁ low and high expressing membranes.

4.1 Introduction

In the previous chapter the effect of receptor density on the G-protein coupling characteristics of the adenosine $\text{A}_1$ receptor was investigated indirectly by examining the effects of agonists on the binding of a radiolabelled agonist and a radiolabelled antagonist in the absence and presence of GDP. As a measure of function, G-protein activation can be measured directly by utilising the $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ assay. In contrast to GTP, $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ is resistant to hydrolysis by the GTP-ase activity of the G-protein $\alpha$ subunit and $\text{G}\alpha[^{35}\text{S}]\text{GTP}\gamma\text{S}$ accumulates in membranes following G-protein activation (Lazareno, 1999). $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding provides a measure of agonist activity that is proximal to the activated receptor and is not dependent on the activity of the components of biochemical signalling cascades. The $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding response is therefore less amplified than second messengers such as cAMP or $\text{Ca}^{2+}$. $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding responses in cell membranes should, in principle, be less prone to receptor desensitisation than whole cell functional responses.

Activation of the endogenous (Lorenzen et al., 1993) or recombinant (Cohen, 1995) adenosine $\text{A}_1$ receptors in membranes leads to an accumulation of $[^{35}\text{S}]\text{GTP}\gamma\text{S}$. In CHO cells activation of the human recombinant $\text{A}_1$ receptor has
been shown by co-immunoprecipitation experiments to result in [\(^{35}\)S]GTP\(\gamma\)S labelled \(\alpha_i\), \(\alpha_{q/11}\) and \(\alpha_q\) G-protein subunits (Cordeaux et al., 2000). However, adenosine A\(_1\) receptor stimulated accumulation of [\(^{35}\)S]GTP\(\gamma\)S in CHO cell membranes is completely abolished by pertussis toxin, suggesting that [\(^{35}\)S]GTP\(\gamma\)S is binding to G-protein \(\alpha_i\) and \(\alpha_q\) subunits. Furthermore, purified bovine A\(_1\) receptors have been shown to activate \(\alpha_{i1}\), \(\alpha_{i2}\) and \(\alpha_q\) subunits when reconstituted into phospholipid vesicles (Munshi et al., 1991) and the A\(_1\) receptor, when fused with either G \(\alpha_{i1}\), \(\alpha_{i2}\), \(\alpha_{i3}\) or \(\alpha_q\), stimulates [\(^{35}\)S]GTP\(\gamma\)S binding with similar agonist potencies (Wise et al., 1999). The stimulation of [\(^{35}\)S]GTP\(\gamma\)S binding in CHO cell membranes by the adenosine A\(_1\) receptor is therefore mediated primarily by G-proteins of the G\(\alpha_{i/q}\) family.

The aim of these studies was to compare and contrast a number of pharmacological parameters of the [\(^{35}\)S]GTP\(\gamma\)S binding response following activation of the adenosine A\(_1\) receptor when expressed at different densities. These parameters include basal [\(^{35}\)S]GTP\(\gamma\)S binding, agonist potency, agonist maximum response, antagonist affinity and the effect of the enhancer PD 81,723. In preliminary studies it became clear that agonist concentration response curves were biphasic in high expressing membranes, stimulating [\(^{35}\)S]GTP\(\gamma\)S binding at low concentrations and inhibiting at high concentrations. An inhibitory [\(^{35}\)S]GTP\(\gamma\)S binding response had not been previously described in the literature and the mechanism responsible for this effect was intriguing. In studies undertaken to pharmacologically characterise this inhibitory response, it was demonstrated that this effect was mediated by the adenosine A\(_1\) receptor and was
related to the extent of G-protein activation. To attempt to explain the nature of this inhibitory phenomenon further studies were carried out, including an investigation of the kinetics of $[^{35}\text{S}]\text{GTP} \gamma\text{S}$ association to CHO cell membranes and attempts to measure the release of G-protein $\alpha$ subunits from the membranes.

### 4.2 Results

#### 4.2.1 Agonist stimulation of $[^{35}\text{S}]\text{GTP} \gamma\text{S}$ binding in CHO A$_1$ low and high expressing membranes.

The binding of $[^{35}\text{S}]\text{GTP} \gamma\text{S}$ to CHO A$_1$ membranes was measured after a 30 min. incubation with 0.1nM $[^{35}\text{S}]\text{GTP} \gamma\text{S}$ at 30°C and the method is described in detail in the experimental section. In LE membranes which expressed 0.5 - 1 pmol.mg$^{-1}$ of the A$_1$ receptor, the binding of $[^{35}\text{S}]\text{GTP} \gamma\text{S}$ in the absence of agonist was 0.045±0.003 fmol.mg$^{-1}$ membrane protein. In HE membranes which expressed 5 - 20 pmol.mg$^{-1}$ of the A$_1$ receptor, basal binding was 2.2±0.2 fold higher at 0.10±0.01 pmol.mg$^{-1}$ (n=17; Fig. 4.1). The increase in basal $[^{35}\text{S}]\text{GTP} \gamma\text{S}$ binding in HE membranes persisted in the presence of a 10-fold higher concentration of adenosine deaminase and the membrane permeabilising agent saponin (10μg.ml$^{-1}$; data not shown) which suggests that it is not due to the presence of cryptic pools of endogenous adenosine. These data suggest that in HE membranes the level of expression of the adenosine A$_1$ receptor is sufficient to allow the detection of constitutively activated G-proteins.
4.1. CHA concentration-effect curves for the modulation of $[^{35}\text{S}]{\text{GTP}}_{\gamma}\text{S}$ binding to adenosine A$_1$ LE and HE membranes.

CHO adenosine A$_1$ LE and HE membranes (20μg) were pre-treated with adenosine deaminase (3U.ml$^{-1}$) and incubated with 0.1nM $[^{35}\text{S}]{\text{GTP}}_{\gamma}\text{S}$, 10μM GDP and increasing concentrations of CHA for 30 min. at 30°C. Data are the mean of triplicate points and representative of 17 experiments. The lines represent the non-linear regression best fits of the data to either a one component (LE) or two component (HE) logistic equation as described in Chapter 2.

The mean values for the basal binding of $[^{35}\text{S}]{\text{GTP}}_{\gamma}\text{S}$ were 0.045 ± 0.003 and 0.10 ± 0.01 fmol.mg$^{-1}$ membrane protein in low and high expressing membranes respectively.
The agonist N\(^6\)-cyclohexyladenosine (CHA) stimulated the binding of 0.1nM [\(^{35}\)S]GTP\(_\gamma\)S to both CHO A\(_1\) LE and HE membranes in a concentration dependent manner (Fig. 4.1 and Table 4.1). The potency (pEC\(_{50}\)) of CHA was increased 10 fold, from 8.10±0.02 in CHO A\(_1\) LE membranes to 9.11±0.08 in HE membranes (n=17). This confirmed the results in Chapter 3 which suggested a ratio of R\(_T\):G\(_T\)>1 in these membranes.

The increase in potency of CHA was accompanied by an increase in the maximum response from 0.13±0.01 fmol.mg\(^{-1}\) in LE membranes to 0.33±0.03 pmol.mg\(^{-1}\) membrane protein in HE membranes (n=17). The increase in the maximum amount of CHA stimulated [\(^{35}\)S]GTP\(_\gamma\)S binding in HE membranes persisted even when membranes were incubated for four hours (data not shown) which indicates that the difference is due to an increase in the total number of G-proteins available for activation, and not a kinetic artefact which may be apparent at shorter incubation periods. These data are therefore consistent with the hypothesis proposed in Chapter 3 that the A\(_1\) receptor in HE membranes is able to interact with, or recruit, more G-proteins than in LE membranes.

Responses to CHA were not observed in CHO cells which had not been transfected with the recombinant adenosine A\(_1\) receptor. Furthermore, the responses to CHA were completely abolished in membranes from CHO A\(_1\) LE and HE cells which had been treated overnight with 50ng.ml\(^{-1}\) pertussis toxin (data not shown), confirming previous reports that the A\(_1\) receptor primarily activates the \(\alpha_{\psi_6}\) subfamily of G-protein \(\alpha\) subunits.
Table 4.1 Summary of agonist concentration-response parameters for the modulation of $[^{35}\text{S}]$GTP$\gamma$S binding to CHO A$_1$ low expression and high expression membranes.

| Agonist   | Low expression | High expression | | | | | | |
|-----------|----------------|----------------|---|---|---|---|---|---|---|---|---|
|           | pEC$_{50}$     | n$_H$          | i.a. | pEC$_{50}$ | n$_H$ | i.a. | pIC$_{50}$ | % inhibition | i.a. | (inhib.) |
| CHA       | 8.12±0.03      | 0.9±0.1        | 1.0 | 9.11±0.03 | -     | 1.0 | 7.10±0.04 | 62±3 | 1.0 |          |
| NECA      | 8.01±0.01      | 0.9±0.1        | 1.0±0.1 | 9.09±0.01 | -     | 0.9±0.1 | 6.65±0.24 | 58±7 | 0.9±0.1 |          |
| R-PIA     | 8.28±0.04      | 0.7±0.1        | 1.0±0.1 | 9.42±0.03 | -     | 0.9±0.1 | 7.08±0.03 | 53±5 | 0.9±0.1 |          |
| GR190178  | 7.34±0.06      | 1.0±0.1        | 1.0±0.3 | 8.29±0.04 | -     | 1.1±0.1 | 6.62±0.10 | 45±3 | 0.8±0.1 |          |
| GR161144  | 7.33±0.03      | 1.1±0.1        | 0.8±0.1 | 8.21±0.06 | 1.3±0.1 | 0.9±0.1 | 6.56±0.26 | 19±5 | 0.3±0.1 |          |
| GR162900  | 7.38±0.09      | 1.0±0.1        | 0.5±0.1 | 7.99±0.06 | 1.0±0.1 | 0.8±0.1 | -     | -     | -     |          |

Data are the mean ± s.e.mean of 17 CHA experiments, 5 GR162900, GR161144 and GR190178 experiments, and mean±range/2 of 2 NECA and R-PIA experiments.

i.a.: intrinsic activity, expressed as the maximum response of the test agonist as a ratio of the maximum response to CHA determined in the same experiment.

n$_H$: Hill slope determined by fitting monophasic data to a four-parameter logistic equation.

a: responses to GR161144 were biphasic in two out of five experiments and data are expressed as mean±range/2.

b: responses to GR162900 were monophasic in all experiments.
The responses to CHA in HE membranes were biphasic. Concentrations greater than 10nM CHA produced a concentration-dependent decrease in [\(^{35}\)S]GTP\(_\gamma\)S binding with a potency which was 100 fold higher than the stimulatory component, and a maximum inhibitory response of 62±3% of the total amount of stimulated [\(^{35}\)S]GTP\(_\gamma\)S binding (Fig. 4.1 & Table 4.1). In contrast, CHA responses in LE membranes were monophasic. The increases in potency and maximum response and the biphasic nature of the CHA concentration-response curve were also observed for the adenosine agonists R-PIA and NECA (Table 4.1). The stimulatory and inhibitory responses have been characterised in detail.

4.2.2 The effect of the antagonist DPCPX on the responses to CHA in CHO A\(_1\) low and high expressing membranes.

The effects of the adenosine A\(_1\) receptor antagonist DPCPX on agonist responses in CHO A\(_1\) LE and HE membranes were investigated. Increasing concentrations of DPCPX (10-100nM) produced a parallel, concentration dependant rightward shift in the responses to CHA in both LE and HE membranes with no effect on the maximum response to CHA (n=3; Fig. 4.2 a & b). Schild analyses produced a straight line with a slope which was not significantly different from unity for both LE and HE membranes, which is indicative of a simple competitive interaction between DPCPX and CHA (Fig. 4.2c). The pA\(_2\) values for DPCPX were determined after the slopes of the Schild plots had been constrained to unity. There was no difference in the pA\(_2\) values for DPCPX of 8.53±0.19 and 8.52±0.15 for LE and HE membranes respectively. The inhibitory response to CHA in HE membranes was also shifted in a parallel, rightward manner (Fig. 4.2b). The pA\(_2\)
Figure 4.2. Antagonism of CHA mediated modulation of $[^{35}S]GTP\gamma S$ binding to adenosine A₁ LE and HE membranes by the adenosine A₁ receptor antagonist DPCPX.

CHO adenosine A₁ LE (a) or HE (b) membranes (20μg) were pretreated with adenosine deaminase (3U.ml⁻¹) and then incubated for 30 min. at 30°C with 10μM GDP, increasing concentrations of CHA, and the stated concentrations of DPCPX. 0.1nM $[^{35}S]GTP\gamma S$ was then added and the membranes incubated for a further 30 min. at 30°C. Data are the mean ± s.e.mean of triplicate data points and representative of four similar experiments. The lines represent the non-linear regression best fits of the data to either a one-component (LE) or two-component (HE) logistic equation as described in Chapter 2.

Panel c: Schild plots for LE and HE membranes (stimulatory and inhibitory responses). The lines represent the best fit to a straight line as described in Chapter 2.

Panel d: The effect of DPCPX on basal $[^{35}S]GTP\gamma S$ binding in HE and LE membranes.
a. Total[^35S]GTP-S bound (CPM)

- control
- 10nM DPCPX
- 30nM DPCPX
- 100nM DPCPX

b. Total[^35S]GTP-S bound (CPM)

- [CHA] log M

C. log (DR-1)

- low expression
- high expression - stimulation
- high expression - inhibition

D. Total[^35S]GTP-S bound (CPM)

- [DPCPX] log M

- low exp
- high exp
for antagonism of the inhibitory component by DPCPX was 8.42±0.14, which was similar to the pA\textsubscript{2} for antagonism of the stimulatory effects of CHA. This demonstrates that the inhibitory response observed in HE membranes is mediated specifically by the adenosine A\textsubscript{1} receptor.

In HE membranes DPCPX inhibited basal $[^{35}\text{S}]$GTP\textgamma{S} binding in a concentration dependent manner with a maximum inhibition of 36±4% (n=4; Fig. 4.2d). Concentration-effect curves to DPCPX were generated with a pIC\textsubscript{50} of 8.32±0.21 which was similar to the pA\textsubscript{2} for antagonism of the responses to CHA. Constitutive receptor activity was also observed, but to a lesser extent, in LE membranes as DPCPX (100nM) inhibited basal $[^{35}\text{S}]$GTP\textgamma{S} binding by 10±3% (n=4). The small magnitude of this response precluded an estimation of the potency of DPCPX (Fig. 4.2d)

Following an incubation with a 10 fold higher concentration of adenosine deaminase and in the presence of 10μg.ml\textsuperscript{-1} of the membrane permeabilising agent saponin, basal $[^{35}\text{S}]$GTP\textgamma{S} binding in HE membranes was inhibited by DPCPX (1μM) to a similar extent as control membranes (data not shown) Taken together, these data suggest that the inhibitory effect of DPCPX results from inverse agonism of a constitutively active adenosine A\textsubscript{1} receptor and is not due to the presence of endogenous adenosine. In addition, the binding of $[^{35}\text{S}]$GTP\textgamma{S} to HE membranes in the presence of DPCPX was higher than the basal binding in LE membranes (Fig. 4.2d) which suggests either that DPCPX is a partial inverse agonist, or that there is an intrinsic difference in the $[^{35}\text{S}]$GTP\textgamma{S} binding properties between the two cell lines.
4.2.3 The effect of PD 81,723 on the CHA mediated modulation of $[^{35}S]GTP\gamma S$
binding to CHO adenosine $A_1$ low and high expressing membranes.

In both LE and HE adenosine $A_1$ membranes the allosteric enhancer PD 81,723
(PD, $3\mu M$) increased the potency (pEC$_{50}$) of CHA three fold (Fig. 4.3). The
pEC$_{50}$ for CHA was increased from $8.08\pm0.02$ to $8.54\pm0.04$ in LE membranes and
from $8.99\pm0.05$ to $9.41\pm0.07$ in high expressing membranes ($n=7$). At a
concentration of $3\mu M$, PD also produced a small decrease in the maximum
response to CHA of $12\pm2\%$ and $11\pm4\%$ in LE and HE membranes respectively.
The inhibitory effect of PD on the maximum response to CHA was more marked
at $10\mu M$ PD. In case this was due to a non-specific effect, a concentration of $3\mu M$
PD was used in all subsequent studies. In the presence of $3\mu M$ PD the inhibitory
response to CHA in HE membranes was also potentiated three fold from
$7.22\pm0.08$ to $7.74\pm0.06$ whilst the magnitude of the inhibitory response was
increased by $5\pm1\%$ ($n=7$). These data provide further evidence that the inhibitory
response is mediated by the adenosine $A_1$ receptor.

PD ($3\mu M$) had no effect on the basal level of $[^{35}S]GTP\gamma S$ binding in LE
membranes but increased the basal binding in HE membranes by $38\pm6\%$ ($n=7$).
An increase in basal $[^{35}S]GTP\gamma S$ binding of similar magnitude was observed in
membranes treated with a 10 fold higher concentration of adenosine deaminase
and in the presence of saponin ($10\mu g.ml^{-1}$), which suggests that the stimulatory
effect of PD results from the intrinsic agonist activity of the compound and not
from the potentiation of endogenous adenosine.
Figure 4.3. The effect of the allosteric enhancer PD 81,723 on the modulation of $[^{35}\text{S}]\text{GTP}_{\gamma}\text{S}$ binding by CHA in adenosine A$_1$ LE and HE membranes.

CHO adenosine A$_1$ LE (a) and HE (b) membranes (20 µg) were pre-treated with adenosine deaminase (3 U mL$^{-1}$) and then incubated with 10 µM GDP, 0.1 nM $[^{35}\text{S}]\text{GTP}_{\gamma}\text{S}$ and increasing concentrations of CHA for 30 min. at 30°C in the absence or presence of PD 81,723 (3 µM) as stated. Data are the mean ± s.e.mean of triplicate points and are representative of 7 experiments. The lines represent the non-linear regression best fits of the data to either a one component (LE) or two component (HE) logistic equation as described in Chapter 2.
4.2.4. The effect of lower efficacy agonists on \[^{35}\text{S}]\text{GTPyS}\) binding to CHO adenosine A\(_1\) LE and HE membranes.

A series of partial agonists were available which had been demonstrated to have a range of intrinsic activities at the adenosine A\(_1\) receptor in a number of functional assays (Sheehan et al., in press; Figure 2.1). In the previous chapter, the binding properties of these agonists were determined and it was also shown that the rank order of ‘GDP shifts’ for these compounds in radioligand binding assays was the same as the expected rank order of intrinsic activity. In addition, these compounds appeared to label fewer high affinity binding sites than CHA.

Partial agonists are characterised by an inability to achieve the same maximum response as a full agonist by virtue of their lower efficacy. The ability of these compounds to stimulate \[^{35}\text{S}]\text{GTPyS}\) binding in LE and HE membranes was therefore investigated in order to evaluate the role of agonist efficacy in the generation of the inhibitory \[^{35}\text{S}]\text{GTPyS}\) response.

GR190178, GR161144 and GR162900 produced a monophasic concentration dependent increase in \[^{35}\text{S}]\text{GTPyS}\) binding in LE adenosine A\(_1\) membranes (Fig. 4.4). GR190178 produced a similar maximum response to CHA whilst GR161144 and GR162900 were partial agonists, with a rank order of intrinsic activities of CHA\(\geq\) GR190178 \(>\) GR161144 \(>\) GR162900. This result confirmed previous data which demonstrated that this series of compounds possessed a spectrum of intrinsic activities (Sheehan et al., in press).
Figure 4.4. The effect of agonists of different efficacy on the binding of $[^{35}\text{S}]\text{GTPyS}$ to adenosine A$_1$ LE and HE membranes.

CHO adenosine A$_1$ LE (a) and HE (b) membranes (20µg) were pre-treated with adenosine deaminase (3U.ml$^{-1}$) and then incubated with 10µM GDP, 0.1nM $[^{35}\text{S}]\text{GTPyS}$ and increasing concentrations of agonist for 30 min. at 30°C. Data are the mean ± range/2 of duplicate points and are representative of 5 experiments. The lines represent the non-linear regression best fits of the data to either a one component (LE) or two component (HE) logistic equation as described in Chapter 2. See Table 4.2.1 for mean agonist pEC$_{50}$ and intrinsic activity values.
In HE membranes, all three agonists produced similar maximum responses to CHA, although the data suggests that GR 162900 may still be a partial agonist. The other lower efficacy agonists demonstrated an ability to decrease $[^{35}S]GTP\gamma S$ binding in a similar manner to CHA, but the magnitude of the inhibitory response was dependent on the efficacy of the ligand (Fig. 4.4, Table 4.1). GR190178 produced an inhibitory component of $[^{35}S]GTP\gamma S$ binding which was 75% of the magnitude of the inhibitory response to CHA, whilst GR161144 produced an inhibitory response (in 2 out of 5 experiments) of 28% of the CHA response. The responses to the lowest efficacy agonist, GR162900, were monophasic in HE membranes. These results demonstrate that the magnitude of the inhibitory $[^{35}S]GTP\gamma S$ binding response in HE membranes is dependent on the efficacy of the agonist.

4.2.5 Attempts to detect agonist mediated G-protein translocation from CHO cell membranes.

Following agonist activation, the topological fate of activated $G\alpha$ subunits is unclear. The release of $G\alpha$ subunits from CHO cell membranes could provide a mechanism for the inhibitory $[^{35}S]GTP\gamma S$ response. Ransnas and Insel (1988b) have shown that G-protein activation leads to a release of $G\alpha_q$ from S49 cells membranes whilst Arthur and co-workers (1999) have demonstrated $G\alpha_q$ release from MDCK cell membranes following stimulation with bradykinin, a process which was accompanied by depalmitoylation of the $G\alpha$ subunit. In contrast, Huang et al. (1999) have shown that activated G-proteins concentrate in
subdomains of the plasma membrane and are not released even after depalmitoylation.

CHO cell membranes were incubated with 10μM GDP, 0.1nM [³⁵S]GTPγS and vehicle, 10nM or 10μM CHA for 30 min. at 30°C. The membranes were cooled to 4°C to minimise [³⁵S]GTPγS dissociation and centrifuged. 0.2ml of the supernatant was applied to a Sephadex gel filtration column and eluted with repeated 0.2ml aliquots of buffer, as described in the methods section. In control experiments, [³⁵S]GTPγS which had not been exposed to CHO membranes appeared in the eluate in fraction 5-16 with a peak at fractions 10-11 (Fig. 4.5g) and was completely recovered from the column. In the supernatant from membranes treated with [³⁵S]GTPγS a ‘shoulder’ of [³⁵S]GTPγS became evident in fraction 4-5 (Fig. 4.5 a - f). However, this ‘shoulder’ was observed in both LE and HE and was independent of agonist treatment and therefore does not appear to be related to the inhibitory [³⁵S]GTPγS binding response observed in CHO A₁ HE membranes. The nature of this ‘shoulder’ is unknown but it is not related to the non-specific [³⁵S]GTPγS labelling of adenosine deaminase as no shoulder was observed when [³⁵S]GTPγS and ADA were incubated in the absence of membranes. Further experiments are required to understand the nature of this response. Using non-transfected CHO cells would reveal if the shoulder is related to the A₁ receptor whilst incubating recombinant or purified G-protein Gα subunits with [³⁵S]GTPγS would establish whether [³⁵S]GTPγS labelled Gα subunits elute at the same position.
Figure 4.5. Gel filtration of supernatant from $[^{35}\text{S}]\text{GTP}_{\gamma}\text{S}$ labelled adenosine A$_1$ LE and HE membranes.

Following a 30min. incubation with adenosine deaminase (3U.ml$^{-1}$) CHO A$_1$ low expressing (A, B & C) or high expressing (D, E & F) membranes were incubated with 10µM GDP, 0.1 nM $[^{35}\text{S}]\text{GTP}_{\gamma}\text{S}$ and either vehicle, 10nM or 10µM CHA for 30 min. at 30°C. Membranes were then centrifuged and 0.2ml of the supernatant eluted down a 4ml Sephadex G50M column at 4°C as described in Chapter 2. Panel G shows the elution profile for $[^{35}\text{S}]\text{GTP}_{\gamma}\text{S}$ alone. Panel H shows the basal and agonist stimulated $[^{35}\text{S}]\text{GTP}_{\gamma}\text{S}$ binding to CHO A$_1$ LE and HE membranes determined by filtration. Data are the mean ± range/2 of duplicate points, with the exception of panels B and G which are single points, and are representative of two experiments.
The involvement of G-protein translocation in the inhibitory $[^{35}S]GTP\gamma S$ binding response was further investigated by examining the effect of adding the adenosine A$_1$ receptor antagonist DPCPX after $[^{35}S]GTP\gamma S$ accumulation had already been stimulated by agonist. CHO A$_1$ LE and HE membranes were first incubated for 30 min. at 30°C with 10µM GDP, 0.1nM $[^{35}S]GTP\gamma S$ and concentrations of CHA which produced the maximum stimulatory (10nM) and inhibitory (10µM) $[^{35}S]GTP\gamma S$ binding responses. DPCPX was then added at a concentration of 1µM, which was approximately 300 fold above its dissociation constant, and membranes were incubated for a further 30 min.

In control experiments LE or HE membranes were incubated with GDP, $[^{35}S]GTP\gamma S$, and CHA, in the absence or presence of DPCPX, for 60 min. DPCPX abolished the response of 10nM CHA in both LE and HE membranes (Fig. 4.6 a & c). The response to 10µM CHA in LE membranes was unaffected by DPCPX whilst in HE membranes the response to 10µM CHA was increased to a similar level to that of 10nM CHA (Figure 4.6 a & c). These results are entirely consistent with a simple, competitive action of DPCPX, with a 300-fold shift in the concentration effect curve to CHA as expected from the Schild studies described in section 4.2.2.

When DPCPX was added to membranes following a 30 min. incubation with agonist, GDP and $[^{35}S]GTP\gamma S$, the responses to 10nM and 10µM CHA in LE membranes, and 10nM CHA in HE membranes, were essentially unchanged (Figure 4.6 b & d). DPCPX therefore had no effect on agonist stimulated $[^{35}S]GTP\gamma S$ which had already accumulated in the membrane. In contrast,
Figure 4.6. The effect of DPCPX on the binding of $[^{35}S]GTP\gamma S$ to adenosine $A_1$ LE and HE membranes following a 30 min. pre-incubation with agonist.

CHO adenosine $A_1$ LE (A & B) or HE (C & D) membranes were pre-treated with adenosine deaminase (3U.ml$^{-1}$) for 30 min.

Panels A & C: Membranes were incubated with the stated concentrations of CHA, GDP (10µM), $[^{35}S]GTP\gamma S$ (0.1nM) in the presence or absence of DPCPX (1µM) for 60 min. and then filtered as described in Chapter 2.

Panels B & D: Membranes were incubated with the stated concentrations of CHA, GDP (10µM) and $[^{35}S]GTP\gamma S$ (0.1nM) for 30 min. after which time DPCPX (1µM) or vehicle was added as stated and the incubation continued for a further 30 min. before filtering, as described in Chapter 2. Data are the mean ± range/2 of duplicate points and are representative of three experiments.
DPCPX added after the pre-incubation period increased the response of 10μM CHA to a similar level observed with 10nM CHA in the absence of antagonist. DPCPX was therefore able to 'recover' the \[^{35}\text{S}]\text{GTPyS} binding which was apparently inhibited by 10μM CHA in control experiments.

These data demonstrate that in HE membranes the inhibitory \[^{35}\text{S}]\text{GTPyS} binding response is fully reversible. The adenosine A\(_1\) receptor remains capable of maximally stimulating \[^{35}\text{S}]\text{GTPyS} binding despite a pre-exposure to an inhibitory concentration of CHA. This result is also inconsistent with the hypothesis of agonist mediated G-protein translocation from CHO cell membranes, as it would be expected that the concentration of G-protein \(\alpha\) subunits, once detached from the membrane, would be infinitely dilute and therefore unavailable for activation by agonist occupied receptor. Both the gel filtration and the DPCPX recovery experiments therefore suggest that the inhibitory \[^{35}\text{S}]\text{GTPyS} binding response in CHO A\(_1\) HE membranes is not a result of G-protein translocation from the membranes. It should be emphasised that in HE and LE membranes, once G\(\alpha\)-\[^{35}\text{S}]\text{GTPyS} has been formed as a result of agonist stimulation, this complex cannot be disrupted by adding antagonist. That is, the \[^{35}\text{S}]\text{GTPyS} remains essentially irreversibly bound to the G-protein \(\alpha\) subunit.

4.2.6 Kinetics of \[^{35}\text{S}]\text{GTPyS} binding to CHO adenosine A\(_1\) LE and HE membranes.

The nature of the inhibitory \[^{35}\text{S}]\text{GTPyS} binding response was further investigated by examining the kinetics of \[^{35}\text{S}]\text{GTPyS} association to CHO A\(_1\) LE and HE
membranes following incubation with increasing concentrations of agonist. The
dissociation of GDP from the G-protein heterotrimer is thought to be the rate
limiting step for G-protein activation. Thus, agonists catalyse GDP-GTP
exchange by dramatically increasing the rate of GDP dissociation (Gilman, 1987).
This simple catalytic mechanism predicts that agonists will increase the observed
rate of $[^{35}\text{S}]\text{GTP}$$\gamma$$\text{S}$ association but have no effect on the maximum amount of
$[^{35}\text{S}]\text{GTP}$$\gamma$$\text{S}$ binding. Thus basal and agonist stimulated $[^{35}\text{S}]\text{GTP}$$\gamma$$\text{S}$ binding
would expect to converge at a certain time after G-protein activation.

The objective of these studies was to address two main questions. Firstly, is the
inhibitory response of high concentrations of CHA associated with a decrease in
the initial rate of $[^{35}\text{S}]\text{GTP}$$\gamma$$\text{S}$ binding? This should provide information regarding
the mechanism of the inhibitory response. Secondly and more generally, are the
kinetics of $[^{35}\text{S}]\text{GTP}$$\gamma$$\text{S}$ binding under a variety of conditions (different expression
levels, different concentrations of $[^{35}\text{S}]\text{GTP}$$\gamma$$\text{S}$, increasing concentrations of
agonist) consistent with the general scheme for G-protein activation described in
the G-protein activation cycle (Gilman, 1987).

In order to address these issues, the kinetics of $[^{35}\text{S}]\text{GTP}$$\gamma$$\text{S}$ association to CHO A$_1$
LE and HE membranes were measured by first pre-incubating membranes with
10µM GDP and either vehicle (basal), 1µM DPCPX, or increasing concentrations
of CHA (0.1nM - 10µM) for 30 min. at 30°C. $[^{35}\text{S}]\text{GTP}$$\gamma$$\text{S}$ (0.1 or 1nM) was then
added and membranes incubated for increasing periods from 1 – 60 min. Two
concentrations of $[^{35}\text{S}]\text{GTP}$$\gamma$$\text{S}$ were used because at low concentrations, the
observed association of a radioligand may be independent of its $k_{on}$ and actually
reflect its $k_{\text{off}}$, as indicated by the equation $k_{\text{obs}} = k_{\text{on}}[L] + k_{\text{off}}$. Preliminary radioligand binding studies had indicated an IC$_{50}$ of approximately 0.3nM for inhibition by GTP$\gamma$S of $[^3\text{H}]$CHA binding to HE and LE membranes (data not shown).

Association data were analysed by obtaining the best fit from either a straight line or monophasic exponential association curve. In general, at 0.1nM $[^3\text{S}]$GTP$\gamma$S and at low concentrations of agonist the association of $[^3\text{S}]$GTP$\gamma$S with CHO A$_1$ membranes was best fit to a straight line whilst at 1nM $[^3\text{S}]$GTP$\gamma$S and at high concentrations of CHA, $[^3\text{S}]$GTP$\gamma$S association was best fit to an exponential association curve. The gradient of the straight line fit provided an estimate of the initial rate of $[^3\text{S}]$GTP$\gamma$S association whilst the initial rate of $[^3\text{S}]$GTP$\gamma$S association from exponential curves was calculated from the product of observed rate constant ($k_{\text{obs}}$) and the maximum level of binding. Three data sets were generated for analysis in this manner: the raw data alone (Fig. 4.7); the raw data following subtraction of the respective basal $[^3\text{S}]$GTP$\gamma$S binding (Fig. 4.8); the raw data following subtraction of the respective LE $[^3\text{S}]$GTP$\gamma$S bound in the presence of DPCPX (Fig. 4.9).

In LE membranes at 0.1nM $[^3\text{S}]$GTP$\gamma$S, the basal initial rate of $[^3\text{S}]$GTP$\gamma$S binding was 1.8$\pm$0.5 fmol.mg$^{-1}$min$^{-1}$ whilst in HE membranes the initial rate was 2.5 fold higher at 4.3$\pm$1.3 fmol.mg$^{-1}$min$^{-1}$ (Fig. 4.7, Table 4.2). At 1nM $[^3\text{S}]$GTP$\gamma$S the basal initial rate was 10 fold higher than at 0.1nM $[^3\text{S}]$GTP$\gamma$S in both LE and HE membranes, which indicates that at the concentrations of $[^3\text{S}]$GTP$\gamma$S used, the initial rates reflect $[^3\text{S}]$GTP$\gamma$S association and not the
Figure 4.7. Association kinetics for the binding of $[^{35}\text{S}]\text{GTP}_{\gamma}\text{S}$ to adenosine A$_1$ LE and HE membranes.

Following pre-treatment with 3U.ml$^{-1}$ ADA, CHO A$_1$ low (a & b) or high (c & d) expressing membranes were incubated with 10$\mu$M GDP, and either vehicle (basal), DPCPX or CHA at the concentrations shown. $[^{35}\text{S}]\text{GTP}_{\gamma}\text{S}$ was then added (0.1nM panels a & b; 1nM panels c & d) and incubated for increasing times. Data are single points and are representative of three experiments. The lines represent the computer generated best fits to an equation for either a straight line or monophasic exponential association as described in Chapter 2.
Figure 4.8. Association kinetics for the stimulated binding of \[^{35}\text{S}]\text{GTPyS}\) to CHO adenosine A\textsubscript{1} LE and HE membranes - basal activation subtracted.

Following pre-treatment with 3U.ml\(^{-1}\) ADA, CHO A\textsubscript{1} low (a & b) or high (c & d) expressing membranes were incubated with 10\(\mu\text{M}\) GDP, and either vehicle (basal), DPCPX or CHA at the concentrations shown. \[^{35}\text{S}]\text{GTPyS}\) was then added (0.1nM panels a & b; 1nM panels c & d) and incubated for increasing times.

The stimulated \[^{35}\text{S}]\text{GTPyS}\) binding was calculated by subtracting the binding in the presence of DPCPX for each cell line and for each concentration of \[^{35}\text{S}]\text{GTPyS}, from the total binding data.

Data are single points and are representative of three experiments. The lines represent the computer generated best fits to an equation for either a straight line or monophasic exponential association as described in Chapter 2.
Figure 4.9. Association kinetics for the binding of \[^{35}\text{S}]\text{GTP}\gamma\text{S}\) to CHO adenosine A\(_1\) LE and HE membranes - low expression basal subtracted.

Following pre-treatment with 3U.ml\(^{-1}\) ADA, CHO A\(_1\) low (a & b) or high (c & d) expressing membranes were incubated with 10\(\mu\)M GDP, and either vehicle (basal), DPCPX or CHA at the concentrations shown. \[^{35}\text{S}]\text{GTP}\gamma\text{S}\) was then added (0.1nM panels a & b; 1nM panels c & d) and incubated for increasing times.

The stimulated \[^{35}\text{S}]\text{GTP}\gamma\text{S}\) binding was calculated by subtracting the binding in the presence of DPCPX in LE membranes, at the appropriate concentration of \[^{35}\text{S}]\text{GTP}\gamma\text{S}\), from the total \[^{35}\text{S}]\text{GTP}\gamma\text{S}\) binding at that time.

Data are single points and are representative of three experiments. The lines represent the computer generated best fits to an equation for either a straight line or monophasic exponential association as described in Chapter 2.
[\textsuperscript{35}S]GTP\gamma S dissociation rate constant, despite the use of low concentrations of [\textsuperscript{35}S]GTP\gamma S and high concentrations of GDP in the assay.

In the presence of 1\mu M DPCPX there was a small inhibition which was approximately 10% of the initial rate of [\textsuperscript{35}S]GTP\gamma S association in control LE membranes, at both 0.1 and 1nM [\textsuperscript{35}S]GTP\gamma S (Fig. 4.7 a & b). In contrast, in HE membranes, DPCPX (1\mu M) decreased the initial rate of [\textsuperscript{35}S]GTP\gamma S by 40-50% at both 0.1 and 1nM [\textsuperscript{35}S]GTP\gamma S (Fig. 4.7 c & d) which is consistent with the inverse agonism of DPCPX reported in section 4.2.2. However, in HE membranes the initial rate of [\textsuperscript{35}S]GTP\gamma S association in the presence of DPCPX remained 40-50% higher than the initial rate in the presence of DPCPX in LE membranes. If the background of G–protein activity in both LE and HE membranes is assumed to be similar, DPCPX would appear to act as a partial inverse agonist of constitutive \textsubscript{AI} receptor activity.

At maximal concentrations of CHA in LE membranes at 0.1 and 1nM [\textsuperscript{35}S]GTP\gamma S, the total amount of [\textsuperscript{35}S]GTP\gamma S bound increased throughout the time course of the experiment (Fig. 4.7 a & b). However, the amount of 1nM [\textsuperscript{35}S]GTP\gamma S stimulated above DPCPX levels by agonist appeared to be constant at 30-60 min. (Fig. 4.7 b) which suggested that stimulated [\textsuperscript{35}S]GTP\gamma S may approach an equilibrium. Subtraction of the [\textsuperscript{35}S]GTP\gamma S binding observed in the presence of DPCPX from the total amount of [\textsuperscript{35}S]GTP\gamma S observed in the presence of increasing concentrations of CHA provided an estimate of the total amount of [\textsuperscript{35}S]GTP\gamma S stimulated by agonist (and receptor; Fig. 4.8).
In HE membranes using 0.1nM $[^{35}\text{S}]\text{GTP}\gamma\text{S}$, CHA (0.1nM-10μM) also increased $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding as a function of time, reaching a constant level of stimulation above the DPCPX level (Fig. 4.8 c; 4.9 c). However, using 1nM $[^{35}\text{S}]\text{GTP}\gamma\text{S}$, the inhibitory concentrations of CHA (1-10μM) gave a different pattern. Total $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding reached a maximum after 10-15 minutes and then decreased below ‘basal’ levels and towards DPCPX levels at 60 minutes (Fig. 4.8 d). The decrease in stimulated $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding at long time periods may reflect the partial inverse agonist nature of DPCPX and its consequent failure to define ‘true’ basal in HE membranes.

When the binding of $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ in the presence of DPCPX in LE membranes was subtracted from the total levels of $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding in HE membranes (at the relevant concentration of $[^{35}\text{S}]\text{GTP}\gamma\text{S}$), stimulated $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding reached constant levels at long time periods at all concentrations of CHA and at both expression levels (Fig. 4.9 b & d). Binding in the presence of DPCPX in LE membranes therefore appeared to be the best estimate of ‘true’ basal $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding which is independent of the concentration and constitutive activity of the adenosine A1 receptor.

Stimulated $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding above true basal was fitted to either a straight line or monophasic exponential curve to obtain an estimate of the initial, stimulated rate of $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ association. CHA increased the initial rate of stimulated $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding in a concentration dependent manner (Fig. 4.10, Table 4.2) in both LE and HE membranes and at both concentrations of $[^{35}\text{S}]\text{GTP}\gamma\text{S}$. The
Figure 4.10. The effect of CHA on the stimulated initial rate, maximal binding and kobs of $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding to CHO adenosine $A_1$ low and high expression membranes.

The stimulated $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ association data, as represented in Fig. 4.9, were analysed by non-linear regression analysis, comparing the best fits of the data to equations for either a straight line or monophasic exponential association for estimation of the stimulated initial rate (panels a & b), maximum binding (panels c & d) and kobs (panels e & f) as described in Chapter 2 and Section 4.2.5.

Panels a, c and e are data from association experiments using 0.1nM $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ whilst panels b, d and f are data from experiments using 1nM $[^{35}\text{S}]\text{GTP}\gamma\text{S}$.

The data are the mean±s.e.mean of three separate experiments. The lines of best fit were determined from non-linear regression analysis with a logistic equation fit to the data from all three experiments.

For the stimulation of the initial rate of $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ association in LE membranes, the pEC$_{50}$ estimates for CHA were 8.34±0.08 and 8.19± 0.05 at 0.1nM and 1nM $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ respectively. In HE membranes the pEC$_{50}$ estimates were 9.41±0.35 and 9.03±0.18.

For the inhibition of stimulated maximum $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding in HE membranes the pIC$_{50}$ values for CHA were 7.17±0.13 7.42±0.19 at 0.1nM and 1nM $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ respectively. The negative log potency values for CHA are the mean±s.e.mean from fits to each of the three separate experiments. Where points are missing from the stimulated maximum binding and kobs graphs, these data were best fit to a straight line.
Table 4.2 Kinetic parameters for the agonist stimulated association of [$^{35}$S]GTPγS binding to CHOA₁ LE and HE membranes.

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>[CHOA] concentration</th>
<th>Initial rate</th>
<th>$E_{\text{max}}$</th>
<th>$k_{\text{obs}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>[fmol mg⁻¹ min⁻¹]</td>
<td>[fmol mg⁻¹]</td>
<td>[min⁻¹]</td>
<td></td>
</tr>
<tr>
<td>Low expression</td>
<td>0.1nM 0</td>
<td>0.15±0.03</td>
<td>a</td>
<td>a</td>
</tr>
<tr>
<td></td>
<td>10nM 2.0±0.3</td>
<td>230b</td>
<td>0.011b</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10μM 3.0±0.5</td>
<td>210b</td>
<td>0.019b</td>
<td></td>
</tr>
<tr>
<td>Low expression</td>
<td>1nM 2.0±0.6</td>
<td>a</td>
<td>a</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10nM 29±6</td>
<td>610±110</td>
<td>0.052±0.13</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10μM 48±11</td>
<td>690±110</td>
<td>0.073±0.016</td>
<td></td>
</tr>
<tr>
<td>High expression</td>
<td>0.1nM 2.8±0.6</td>
<td>170b</td>
<td>0.024b</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10nM 14.0±1.7</td>
<td>260±70</td>
<td>0.056±0.006</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10μM 12.7±1.2</td>
<td>120±10</td>
<td>0.108±0.015</td>
<td></td>
</tr>
<tr>
<td>High expression</td>
<td>1nM 34±9</td>
<td>1260±200c</td>
<td>0.032±0.004c</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10nM 190±10</td>
<td>1030±20</td>
<td>0.21±0.05</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10μM 210±10</td>
<td>480±110</td>
<td>0.48±0.09</td>
<td></td>
</tr>
</tbody>
</table>

Following a 30 min. pre-incubation with 3U.ml⁻¹ ADA, membranes were incubated with either DPCPX (1μM) or the stated concentration of CHA, and 10μM GDP for 30 min. at 30°C. [$^{35}$S]GTPγS (0.1 or 1nM) was then added and the membranes incubated for increasing time (1-60 min.). Agonist stimulated [$^{35}$S]GTPγS binding was determined by subtracting the binding of [$^{35}$S]GTPγS in LE membranes in the presence of DPCPX (1μM) at the relevant concentration of [$^{35}$S]GTPγS. The data were then analysed by obtaining the best fit to equations for a straight line and monophasic exponential association. Data are the mean ± s.e.mean from three separate experiments. Data were best fit to a straight line in (a) all three experiments; (b) one out of three experiments; (c) two out of three experiments.
potency values of CHA for increasing the initial rate of $[^{35}S]GTP_\gamma S$ association were similar for the two concentrations of $[^{35}S]GTP_\gamma S$ and CHA was approximately 10-fold more potent in HE membranes. In LE membranes, the pEC$_{50}$ values for CHA were 8.34±0.08 and 8.19±0.05, and in HE membranes were 9.41±0.35 and 9.03±0.18 at 0.1 and 1nM $[^{35}S]GTP_\gamma S$ respectively (n=3). The maximum stimulated rate was approximately 10-fold higher at the higher concentration of $[^{35}S]GTP_\gamma S$ (Fig. 4.10 a & b). High concentrations of CHA did not appear to have an inhibitory effect on the initial rate of $[^{35}S]GTP_\gamma S$ association in either LE or HE membranes (Fig. 4.10 a & b). Surprisingly, the maximum stimulated initial rate in HE membranes was only 3-4 fold that observed in LE membranes, which is not consistent with the 20-fold difference in receptor expression levels.

For $[^{35}S]GTP_\gamma S$ association data which were best fit to a monophasic exponential association curve, the asymptotic, or maximum, level of $[^{35}S]GTP_\gamma S$ binding could be determined. In LE membranes increasing concentrations of CHA produced similar levels of stimulated maximum $[^{35}S]GTP_\gamma S$ binding (Fig. 4.10 c & d). In contrast, in HE membranes CHA produced a concentration dependent decrease in the maximum level of $[^{35}S]GTP_\gamma S$ binding, with similar pIC$_{50}$ values of 7.17±0.13 and 7.42±0.19 at 0.1 and 1nM $[^{35}S]GTP_\gamma S$ respectively (Fig. 4.10 c & d).

In LE membranes at 1nM $[^{35}S]GTP_\gamma S$ the maximum level of $[^{35}S]GTP_\gamma S$ binding of approximately 0.7 pmol.mg$^{-1}$ (Fig.4.10) was similar to the receptor expression
level in this cell line. In contrast, in HE membranes, the maximum amount of stimulated $[^{35}\text{S}]\text{GTP} \gamma \text{S}$ binding, of approximately 1.3 pmol.mg$^{-1}$ was 10-fold lower than the receptor density in this cell line. These data are again consistent with the adenosine $A_1$ receptor being able to interact with more G-proteins in HE membranes, but the amount of interacting G-protein is less than would be predicted from the differences in receptor expression levels. In addition, at 1nM $[^{35}\text{S}]\text{GTP} \gamma \text{S}$ the maximum levels of stimulated $[^{35}\text{S}]\text{GTP} \gamma \text{S}$ binding were approximately four fold higher than at 0.1nM $[^{35}\text{S}]\text{GTP} \gamma \text{S}$. This suggests that these concentrations of $[^{35}\text{S}]\text{GTP} \gamma \text{S}$ are in the region of the apparent $K_d$ for the radioligand.

The observed rate constant ($k_{obs}$) was determined using the equation $k_{obs} = \frac{\text{initial rate}}{E_{max}}$ and is related to the half time ($t_{1/2}$) for the exponential association of $[^{35}\text{S}]\text{GTP} \gamma \text{S}$ binding. In LE membranes the effect of low concentrations of CHA (0.01-1nM) on $k_{obs}$ could not be determined because the increase in $[^{35}\text{S}]\text{GTP} \gamma \text{S}$ binding was linear, whilst high concentrations of CHA (10nM-10μM) had little effect on $k_{obs}$ as the initial rate and $E_{max}$ of $[^{35}\text{S}]\text{GTP} \gamma \text{S}$ binding appeared to be maximal over this agonist concentration range. In HE membranes CHA increased $k_{obs}$ for $[^{35}\text{S}]\text{GTP} \gamma \text{S}$ association in a concentration-dependent manner. However, the responses to CHA were observed over 4-5 orders of magnitude of CHA concentration, which is consistent with the presence of two separate kinetic processes, and which may represent the stimulatory and inhibitory components of CHA mediated $[^{35}\text{S}]\text{GTP} \gamma \text{S}$ binding.
In summary, CHA produced a monophasic, concentration dependent increase in the initial rate of \[^{35}\text{S}]\text{GTPyS}\) binding to CHO A1 LE and HE membranes. In addition, the stimulated level of \[^{35}\text{S}]\text{GTPyS}\) binding appeared to be constant from between 30-60 min, which may not be consistent with a simple catalytic mechanism for agonist stimulated G-protein activation. The inhibitory effect of CHA was not evident in measurements of the initial rate of \[^{35}\text{S}]\text{GTPyS}\) association but appeared to reflect a decrease in the amount of activated, \[^{35}\text{S}]\text{GTPyS}\) bound, G-protein \(\alpha\) subunits.

4.3 Simulation of the effects of receptor density on functional responses using the Ternary Complex Model.

The effects of changing \(R_T\) on the potency and maximum response of an agonist were simulated using the ternary complex model as described in Appendix 1. The theoretical functional response was taken as the sum of the constitutive (RG) and the agonist activated (ARG) receptor-G-protein complexes. To maintain consistency with previous simulations, the agonist affinity was given a fixed value of \(1 \times 10^6 \text{M}^{-1}\), and the cooperativity constant \(\alpha\) was given a value of 1000. \(G_T\) was fixed at 1 and \(R_T\) was varied over the range 0.1 - 100.

When \(R_T\) was varied over the range such that \(R_T:G_T < 1\), the maximum response to the agonist was increased, with no apparent change in agonist potency (Fig. 4.11 a). As \(R_T\) exceeded \(G_T\), the maximum response became limited to the value of \(G_T\), and increases in \(R_T\) resulted in an increase in agonist potency with no change in maximum response (Fig. 4.11 b). At higher values of \(R_T\), the basal response also
Figure. 4.11. Simulation of functional responses using the Ternary Complex Model: Changing the $R_T:G_T$ stoichiometry.

The effect of $R_T:G_T$ stoichiometry were simulated by keeping $G_T$ fixed at a value of 1, and varying $R_T$ from 0.1-3.2 (panel a) and 10 - 320 (panel b). Other parameter values were as follows: agonist affinity $1 \times 10^6 \text{M}^{-1}$, $\alpha=1000$; $K_G=0.001$.

The simulation in panel c demonstrates that the TCM is able to qualitatively describe the data obtained from $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding studies in LE and HE membranes when $R_T$ is increased by 20-fold only if there is a concomitant two-fold increase in $G_T$.

The lines represent the computer-generated best fits of the simulated data to a four parameter logistic equation as described in Chapter 2. In all cases the Hill slopes were not significantly different from unity.
increased, which was a result of the generation of a significant amount of RG complexes in the absence of agonist.

In order to simulate the relative potencies and maximum responses of CHA in LE and HE membranes using the ternary complex model of agonism, it is therefore necessary to increase both the total number of receptors and the total number of G-proteins (Fig. 4.11 c). It should be noted however, that the measured receptor densities and $R_T:G_T$ ratios described in Sections 3.2.1 and 3.2.2 are not able to describe the functional responses in LE and HE membranes when they are inserted into the TCM. The model therefore provides only a qualitative description of the experimental data. It is also noteworthy that this simple model of agonist, receptor and G-protein interactions is not sufficient to predict biphasic agonist concentration-effect curves.

4.4 Discussion

The purpose of this series of experiments was to compare and contrast the pharmacological and functional properties of the adenosine $A_1$ receptor expressed in CHO cell membranes with a 20-fold difference in expression levels. During the initial characterisation of the functional responses in HE membranes, an inhibitory response to agonists was observed. This response was pharmacologically characterised and experiments were performed to investigate the mechanisms responsible for the inhibitory response.
4.4.1 The comparison of basal and agonist stimulated $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding responses in CHO A1 LE and HE membranes.

In HE membranes both the potency of CHA and the maximum amount of CHA stimulated $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding were greater than that observed in LE membranes. Whilst an increase in the maximum response to an agonist would be intuitively expected when the receptor density is increased, the magnitude of the increase in the maximum amount of $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding in HE membranes was only 2.5 fold higher than that observed in LE membranes, which was in contrast to the 20-fold difference in receptor density determined from radiolabelled antagonist binding (Chapter 3). These data therefore suggest that in HE membranes more G-proteins are available for activation by the A1 receptor, but these additional G-proteins are not sufficient to maintain a constant $R_T:G_T$ ratio in the two cell lines. This provides further qualitative support for the differences in the ratio $R_T:G_T$ observed in radioligand binding studies. In addition, CHA was 10-fold more potent in stimulating $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ in HE membranes which is predicted by the TCM to occur when the concentration of activatable G-proteins is limiting (Fig. 4.11).

The effect of receptor density on agonist responses have been traditionally studied using isolated, intact tissues, either by using an irreversible antagonist to decrease the receptor number (Furchgott, 1966) or by determining agonist responses in tissues with different receptor densities (Kenakin & Beek, 1980). In general, at high levels of receptor density, alterations in $R_T$ result only in changes in agonist potency and not maximum response, whilst at low levels of receptor density, changes in $R_T$ result in changes to the maximum response of an agonist, with little
change in agonist potency. These effects are entirely consistent with the predictions of simple models of drug-receptor interaction when a component of the signal transduction cascade becomes limiting (Fig. 4.11). The relationship between receptor density, agonist potency and maximum response has also been demonstrated in cell lines where the expression of recombinant receptors has been varied, either by selecting clones of different receptor expression levels (Horie & Tsujimoto, 1995; McDonnell et al., 1998; Whaley et al., 1993), the use of the gene expression enhancer sodium butyrate (Gazi et al., 1999) or by the use of an inducible promoter (Esbenshade et al., 1995; Hermans et al., 1999).

Agonist stimulated $[^{35}S]$GTP$\gamma$S binding responses in membranes from the cell lines used in this thesis have been previously described (Cordeaux et al., 2000), and demonstrated to have similar agonist potencies, but different maximum response in LE and HE membranes. In contrast to this thesis, Cordeaux et al demonstrated that a 20-fold increase in expression of the $A_1$ receptor (0.2 to 3.4pmol.mg$^{-1}$) led to a 20-fold increase (0.01 to 0.2 pmol.mg$^{-1}$) in the maximum level of $[^{35}S]$GTP$\gamma$S binding, and that therefore the ratio of receptor density to activatable G-protein was constant. The biggest difference between the data presented in the two studies is in the receptor densities determined in radioligand binding studies, which were approximately 2-5 fold higher in this thesis. Whilst the differences in expression levels may be due to differences in the methods of tissue culture or membrane preparation, the lower receptor densities in both LE and HE membranes determined by Cordeaux can adequately explain the differences in functional responses reported in the two studies. Hence, the lower receptor densities reported by Cordeaux would be less likely to saturate the total
pool of available G-proteins, and it would be expected that agonist maximum response, and not potency, would be different between the two cell lines. The absolute concentration of receptors and the ratio of $R_T:G_T$ are therefore important in determining both the potency and maximum response of an agonist.

A number of other studies have investigated the role of receptor/G-protein stoichiometry in agonist stimulation of $[^{35}\text{S}]\text{GTP}_\gamma\text{S}$ binding. Using the radiolabelled inverse agonist $[^3\text{H}]\text{SB224,289}$ Newman-Tancredi et al. (2000) demonstrated that the ratio of receptor density to activatable G-proteins was 2.8 and 0.3 in CHO cells expressing 8.5 and 0.4 pmol.mg$^{-1}$, respectively, of the serotonin h5-HT$_{1B}$ receptor. Interestingly, in this study no difference in agonist potency was observed for the stimulation of $[^{35}\text{S}]\text{GTP}_\gamma\text{S}$ binding in membranes from these cell line. In intact digitonin treated C6 rat glioma cells, a reduction of 70% of the receptor density of the stably transfected $\mu$-opioid receptor, using an irreversible alkylating agent, resulted in a similar reduction in the level of maximum, agonist stimulated $[^{35}\text{S}]\text{GTP}_\gamma\text{S}$ binding (Alt et al., 2001). However, the effect of the reduction in the receptor number on agonist potency was not reported in this study.

4.4.2 Pharmacological characterisation of the stimulatory $[^{35}\text{S}]\text{GTP}_\gamma\text{S}$ binding response in LE and HE membranes.

The affinity of the antagonist DPCPX for the adenosine A$_1$ receptor in LE and HE membranes was determined by Schild analysis. Similar $pA_2$ values for antagonism of the CHA stimulated $[^{35}\text{S}]\text{GTP}_\gamma\text{S}$ binding response by DPCPX of 8.53 and 8.52 in LE and HE membranes respectively were obtained. These values
were not significantly different from the binding affinities for DPCPX obtained in saturation binding studies in Chapter 3 (P>0.05, one-way analysis of variance), and confirmed that the affinity of DPCPX for the adenosine A₁ receptor was independent of receptor expression.

The basal level of [³⁵S]GTPγS binding was 2.2 fold higher in HE, compared with LE membranes indicating that constitutive G-protein activation by the A₁ receptor was detectable in HE membranes. These experiments were performed in the presence of adenosine deaminase at a concentration which maximally inhibited basal [³⁵S]GTPγS binding in the absence of any pharmacological ligands, which would indicate that endogenous adenosine had been completely removed by ADA. In the study of Cordeaux et al. (2000) basal [³⁵S]GTPγS binding was 10-fold higher in high, compared with low, expressing A₁ membranes.

Constitutive activity has been widely reported to be a function of receptor density for an extensive range of 7TM GPCRs (for a review see De Ligt et al., 2000). For wild type receptors, which would be expected to demonstrate low levels of constitutive activity, the ability to observe agonist independent activity arises from the increased amount of RG complexes generated at high levels of receptor expression. This is in contrast to constitutively active mutant GPCRs, which activate G-proteins in the absence of agonists as a result of the mutation-induced increase in affinity for the G-protein.

DPCPX appeared to act as an inverse agonist in both LE and HE cell lines, which suggests that the A₁ receptor is constitutively active in both cell lines. In LE membranes however, the inhibition was small (~10%) and as a result the
concentration dependence for DPCPX could not be quantitated. In contrast in HE membranes, DPCPX inhibited basal[^35]S]GTPγS binding by 36% in a concentration dependent manner, with a pIC$_{50}$ of 8.3, which was similar to the pA$_2$ estimates determined from Schild analysis. Interestingly, DPCPX was unable to inhibit basal[^35]S]GTPγS binding to the levels observed in LE membranes, suggesting that DPCPX is a partial inverse agonist. Similarly, Cordeaux et al (Cordeaux et al., 2000) demonstrated that DPCPX, and the adenosine receptor antagonist xanthine amine congener, inhibited basal[^35]S]GTPγS binding to a similar extent in HE but had no inhibitory response in LE membranes.

DPCPX and a number of other adenosine receptor antagonists have been shown to inhibit both basal[^35]S]GTPγS binding and cAMP levels in CHO cells expressing 4-8 pmol.mg$^{-1}$ of the human recombinant A$_1$ receptor (Shryock et al., 1998). In detergent permeabilised embryonic chick ventricular myocytes, DPCPX and the antagonist BW-A844U, increased adenylyl cyclase activity by 50% (the opposite effect to A$_1$ receptor agonists). All of these studies were performed in the presence of ADA, which suggests that the effect of antagonists is independent of endogenous adenosine and can be attributed to the inverse agonist properties of these ligands. However, Prater et al (1992) have demonstrated that in bovine brain membranes, endogenous adenosine can be found in small membrane vesicles which is resistant to ADA, even after solubilisation. Endogenous adenosine may therefore not completely removed by the presence of ADA and therefore cannot be ruled out in some experimental protocols as an explanation for the inverse agonist properties of adenosine receptor antagonists.
In this study, the inhibitory response to DPCPX was still observed at 10-fold higher concentrations of ADA and in the presence of the membrane permeabilising agent saponin, which suggests that the presence of endogenous adenosine at a concentration which is sufficient to activate the A1 receptor is unlikely. Furthermore, the increase in basal \([^{35}\text{S}]\text{GTP}\gamma\text{S}\) binding in HE membranes in the presence of PD is too small to be a result of the enhancement of endogenous adenosine (Cohen, 1995), but is consistent with an agonist action of PD.

4.4.3 Pharmacological characterisation of agonist mediated inhibition of \([^{35}\text{S}]\text{GTP}\gamma\text{S}\) binding in HE membranes.

In HE membranes increasing concentrations of agonist first stimulated and then inhibited the binding of \([^{35}\text{S}]\text{GTP}\gamma\text{S}\). Agonist mediated inhibition of G-protein activation is of interest because of its potential as a novel control mechanism for negative feedback of receptor activation. This study has characterised the detailed pharmacology of the inhibitory response using a standard 30 min. \([^{35}\text{S}]\text{GTP}\gamma\text{S}\) incubation period. The inhibitory response was not observed in LE membranes and is therefore related to the adenosine A1 receptor density. The inhibitory response was antagonised by the A1 receptor antagonist DPCPX with a pA2 value that confirmed the response was mediated by the A1 receptor. Furthermore, the presence of 3μM PD potentiated the inhibitory response to a similar extent as the stimulatory response which also supports an A1 receptor mediated inhibition of \([^{35}\text{S}]\text{GTP}\gamma\text{S}\) binding. It should be noted that the antagonist produces a paradoxical stimulation of \([^{35}\text{S}]\text{GTP}\gamma\text{S}\) binding at high agonist concentrations but conventional inhibition at low agonist concentrations (see Fig. 4.2 b). Equally, the allosteric
enhancer PD 81,723 produces a paradoxical inhibition of $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding stimulated by high concentrations of CHA (see Fig. 4.3 b). The inhibitory response was unrelated to the maximum level of agonist stimulated $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ but was related to the efficacy of the agonist used. The agonists GR190178, GR161144 and GR162900, which demonstrated partial agonism in LE membranes, achieved a similar level of stimulation of $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding as CHA in HE membranes, but differed in the magnitude of the inhibitory response. The agonist mediated inhibition of $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding is therefore mediated by the adenosine $A_1$ receptor and is related to the density of receptor expression and agonist efficacy.

An initial hypothesis for the inhibitory response was that the reduction of $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding was a result of the instability of activated G-protein $\alpha$ subunits and subsequent release of these subunits from the membrane fragments. Whilst G-protein translocation has been previously proposed (Arthur et al., 1999; Ransnas & Insel, 1988b), this research area remains controversial (Huang et al., 1999). Following activation with an inhibitory concentration of agonist, gel filtration was performed on the supernatant of HE membranes. Activity was detected in the excluded fraction, which was consistent with the mass of a G-protein $\alpha$ subunit. However, this activity was observed in both LE and HE membranes and was not dependent on agonist concentration. It seems unlikely, therefore, that this can account for the inhibitory $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding response.

When the antagonist DPCPX was added to membranes which had been previously exposed to both an inhibitory concentration of agonist and $[^{35}\text{S}]\text{GTP}\gamma\text{S}$, the
binding of $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ was restored to the maximal level of $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding. It would be expected that G-protein $\alpha$ subunits, once released from the membrane, would not be available for re-activation and this result therefore argues against G-protein translocation as a mechanism for agonist stimulated inhibition of $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding. The ability of DPCPX to restore the maximal $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding response also suggests that the inhibitory response is not a result of non-specific membrane instability, as has been used to explain some of the effects of $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding to muscarinic receptors (Waelbroeck, 2001) but is a property of the receptor system itself. Once $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ is added, the inhibitory response develops reversibly, and with time.

This study is the first detailed characterisation of an agonist mediated inhibitory $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ response mediated by the adenosine $A_1$ receptor. An inhibitory $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding response is present in data for the wild type human recombinant $A_1$ receptor alone and also fused to a pertussis toxin resistant $G\alpha_{41}$ subunit (Bevan et al., 1999) but has not been quantified or discussed. It has been previously observed for the muscarinic $M_1$ receptor (S. Lazareno, personal communication), adrenergic $\alpha_{2A}$ receptor (R. Leppik, personal communication) and the neuropeptide NPY receptor (L. Clark, personal communication) but not characterised.

Agonist induced, inhibition of $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding has also been recently demonstrated, using an antibody capture technique, for human recombinant 5-HT$_{1A}$ receptors overexpressed in CHO cells, which are mediated by $G\alpha_{43}$ subunits (Newman-Tancredi et al., 2002). This response shares some similar features to
the A₁ mediated response described in this thesis, including a dependence on agonist efficacy, and some of the temporal aspects of the development of the inhibitory response. Important differences, however, exist between the studies presented in this thesis and that of Newman-Tancredi et al. Firstly, in their filtration studies, 5HT concentration response curves were comprised of two stimulatory components which were mediated by Go₂ and Go₃. Only upon the addition of unlabelled GTPγS did the curves become bell-shaped, as the low potency 5-HT response was lost. Bell-shaped responses were demonstrated when the G-protein involved in binding [³⁵S]GTPγS was determined using the antibody capture method. However, this procedure involved the addition of detergent to solubilize the membranes. It is therefore difficult to make comparisons between the data presented in this thesis, and that of Newman-Tancredi et al.

Bell-shaped agonist dose response curves are frequently observed for G-protein coupled receptors and have been explained by the activation of a heterogeneous population of receptors and the subsequent generation of functionally antagonistic biochemical signalling pathways (Kenakin & Pike, 1987; Lejeune et al., 1997). As described above, however, both stimulatory and inhibitory [³⁵S]GTPγS binding responses are mediated by the adenosine A₁ receptor. Furthermore, the lack of an agonist response in membranes from wild type CHO cells, together with the monophasic agonist concentration-response curves in LE membranes suggests that a heterogeneous populations of receptors cannot explain the production of bell-shaped data in HE membranes.

Bell-shaped agonist concentration-response curves can also be generated by the generation of multiple biochemical signalling pathways via activation of a single
population of receptors, and can be due to the promiscuous coupling of a receptor with more than one-class of G-protein (for example see Pohjanoksa et al., 1997). The A₁ receptor can co-immunoprecipitate with [³⁵S]GTPγS bound Ga₄ subunits and stimulate adenylyl cyclase in pertussis toxin treated CHO cells (Cordeaux et al., 2000). However, pretreatment of cells with PTX completely abolished agonist stimulated [³⁵S]GTPγS binding in HE membranes. Furthermore, in contrast to measurements of cyclic AMP, the activation of multiple G-protein subunits would not be expected to generate opposing [³⁵S]GTPγS binding responses, even if the different subunits bind [³⁵S]GTPγS with different apparent association rates. In extensive simulations using a modified version of the ternary complex model to include two G-proteins (G₁ and G₂), and assuming that the theoretical response is proportional to the total amount of RG complexes (RG₁ + RG₂ + ARG₁ + ARG₂), a biphasic agonist response could not be described. It therefore seems unlikely that multiple G-protein interactions can explain agonist inhibition of [³⁵S]GTPγS binding.

The loss of an agonist response with time is a common feature of G-protein coupled receptors expressed in intact cells. It is generally considered that receptor desensitization, as observed for the β₂-adrenergic receptor, occurs rapidly after agonist exposure and is a result of G-protein receptor kinase mediated phosphorylation of the receptor, followed by the subsequent binding of β-arrestin proteins, the uncoupling of receptors from G-proteins, and receptor internalisation (Lefkowitz, 1998). However, the adenosine A₁ receptor appears to be resistant to acute desensitisation, which is ascribed to the lack of multiple serine and threonine residues in its carboxyl-terminus tail (Olah & Stiles, 1995). Hence, in CHO cells
expressing the human adenosine A₁ receptor, a 30 minute exposure with agonist has no effect on the potency or maximum response of a subsequent agonist challenge for the inhibition of adenylyl cyclase (Palmer et al., 1996). Similarly, in guinea pig DDT₁ MF-2 cells which natively express the A₁ receptor, the t₁/₂ for desensitisation of the adenylyl cyclase response is 6 – 8 hours (Ramkumar et al., 1991), although Ciruela et al. (1997) have demonstrated rapid (5-15 min.) agonist induced clustering of the A₁ receptor in this cell line. In contrast to the reported slow desensitisation of the A₁ receptor, the inhibitory [³⁵S]GTPyS binding response in HE membranes develops rapidly (2-5 mins; Fig. 4.9 d). Furthermore, desensitisation processes, such as receptor phosphorylation and internalisation are unlikely to occur in a washed membrane preparation, where it would be assumed that the cytoskeletal machinery, catalytic proteins and chemical energy sources required to support such processes would not be present.

Bell-shaped agonist concentration response curves have been observed for the single transmembrane spanning, tyrosine kinase linked growth hormone (Ilondo et al., 1994) and insulin (De Meyts et al., 1995) receptors. Receptors of this class have been demonstrated to form receptor dimers, which represent the functionally relevant complexes. A model of receptor dimerisation has been used to describe this data. Low concentrations of agonist promote the formation of functionally active monoliganded dimers, whilst at higher concentrations biliganded, functionally inactive agonist receptor dimer complexes predominate.

Homo- and hetero- receptor dimerisation are emerging concepts within the field of family A G-protein coupled receptor research and there is experimental evidence to suggest the heterodimerisation of the A₁ receptor with the dopamine D₁ receptor.
Furthermore, the agonist-induced radiolabelled agonist dissociation data for the A<sub>1</sub> receptor presented in Chapter 3 is difficult to explain without invoking receptor-receptor interactions. Whether the bell-shaped \[^{35}\text{S}]\text{GTP}\gamma\text{S}\ binding data presented in this chapter is a consequence of receptor dimerisation, and whether the inhibitory response represents the generation of functionally inactive monomers or dimers remains to be determined.

4.4.4. The kinetics of \[^{35}\text{S}]\text{GTP}\gamma\text{S}\ association to adenosine A<sub>1</sub> LE and HE membranes.

The inhibitory \[^{35}\text{S}]\text{GTP}\gamma\text{S}\ binding response was further investigated by examining the kinetics of \[^{35}\text{S}]\text{GTP}\gamma\text{S}\ binding to CHO A<sub>1</sub> LE and HE membranes following incubation with increasing concentrations of CHA. Whilst the inhibitory \[^{35}\text{S}]\text{GTP}\gamma\text{S}\ binding response was observed at time points in the range 5 - 30 minutes, there was no significant difference in the initial rate of \[^{35}\text{S}]\text{GTP}\gamma\text{S}\ binding following incubation with either 10nM or 10\mu M CHA, which would suggest that high concentrations of CHA are not decreasing the affinity of \[^{35}\text{S}]\text{GTP}\gamma\text{S}\ binding for the relevant G-proteins. However, at 10\mu M CHA, the maximum amount of \[^{35}\text{S}]\text{GTP}\gamma\text{S}\ bound was substantially lower than that observed both for basal \[^{35}\text{S}]\text{GTP}\gamma\text{S}\ binding and in the presence of 10nM CHA. Similar qualitative findings were observed for both 0.1 and 1nM \[^{35}\text{S}]\text{GTP}\gamma\text{S}\ which would also suggest that high concentrations of CHA do not affect the affinity of \[^{35}\text{S}]\text{GTP}\gamma\text{S}\ for the G-protein.

In HE membranes, and at both concentrations of \[^{35}\text{S}]\text{GTP}\gamma\text{S}, CHA stimulated the initial rate of \[^{35}\text{S}]\text{GTP}\gamma\text{S}\ binding in a potent and monophasic manner with a Hill
slope not significantly different from unity. The inhibitory effects of CHA on the maximum level of stimulated $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding was also monophasic, with a Hill slope of unity, but was of considerably lower potency. CHA therefore appears to be activating two distinct kinetic processes, with potencies which differ by approximately two orders of magnitude. This is further confirmed by the effect of CHA on the observed rate constant, which reflects both the initial rate and maximum amount of $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding, and occurs over 4-5 orders of magnitude (Fig. 4.10 e & f).

The inhibitory response is not present at $t = 0$ but it increases with time. It appears to be due to a decrease in the maximum amount of stimulated $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding and not due to a decrease in the initial rate of $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding at high agonist concentrations. It may therefore be viewed as product inhibition, caused by the generation of AR complexes, $\text{G}\alpha-[^{35}\text{S}]\text{GTP}\gamma\text{S}$ or free $\beta\gamma$ subunits. One or more of these components would therefore be expected to inhibit the further binding of $[^{35}\text{S}]\text{GTP}\gamma\text{S}$, possibly by inhibiting the release of GDP. This inhibitory response is only present at high receptor expression levels. Therefore, the generation of adenosine A$_1$ receptor homodimers may explain the slower, inhibitory $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding response if the rate of dimer formation is slower than the activation of monomeric A$_1$ receptors and the subsequent stimulation of $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding. It would also therefore be predicted that the formation of A$_1$ receptor homodimers requires the presence of activated G-protein $\alpha$ or $\beta\gamma$ subunits.
Chapter 5. The mechanism of action of the adenosine A₁ receptor enhancer PD 81,723.

5.1 Introduction

The 2-amino-3-benzoylthiophene PD 81,723 (PD) was first identified as an allosteric enhancer of agonist binding to the adenosine A₁ receptor in rat brain membranes by Bruns and Fergus (1990). In the presence of guanine nucleotides, PD has been demonstrated to increase agonist affinity by up to 10 fold at both the human recombinant A₁ receptor stably expressed in CHO cells (Cohen, 1995) and the A₁ receptor in rat brain membranes (Kourounakis et al., 2000). The allosteric properties of PD have been confirmed in kinetic studies, which have shown PD to slow down the rate of radiolabelled agonist dissociation from the A₁ receptor in rat brain (Bruns & Fergus, 1990), guinea pig forebrain (Kourounakis et al., 2000) and the human recombinant A₁ receptor (Cohen, 1995; Kollias-Baker et al., 1994).

The enhancing effects of PD are also manifest in functional studies. In CHO cells stably expressing the human recombinant A₁ receptor, PD has been demonstrated to potentiate agonist mediated inhibition of forskolin stimulated cAMP levels (Bhattacharya & Linden, 1995; Cohen, 1995) and stimulation of [³⁵S]GTPγS binding (Cohen, 1995). PD has also been demonstrated to potentiate agonist mediated inhibition of cAMP levels at the A₁ receptor endogenously expressed in the rat thyroid cell line FRTL-5 (Bruns & Fergus, 1990). In addition, PD also potentiates agonist-mediated inhibition of the rate of contraction of isolated atria from rat (Mudumbi et al., 1993) and guinea pig (Leung et al., 1995).
The potentiation of agonist responses at the adenosine A₁ receptor may be subject to tissue and species variation. PD (20μM) increased the binding of 1nM \([^3\text{H}]\text{CHA}\) to membranes from dog, human and rat brain by 250, 200 and 150% respectively, but demonstrated only inhibition of \([^3\text{H}]\text{CHA}\) binding to bovine brain membranes (Bruns & Fergus, 1990). Furthermore, the ability of PD to enhance ligand binding to the adenosine A₁ receptor may depend on the tissue distribution of receptor expression. Jarvis et al. (1999) have demonstrated that PD is able to enhance the binding of \([^3\text{H}]\text{CHA}\) to brain membranes from rat, guinea pig and dog whilst having little effect on the binding of the radiolabelled agonist to adipocyte membranes from the same species. PD has also been demonstrated to lower the threshold of ischaemic pre-conditioning in anaesthetised dogs, an effect that was mediated by the A₁ receptor (Mizumura et al., 1996). In contrast, PD had no effect in an in vivo model of cerebral ischaemia/reperfusion injury in gerbil (Cao & Philips, 1995).

Whilst there is a large body of evidence demonstrating that PD can potentiate both the binding and functional responses of agonists at the A₁ receptor, the mechanism of action of PD remains unclear. The ability of PD to potentiate agonist binding appears to be related to the G-protein coupling characteristics of the A₁ receptor. In the absence of guanine nucleotides and in the presence of \(\text{Mg}^{2+}\), PD has little effect on agonist affinity (Kourounakis et al., 2000). In contrast, in the presence of guanine nucleotide, PD is able to increase agonist affinity by up to 10-fold (Birdsall et al., 1994).

In addition to its effects on the binding and functional properties of orthosteric agonists, PD has also been demonstrated to act as an agonist in its own right.
Thus, in FRTL-5 cells endogenously expressing the adenosine A₁ receptor (Bruns & Fergus, 1990) or CHO cells expressing the recombinant A₁ receptor (Bhattacharya & Linden, 1995), PD decreases the effect of forskolin on cAMP levels. This effect is observed at high concentrations of adenosine deaminase (Kollias-Baker et al., 1997; Musser et al., 1999), which suggests that the agonist effect of PD is not due to an enhancement of the actions of endogenous adenosine.

Bruns and Fergus (1990) have proposed a two-state model of the A₁ receptor where PD acts allosterically to shift the equilibrium between ground and active states of the A₁ receptor in favour of the active state. However, the predictions of this model have not been presented. In contrast Birdsall et al. (1994) have directly analysed the effects of PD on agonist binding to the A₁ receptor, in the presence of GTP, using the simple allosteric ternary complex model, and generated estimates of the affinity and cooperativity constants for PD and a range of adenosine agonists. Neither of these models, however, take into account the presence of G-proteins. Kourounakis et al. (2000) have attempted to describe the effect of PD using a cubic model of agonist, receptor and G-protein which incorporates receptor isomerisation from a ground to an active state. This scheme is incomplete, however, because it does not include an allosteric modulator. Furthermore, the predictions of this model have not been described.

The aims of these studies were two-fold. Firstly, to evaluate the effect of PD on the binding and functional properties of agonists of different efficacy at the adenosine A₁ receptor. Secondly, to evaluate the predictions of a number of mathematical models of drug-receptor interactions and attempt to find the simplest scheme able to incorporate the experimental observations of the actions of PD.
5.2 Results

5.2.1 Properties of PD 81,723 in radioligand binding assays

5.2.1.1 The effect of PD 81,723 on \[^3\text{H}\]CHA competition binding in CHO A\textsubscript{1} HE membranes.

In the presence of 10μM PD 81,723 (PD) and the absence of guanine nucleotides, a small increase in the amount of bound \[^3\text{H}\]CHA (1-2nM) specifically bound to the A\textsubscript{1} receptor was observed (7 ± 1%, n=4). This result suggests a small amount of positive cooperativity between PD and CHA at the coupled adenosine A\textsubscript{1} receptor which would be expected to be beyond the limits of detection in competition binding experiments (Fig. 5.1).

In accord with this expectation, in high expressing CHO adenosine A\textsubscript{1} membranes the high efficacy adenosine A\textsubscript{1} receptor agonist CHA inhibited the binding of 1-2nM \[^3\text{H}\]CHA with a pKi of 8.97 ± 0.03 (n=2) in the absence of guanine nucleotides (Fig 5.1; Table 5.1). In the presence of 10μM PD the affinity of CHA to inhibit \[^3\text{H}\]CHA was unaffected (pKi 8.99 ± 0.10).

The lack of effect of PD on the affinity of ligands binding to the coupled state of the A\textsubscript{1} receptor was not restricted to the agonist CHA. PD (10μM) also had no effect on the affinity of the high efficacy agonist NECA, the lower efficacy agonists GR190178, GR161144 and GR162900, and the antagonist/inverse agonist DPCPX (Fig. 5.1, Table 5.1). For all unlabelled ligands, the inhibition of \[^3\text{H}\]CHA was monophasic, with Hill coefficients not significantly different from unity.
Figure 5.1. The effect of PD 81,723 on competition binding of [³H]CHA at the adenosine A₁ receptor.

After incubating with ADA (3U.ml⁻¹) for 30 min., membranes were added to [³H]CHA (1-2 nM), saponin (30µg.ml⁻¹) and increasing concentrations of cold ligand, in the absence (closed circles) or presence (open circles) of 10µM PD 81,723. Data points are the mean ± range/2 of duplicate determinations from one experiment. Curves are computer generated best fits to a 1-site model. Parameter estimates from 2-3 experiments are summarised in Table 5.1.
Table 5.1. Summary of the effect of PD 81,723 on the affinity of ligands for the G-protein uncoupled and coupled states of the adenosine A<sub>1</sub> receptor.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Uncoupled receptor</th>
<th>Coupled receptor</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>+10μM PD 81,723</td>
</tr>
<tr>
<td>CHA</td>
<td>5.6 ± 0.1</td>
<td>6.8 ± 0.1</td>
</tr>
<tr>
<td>NECA</td>
<td>5.4 ± 0.1</td>
<td>6.5 ± 0.03</td>
</tr>
<tr>
<td>GR190178</td>
<td>6.1 ± 0.1</td>
<td>6.2 ± 0.3</td>
</tr>
<tr>
<td>GR161144</td>
<td>7.1 ± 0.1</td>
<td>7.1 ± 0.1</td>
</tr>
<tr>
<td>GR162900</td>
<td>7.5 ± 0.2</td>
<td>7.4 ± 0.3</td>
</tr>
<tr>
<td>DPCPX</td>
<td>8.9 ± 0.2</td>
<td>8.5 ± 0.2</td>
</tr>
</tbody>
</table>

Data are the mean pK<sub>i</sub> ± s.e.mean of 3 (uncoupled) or 2 (coupled) experiments using membranes from CHO A<sub>1</sub> high expressing cells.

PD shift is the mean ± range/2 of the ratio of K<sub>i</sub> values in the absence and presence of 10μM PD 81,723.

Ligand affinity for the G-protein uncoupled receptor was determined by incubating membranes with [³H]DPCPX (0.4-0.6nM) in the presence of 100μM GDP, 30μg.ml<sup>-1</sup> saponin and increasing concentrations of cold ligand.

Ligand affinity for the G-protein coupled receptor was determined by incubating membranes with [³H]CHA (1-2nM), 30μg.ml<sup>-1</sup> saponin and increasing concentrations of cold ligand.
5.2.1.2 The effect of PD 81,723 on \(^{3}\text{H}\)DPCPX competition binding, in the presence of guanine nucleotides, in CHO A1 HE membranes.

In the presence of 100μM GDP the binding of the radiolabelled antagonist/inverse agonist \(^{3}\text{H}\)DPCPX (0.4-0.6nM) to the adenosine A1 receptor was decreased by 38 ± 5% in the presence of 10μM PD (Fig. 5.2), suggesting a degree of negative cooperativity between the binding of DPCPX and PD. These data, and the data of F. Cohen (1995), suggest that the affinity of PD for the unoccupied receptor is \(~10^5\text{M}^{-1}\). In the absence of PD, increasing concentrations of unlabelled DPCPX inhibited the binding of \(^{3}\text{H}\)DPCPX with a pKi of 8.85 ± 0.18, whilst in the presence of 10μM PD the pKi was 8.53 ± 0.21 (n=3; Fig. 5.2, Table 5.1), which may also indicate a negative cooperative binding interaction between PD and DPCPX.

In the presence of 100μM GDP, the binding of \(^{3}\text{H}\)DPCPX (0.4-0.6 nM) was inhibited by CHA with a pKi of 5.60 ± 0.08 (n=3; Fig 5.2, Table 5.1). In the presence of 10μM PD, the affinity of CHA was increased by approximately 15 fold to 6.79 ± 0.14, confirming the positive cooperativity between PD and CHA for the G-protein uncoupled state of the adenosine A1 receptor demonstrated in previous studies (Birdsall et al., 1994). The binding of the high efficacy agonist NECA to the adenosine A1 receptor was also enhanced to a similar degree by the presence of 10μM PD (Fig. 5.2, Table 5.1).

In contrast to CHA and NECA however, the binding of the lower efficacy agonists GR190178, GR161144 and GR162900 were unaffected by the presence of 10μM PD (Fig. 5.2, Table 5.1). The ability of PD to increase the affinity of ligands for
Figure 5.2. The effect of PD 81,723 on competition binding of \(^{3}\text{H}\)!DPCPX at the adenosine A\(_1\) receptor in the presence of GDP.

After incubating with ADA (3U.ml\(^{-1}\)) for 30 min., membranes were added to \(^{3}\text{H}\)!DPCPX (0.4-0.6nM), saponin (30\(\mu\)g.ml\(^{-1}\)), GDP (100\(\mu\)M) and increasing concentrations of cold ligand, in the absence (closed circles) or presence (open circles) of 10\(\mu\)M PD 81,723. Data points are the mean \pm range/2 of duplicate determinations from one experiment. Curves are computer generated best fits to a one-site model. Parameter estimates from 2-3 experiments are summarised in Table 5.1.
the uncoupled state of the A₁ receptor therefore appeared to be restricted to the high efficacy agonist CHA and NECA. A similar result has also been demonstrated for R-PIA and adenosine itself (Birdsall et al., 1994). For all unlabelled ligands, the inhibition of [³H]DPCPX in the presence of GDP was monophasic, with Hill coefficients not significantly different from unity.

5.2.1.3 The effect of PD 81,723 on [³H]DPCPX competition binding in the absence of guanine nucleotide

In the absence of guanine nucleotides the binding of [³H]DPCPX (0.4-0.6nM), was decreased by 42 ± 6% in the presence of 10μM PD (n = 4). The effect of PD on the binding of [³H]DPCPX in the absence of GDP was similar to that observed in the presence of GDP. However, these results cannot strictly be compared as the experiments were not performed in a pairwise fashion. However, previous results from this laboratory indicate that the inhibitory effects of PD on [³H]DPCPX in the absence of guanine nucleotides are greater than those observed in the presence of guanine nucleotides (F. Cohen, A. Martin, unpublished results). These results indicated that the binding properties of PD are subject to a small (1.5-2 fold) ‘GTP shift’ in the same manner as orthosteric agonists of the adenosine A₁ receptor.

In a single experiment, in the absence of guanine nucleotides, the binding of [³H]DPCPX to the A₁ receptor was inhibited by the high efficacy agonist NECA in a biphasic manner (Fig. 5.3). In the presence of PD (10μM), the dissociation constant of NECA for the uncoupled receptor was increased by 5 fold (pKᵋ values of 5.36 ± 0.08 and 6.10 ± 0.13 in the absence and presence of PD, respectively). At the coupled receptor, the estimates of the dissociation constant of NECA
5.3. The effect of PD 81,723 on the competition binding of $[^3H]$DPCPX by NECA at the adenosine $A_1$ receptor, in the absence of GDP.

Membranes from CHO $A_1$ high expressing cells (1-2μg) were incubated with $[^3H]$DPCPX (0.4 - 0.6nM), 30μg.ml$^{-1}$ saponin, and increasing concentrations of the high efficacy agonist NECA in the absence (open circles) or presence (closed circles) of 10μM PD 81,723.

Data are the mean±s.e.mean of triplicate determinations. The line represents the best fit of the data to a two, independent site, binding model determined by non linear regression analysis.
differed by less than two fold in the absence and presence of PD (pK$_{H}$ values of 9.02 ± 0.10 and 8.73 ± 0.20 respectively). A similar result was found for CHA. Thus, the effect of PD on the affinity of an agonist for the coupled and uncoupled states of the A$_1$ receptor as determined by [³H]DPCPX competition binding in the absence of guanine nucleotide, are similar to that determined using radiolabelled agonist (coupled receptor), or [³H]DPCPX in the presence of GDP (uncoupled receptor).

The fraction of high affinity binding sites described by NECA was essentially unchanged by the presence of 10µM PD (0.69 ± 0.02 and 0.64 ± 0.04 in the absence and presence of PD, respectively). The lack of any measurable effect of PD on the proportion of high affinity binding sites is consistent with the small direct effect of PD on [³H]CHA binding described above. This result is largely consistent with data for the rat A$_1$ receptor both in brain membranes and recombinantly expressed in CHO cells, where similar fractions of high affinity agonist binding were observed in the absence and presence of PD (0.52 to 0.55(+PD) and 0.71 to 0.77 (+PD) brain and CHO cell membranes respectively, Musser et al., 1999). In contrast in guinea pig forebrain membranes PD (30µM) increased the fr$_{H}$ for R-PIA from 0.38 to 0.55 (Kollias-Baker et al., 1994).

5.2.1.4 The effect of PD 81,723 on the inhibition of radiolabelled agonist binding by guanine nucleotides.

GTP produced a concentration dependent inhibition of the binding of the radiolabelled agonists [³H]NECA, [³H]CHA and [³H]PIA (1-2nM) with similar pIC$_{50}$ values of 7.52, 7.38 and 7.40 respectively (one experiment, Fig 5.4). In the
Figure 5.4. The effect of PD 81,723 on the inhibition by GTP of the binding of radiolabelled agonists to the adenosine A₁ receptor.

Membranes from CHO A₁ high expressing cells were incubated with 1-2nM of [³H]CHA (a), [³H]NECA (b) or [³H] PIA (c) in the presence of increasing concentrations of GTP, and 30µg.ml⁻¹ saponin, in the absence (filled symbols) or presence (empty symbols) of 10µM PD 81,723 for 60 min. at room temperature. Non-specific binding was determined by the addition of 3mM theophylline. Data are the mean ± s.e. mean of triplicate data points. The curves are the computer generated best fit to a one-site model, allowing the Hill slope to vary.
presence of PD the potency of GTP was reduced 2-3 fold to 7.14, 7.07 and 7.12 respectively (one experiment, Fig 5.4). This effect has been reported for the inhibition of \[^{3}H\]CHA by the guanine nucleotide analogue Gpp(NH)p in guinea pig forebrain membranes (Kollias-Baker et al., 1994) and the inhibition of the radiolabelled agonist \[^{125}I\]ABA (\[^{125}I\]N\(^{6}\)-(3-iodo-4-aminobenzyladenosine) binding to CHO A\(_{1}\) membranes by GTP\(_{\gamma}\)S (Bhattacharya & Linden, 1995) and interpreted as a stabilisation of the G-protein coupled state of the receptor by PD. Another way to portray the data shown in Fig. 5.4 is that the % enhancement of \[^{3}H\]CHA binding by PD increases as the concentration of guanine nucleotide increases.

5.2.2. Effect of PD 81,723 on A\(_{1}\) receptor function measured in \[^{35}S\]GTP\(_{\gamma}\)S binding assays.

The effect of PD on the functional responses of the adenosine A\(_{1}\) receptor agonist CHA have been presented in section 4.2.3. In summary, PD (3\(\mu\)M) increased the potency of CHA by 3 fold in both low (LE) and high (HE) expressing CHO A\(_{1}\) membranes. In addition, PD increased the basal level of \[^{35}S\]GTP\(_{\gamma}\)S binding by 38% in HE membranes but had little or no effect on basal binding in LE membranes. The aim of the present studies was to evaluate the effect of PD on the functional responses to agonists of lower efficacy.

5.2.2.1 Low expressing CHO A\(_{1}\) membranes

In control experiments, GR190178, GR161144 and GR162900 stimulated the binding of \[^{35}S\]GTP\(_{\gamma}\)S to LE membranes with similar potencies (pEC\(_{50}\) 7.3-7.4; Figure 5.5; Table 5.2). The maximum response to GR190178 was similar to that
Figure 5.5. The effect of PD 81,723 on the $[^{35}\text{S}]$GTP$\gamma$S functional responses of agonists of varying efficacy.

Membranes from CHO A1 low expressing (a,c,e) or high expressing (b,d,f) were preincubated with ADA (3U.ml$^{-1}$) for 30 min. before being added to GDP (10μM), $[^{35}\text{S}]$GTP$\gamma$S (0.1nM) and increasing concentrations of agonist in the absence (panels c & d; panels a & b closed symbols) or presence (panels e & f; panels a & b open symbols) of 3μM PD 81,723. Membranes were incubated for a further 30 min. at 30°C before rapid filtration and washing. Data are the mean ± range/2 of duplicate data points. The curves represent the computer generated best fits to either one- or two-component models as described in Chapter 2.
**Legend to Table 5.2:**

Data are the mean ± s.e.mean of 4 experiments.

i.a.: intrinsic activity, expressed as a fraction of the maximum response to CHA in the same experiment.

a: biphasic concentration response curves in two out of four experiments and data expressed as mean ± range/2.

b: control curves were monophasic in all four experiments in the absence of PD, and biphasic in two out of four experiments in the presence of PD (data expressed as mean ± range/2).

The effect of PD is expressed as; c: the ratio of agonist EC$_{50}$ in the absence and presence of PD, or d: the increase in agonist intrinsic activity, expressed as a percentage of the maximum response to CHA.
Table 5.2 Summary of the effects of PD 81,723 on the $[^{35}S]$GTP$\gamma$S binding response of adenosine A$_1$ receptor agonists in CHO A$_1$ low and high expressing membranes.

<table>
<thead>
<tr>
<th>Agonist</th>
<th>Treatment</th>
<th>Low expression</th>
<th>High expression</th>
<th>Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>pEC$_{50}$</td>
<td>i.a.</td>
<td>pEC$_{50}$</td>
</tr>
<tr>
<td>CHA</td>
<td>Control</td>
<td>8.1 ± 0.01</td>
<td>1.00</td>
<td>9.0 ± 0.1</td>
</tr>
<tr>
<td>3μM PD</td>
<td>8.6 ± 0.04</td>
<td>1.00</td>
<td>9.4 ± 0.1</td>
<td>1.00</td>
</tr>
<tr>
<td>Effect of PD</td>
<td>2.8 ± 0.2$^c$</td>
<td>2.7 ± 0.4$^c$</td>
<td>4.0 ± 1.1$^c$</td>
<td></td>
</tr>
<tr>
<td>GR190178</td>
<td>Control</td>
<td>7.4 ± 0.1</td>
<td>0.99± 0.04</td>
<td>8.3 ± 0.1</td>
</tr>
<tr>
<td>3μM PD</td>
<td>7.7 ± 0.1</td>
<td>1.00± 0.05</td>
<td>8.6 ± 0.1</td>
<td>1.00± 0.03</td>
</tr>
<tr>
<td>PD shift</td>
<td>2.3 ± 0.2$^c$</td>
<td>1 ± 3$^d$</td>
<td>1.8 ± 0.3$^c$</td>
<td>4 ± 5$^d$</td>
</tr>
<tr>
<td>GR161144</td>
<td>Control</td>
<td>7.4 ± 0.04</td>
<td>0.82 ± 0.02</td>
<td>8.3 ± 0.2</td>
</tr>
<tr>
<td>3μM PD</td>
<td>7.7 ± 0.1</td>
<td>0.97 ± 0.04</td>
<td>8.6 ± 0.1</td>
<td>0.99 ± 0.03</td>
</tr>
<tr>
<td>Effect of PD</td>
<td>2.1 ± 0.3$^c$</td>
<td>15 ± 4$^d$</td>
<td>1.9 ± 0.4$^c$</td>
<td>15 ± 8$^d$</td>
</tr>
<tr>
<td>GR162900</td>
<td>Control</td>
<td>7.3 ± 0.03</td>
<td>0.52 ± 0.05</td>
<td>8.0 ± 0.1</td>
</tr>
<tr>
<td>3μM PD</td>
<td>7.6 ± 0.1</td>
<td>0.73 ± 0.01</td>
<td>8.4 ± 0.1</td>
<td>0.81 ± 0.06</td>
</tr>
<tr>
<td>Effect of PD</td>
<td>2.3 ± 0.4$^c$</td>
<td>20 ± 5$^d$</td>
<td>2.5 ± 0.7$^c$</td>
<td>4 ± 6$^d$</td>
</tr>
</tbody>
</table>
of CHA whilst GR161144 and GR162900 were partial agonists, with GR162900 having lower efficacy than GR161144. In the presence of 3μM PD, the potency of GR190178, GR161166 and GR162900 was increased by approximately 2 fold (Table 5.2). Both the basal and maximum [35S]GTPγS binding responses to GR190178 were unaffected by the presence of 3μM PD. In contrast, the maximum responses to GR161144 and GR162900 were increased by 15 and 20%, respectively, of the maximum response to CHA. The effect of PD on the functional responses of the low efficacy agonists was therefore both an increase in intrinsic activity and a two-fold increase in agonist potency.

5.2.2.2 High expressing CHO A1 membranes

In CHO A1 high expressing membranes CHA, GR190178, GR161144 and GR162900 had similar potencies for the stimulation of [35S]GTPγS binding, which were approximately 5-10 fold higher than in low expressing membranes (pEC50 8.0-9.0). In the presence of 3μM PD, the potency of all of the agonists studied was increased by 2-3 fold (Fig. 5.5, Table 5.2). In the presence of 3μM PD, the maximum response to GR 161144 and GR 162900 was also increased, although the increase was smaller than that observed in low expressing membranes, possibly because these agonists generated maximum responses which were closer to that of the full agonist CHA.

The concentration-response curves for CHA, GR 190178 and GR 161144 (two out of four experiments) were biphasic, with a concentration dependent decrease in [35S]GTPγS binding observed at higher agonist concentrations. This response has been characterised in detail in the preceding Chapter. The potencies of CHA, GR
190178 and GR 161144 were similar (pECs_50 6.6-7.2; Fig. 5.5, Table 5.2) for this inhibitory response. In the presence of PD (3μM) the potency of the inhibitory component of the responses to CHA, GR190178 and GR161144 was increased by 3-6 fold (Figure 5.5 and Table 5.2). PD also increased the intrinsic activity of this inhibitory response (expressed as a percentage of the maximum CHA inhibitory response) of GR 190178 and GR 161144 by 18 and 33% respectively. Furthermore, whilst control concentration-response curves to GR162900 were monophasic in all four experiments, in the presence of PD (3μM) the concentration-response curve to GR 162900 was biphasic in two out of four experiments. These results in high expressing membranes provide further evidence that PD increases the intrinsic activity of partial agonists of the adenosine A_1 receptor.

5.2.2.3 The effect of adenosine deaminase on the responses of CHA, DPCPX, and PD in CHO A_1 HE membranes.

In the absence of adenosine deaminase (ADA) the basal binding of [^35S]GTP_γ_S represented 67% of the specific [^35S]GTP_γ_S binding defined as the difference between a maximal concentration of CHA (10nM) and DPCPX (1μM; Fig. 5.6). PD (3μM) increased the basal [^35S]GTP_γ_S response to 92% of the specific [^35S]GTP_γ_S binding response, i.e. the response to PD approached that obtained with the full agonist CHA. The marked effects of DPCPX and PD in the absence of ADA are consistent with the presence of a concentration of endogenous adenosine that is sufficient to activate the adenosine A_1 receptor. It is notable, however, that a response to CHA is observed in the absence of ADA, which suggests that the endogenous adenosine is present at a submaximal concentration.
5.6. The effect of adenosine deaminase on basal $[^{35}\text{S}]$GTP$_\gamma$S binding, and responses to CHA, PD 81,723 and DPCPX in CHO adenosine A$_1$ high expressing membranes.

Membranes were incubated with increasing concentrations of adenosine deaminase for 30 min. at room temperature before being added to GDP (10μM), $[^{35}\text{S}]$GTP$_\gamma$S (0.1nM) and either vehicle, DPCPX, PD or CHA at the stated concentrations. Membranes were incubated for a further 30 min. at 30°C before rapid filtration and washing as described in the Methods section.

Specific $[^{35}\text{S}]$GTP$_\gamma$S binding is defined as the difference between the binding in the presence of DPCPX (1μM) and CHA (10nM) in the absence of ADA, the latter value being normalised to 100%. Data are the mean ± range/2 of duplicate data points.
The addition of adenosine deaminase (0.003 – 30 U.ml⁻¹) produced a concentration dependent decrease in the amount of [³⁵S]GTPγS binding in the absence (basal) and presence of PD (3µM). At lower concentrations, the effects of ADA (0.003 – 0.3 U.ml⁻¹) on the amount of basal and PD stimulated [³⁵S]GTPγS binding was marked, suggesting that ADA was readily removing endogenous adenosine. At high concentrations, ADA (3-30 U.ml⁻¹) decreased the responses to DPCPX and CHA, in addition to producing further decreases in basal and PD stimulated [³⁵S]GTPγS binding, suggesting a non-specific inhibitory effect of ADA at these concentrations.

Whilst the effects of ADA at low concentrations suggest that the increase in [³⁵S]GTPγS binding in the presence of PD may be partly due to the presence of endogenous adenosine, the failure of high concentrations of ADA to abolish the effects of PD suggests that, under these conditions, the response to PD is independent of endogenous adenosine. Similar results were obtained in the presence of 10µg.ml⁻¹ of the permeabilising agent saponin, which also indicates that the effects of PD are not due to the presence of cryptic pools of endogenous adenosine (Prater et al., 1992).

5.3 Simulation of the effects of PD 81,723 on the binding and functional properties of the adenosine A₁ receptor.

The effects of PD on the binding and functional properties of the A₁ receptor can be summarised as follows:
(a) An increase in affinity of high efficacy, but not low efficacy agonists, for the G-protein uncoupled A₁ receptor.

(b) Little or no detectable effect on ligand affinity for the G-protein coupled state of the A₁ receptor.

(c) A decrease in the potency of guanine nucleotides to inhibit radiolabelled agonist binding.

(d) An increase in potency of high efficacy agonists.

(d) An increase in the potency and intrinsic activity of partial agonists.

(e) An increase in basal [³⁵S]GTPγS binding in high but not low expressing CHO A₁ membranes.

The following section describes attempts to accommodate the above observations within a mathematical model of ligand, receptor and G-protein interaction.

5.3.1 Ternary complex model

The simple ternary complex model (TCM), as discussed in Section 1.3 and Chapters 3 and 4, was evaluated for its ability to predict the properties of PD. The TCM (Fig. 5.7) describes the cooperative interactions of a ligand A, with a G-protein, G, at a receptor R. The affinity constants of A and G for unoccupied R, are $K_A$ and $K_G$ respectively and the extent of cooperativity between A and G is described by the cooperativity constant $\alpha$. Whilst this scheme does not explicitly contain an allostERIC modulator, the effects of PD were modelled by assuming that PD is capable of modulating $K_A$, $K_G$ and $\alpha$.
Figure 5.7. The Ternary Complex Model

(A) Scheme illustrating receptor R interacting with agonist A and G-protein G. $K_A$ and $K_G$ are the affinity constants for the binding of A to G and R to G, respectively. $\alpha$ is the allosteric cooperativity constant. The magnitude of $\alpha$ describes the extent to which the affinity of R for G is increased following the binding of A. For agonists ($\alpha > 1$) the binding of A leads to an increase in the affinity of R for G. For inverse agonists ($\alpha < 1$), the binding of A leads to a decrease in the affinity of R for G. Simple, competitive antagonists ($\alpha = 1$) bind to the receptor but do not change the affinity of R for G.

The apparent radioligand binding dissociation constants for the G-protein coupled and uncoupled states of the receptor, expressed in terms of the parameters of the model, are shown in panel B. The agonist functional potency, basal and maximum agonist response, expressed in terms of the parameters of the model, are shown in panel C. The derivation of these terms is presented in Appendix 2.
5.3.1.1 Binding

The apparent dissociation constant of a ligand is defined in the TCM by the expression \[ \frac{1 + [G] K_G}{K_A (1 + \alpha [G] K_G)} \] (Appendix 1 and Fig 5.7), where [G] represents the concentration of G-protein, relative to \( K_G \). This expression has the limiting values of \( 1/K_a \) when \( K_G \to 0 \), and \( 1/\alpha K_A \) when \( K_G \to \infty \). These values represent the apparent ligand dissociation constant for the G-protein uncoupled and coupled state of a receptor respectively. The apparent dissociation constant of a ligand for its receptor will take a value between these two limiting values, and will be dependent on the relative values of [G], \( K_G \) and \( \alpha \).

Data presented in Chapter 3 (sections 3.2.1 - 3.2.3 and discussion therein) indicated that in the absence of guanine nucleotides there is significant receptor-G-protein precoupling, and that this is best described when \( K_G = 10 \). The effects of PD on agonist binding to the G-protein coupled receptor were therefore simulated using this value of \( K_G \), at which the apparent agonist dissociation constant is close to its lower limit (\( K_h = 1/\alpha K_A \)). The presence of GDP is assumed to decrease the affinity of R for G by a factor of 10000. This seems reasonable given the ability of GDP to decrease the \( K_l/K_h \) value of high efficacy agonists by at least a factor of 1000 (Chapter 3, sections 3.2.6 - 3.2.7). The agonist cooperativity constant was given a value of 1000, which is predicted for a \( K_l/K_h \) value of 1000 when \( K_G = 10 \). The affinity of the agonist for R (\( K_A \)) was given a value of \( 1 \times 10^6 \) M\(^{-1} \), which is similar to the affinity of CHA for the uncoupled \( A_1 \) receptor. The effect of PD on the apparent agonist dissociation constants for the G-protein coupled and uncoupled receptor was simulated in turn, by assuming that PD increased the
value of $K_A$, $\alpha$ and $K_G$ by 10 fold. The values of $G_T$ and $R_T$ were set to 1 and 0.1 respectively, which allowed the generation of monophasic inhibition curve data.

An increase in the value of $K_A$ induced by an allosteric modulator will result in an equivalent decrease in the apparent dissociation constants for the G-protein coupled and uncoupled receptor (Fig. 5.8a). The effect of the modulator on the value of $K_A$ is therefore predicted by the TCM to be independent from the G-protein coupled state of the receptor. In radioligand binding studies however, PD increased the apparent affinity of agonists for the G-protein uncoupled state of the receptor, but not the coupled state of the receptor, which is not consistent with the predictions of the model.

When the apparent dissociation constant of a ligand is close to the limiting value of $1/\alpha.K_A$ (when $[G].K_G >> 1$), an increase in the value of the cooperativity constant $\alpha$ will be manifest as an equivalent decrease in the apparent dissociation constant for the G-protein coupled state of the receptor (Fig. 5.8b). As the apparent dissociation constant approaches its upper limit of $1/K_A$, the effect of $\alpha$ decreases. Hence, an allosterically induced increase in agonist efficacy ($\alpha$ in the TCM) will be primarily manifested as an increase in apparent ligand affinity for the G-protein coupled, but not uncoupled, state of a receptor. This is not observed experimentally with PD.

Alterations in the value of $K_G$ have no effect on the apparent affinity of an agonist for the coupled state of a receptor when its dissociation constant is close to its lower limit of $1/\alpha.K_A$ (Fig 5.8c). When the apparent dissociation constant for a ligand lies between its upper and lower limits, increases in the value of $K_G$
Figure 5.8. Simulation of the effects of an allosteric ligand using the Ternary Complex Model: Radioligand binding.

The simulations were performed as described in Chapter 2 using the equations derived in Appendix 1. The following model parameters were used:

Agonist affinity ($K_a$) = 1x10^6 M^-1

Agonist cooperativity constant ($\alpha$): 1000.

The radioligand was assumed to be a silent antagonist and present at a concentration (relative to its affinity) at which the Cheng-Prusoff correction was negligible. The model parameters used for the radioligand were as follows:

Radioligand affinity: 1

Radioligand concentration: 0.01

Cooperativity constant: 1

The total concentration of G-protein ($G_T$) and receptor ($R_T$) were 1 and 0.1 respectively (i.e. $G_T >> R_T$). In all cases the inhibition curves were monophasic with Hill slopes of unity.

The effect of receptor-G-protein coupling (curve 3) and uncoupling (curve 1) was simulated by setting the value of $K_G$ to 10 and 1x10^-3 respectively. Using these values, the apparent affinity constants for the G-protein coupled and uncoupled receptor were close approximations to $\alpha.K_a$ and $K_a$, respectively.

Three different ways that an allosteric ligand can affect agonist binding to the G-protein coupled and uncoupled receptor were simulated by increasing the value of $K_a$ (agonist affinity), $\alpha$ (agonist efficacy) or $K_G$ (receptor - G-protein affinity) by 10-fold (panels a, b and c respectively).

The simulated data were analysed by non-linear least squares regression as described in Chapter 2. Data were analysed by fitting to a one-site model for the estimation of the apparent ligand dissociation constant.
produce corresponding changes in the apparent dissociation constant. When the model parameters are set such that the apparent dissociation constant is close to the limiting value $1/K_A$, (when $\alpha[G].K_G < 1$, but is not zero) the agonist $K_L$ value may still be sensitive to changes in $K_G$ (Fig 5.8c).

Hence, the TCM predicts that changes in the value of either $\alpha$ or $K_G$, but not $K_A$ may produce changes in the apparent affinity constant of a ligand which are dependent on the G-protein coupled state of a receptor. However, changes to $\alpha$ will have a greater influence on the affinity of an agonist for the G-protein coupled state of a receptor. The ligand binding properties of PD are therefore predicted by the TCM to be consistent only with an increase in $K_G$, the affinity of the unoccupied receptor for its G-protein.

5.3.1.2 Function

When $R_T << G_T$ the mid-point curve location for an agonist concentration-response curve is predicted by the TCM to be identical to the apparent dissociation constant, i.e. $\frac{1 + [G]K_G}{K_A(1 + \alpha[G]K_G)}$ (Appendix 1 and Fig. 5.7). Hence, an allosterically induced increase in $K_A$ is predicted to produce an equivalent increase in agonist potency (Fig 5.9a) which is in keeping with the experimental observations with PD. The maximum response to an agonist is defined in the TCM as $\frac{\alpha[G]K_G}{1 + \alpha[G]K_G}$ (Appendix 1 and Fig. 5.7), and it can be seen from this expression, and illustrated in Fig. 5.9a, that an increase in the value of $K_A$ will have no effect on the maximum response of an agonist. Whilst this prediction is observed experimentally with PD and full agonists such as CHA, PD is observed to increase
Figure 5.9. Simulation of the effects of an allosteric ligand using the Ternary Complex Model: Function.

The simulations were performed as described in Chapter 2 using the equations derived in Appendix 1. The following model parameters were used:

Agonist affinity ($K_a$) = $1 \times 10^6$ M$^{-1}$

Agonist cooperativity constant ($\alpha$): 1000 (high efficacy, curves 1 and 2)

30 (low efficacy, curves 3 and 4)

The effects of an allosteric ligand on agonists of high and low efficacy (curves 2 and 4 relative to control curves 1 and 3) were simulated in three ways, by increasing the values of $K_a$, $\alpha$ and $K_G$ by 10-fold (panels a, b & c respectively).

The concentration of $G_T$ and $R_T$ were 1 and 10 respectively and $K_G$ was given a value of $1 \times 10^3$. Using these values, the simulated basal response was close to zero, but was able to be increased when $K_G$ was increased by 10 fold (panel c). The simulated data were analysed by non-linear least squares regression as described in Chapter 2, by fitting to a sigmoidal dose-response curve for the estimation of agonist potency and maximum response. In all cases the curves were monophasic with Hill slopes of unity.
the maximum response of partial agonists. Hence, the experimental observations are not consistent with the predictions of the TCM for a simple increase in $K_A$.

When $G_T$ is limiting ($R_T > G_T$), the agonist $EC_{50}$ is an underestimate of

$$\frac{1 + [G]K_G}{K_A(1 + \alpha[G]K_G)}$$

although the limiting values of the $EC_{50}$ will be $1/K_A$ and $1/\alpha K_A$, depending on the value of $K_G$. However, the $EC_{50}$ is still sensitive to changes in $\alpha$, $K_A$ and $K_G$ (Fig. 5.9) under conditions where $R_T > G_T$.

The effect of increasing either $\alpha$ or $K_G$ on the $EC_{50}$ and the maximum response of an agonist are similar (Fig. 5.9b and c). For a high efficacy agonist, an increase in the value of either $\alpha$ or $K_G$ produces an increase in agonist potency of similar magnitude. The effect of increasing $\alpha$ or $K_G$ on the maximum response of a high efficacy agonist is small when the limiting value of $\frac{\alpha[G]K_G}{1 + \alpha[G]K_G}$ is approached, which is equivalent to $G_T$ when $[G]$ is conserved. Conversely, for lower efficacy agonists the maximum response is more sensitive to changes in either $\alpha$ or $K_G$, whilst smaller changes in agonist $EC_{50}$ are predicted.

In the absence of agonist, the basal response is defined by the TCM as $\frac{[G]K_G}{1 + [G]K_G}$ (Appendix 1 and Fig. 5.7). From this expression, it can be seen that the basal response will vary with $K_G$ but is independent of $\alpha$. An increase in the value of $K_G$, but not $\alpha$, is therefore consistent with the experimental observation that PD possesses intrinsic efficacy (Fig. 5.9c).
In summary, the effects of PD on the potency and maximum response of agonists of varying efficacy can be predicted by the TCM in terms of an increase in $\alpha$ or $K_G$, but not $K_A$. However, only an increase in $K_G$ can predict the agonist independent effect of PD on the basal functional response.

5.3.2. The Quaternary Complex Model

In order to further explore the mechanistic implication of allosteric modulation on $K_G$, a quaternary complex model (QCM) was derived, which describes the full interaction between agonist (A), receptor (R), G-protein (G) and allosteric ligand (PD, fig. 5.10). In contrast to the TCM, the quaternary complex model explicitly describes the effect of PD on the ligand binding and functional properties of a receptor. PD can influence the behaviour of a receptor through three cooperativity constants, $\beta$, $\gamma$ and $\delta$. The influence of each of these cooperativity constants of PD, on the binding and functional properties of a receptor will now be considered, using simulations of the quaternary complex model.

5.3.2.1 Binding

The apparent agonist affinity for the G-protein coupled state of the receptor is given by the term


(Appendix 2 and Fig. 10). With respect to the G-protein coupling state of the receptor, the apparent agonist dissociation constant has the limiting values

$$\frac{1 + [PD]K_{PD}}{K_A(1 + \beta[PD]K_{PD})} \quad \text{and} \quad \frac{1 + \gamma[PD]K_{PD}}{\alpha K_A(1 + \beta\gamma\delta[PD]K_{PD})}$$

which represent the agonist dissociation constants for the G-protein uncoupled ([G]K_G <<1) and
Figure 5.10. The Quaternary Complex Model.

Panel A. Scheme illustrating the interactions of receptor R, agonist A, G-protein G, and allosteric modulator PD. $K_A$, $K_G$ and $K_{PD}$ are the affinity constants for the binding to the receptor of A, G, and PD, respectively. $\alpha$, $\beta$ and $\gamma$ are the allosteric cooperativity constants for the formation of the ternary species A.R.G, A.R.PD and PD.R.G, respectively. $\delta$ is the cooperativity constant which determines the extent of formation of the quaternary species A.PD.R.G from each of the three ternary species.

Panel B. The apparent radioligand binding dissociation constants for the G-protein coupled and uncoupled states of the receptor, in terms of the parameters of the QCM.

Panel C. The agonist functional potency, basal and maximum agonist response, in terms of the parameters of the QCM.
coupled ([G].K_G > 1) state of the receptor respectively. The apparent agonist
dissociation constant will therefore have some value between these two limiting
values and will depend on the magnitude of the cooperativity constants and the
values of [G].K_G.

The effects of PD were simulated using the values of K_A, K_G, α, G_T and R_T as
described in section 5.3.1.1, and it was assumed that GDP acted in the same
manner, by decreasing the value of K_G by 10000-fold. When the presence of PD
was simulated, it was assumed to be present at a concentration 10-fold higher than
its dissociation constant for the uncoupled receptor (1/K_PD). The influence of each
of the three cooperativity constants, β, γ and δ, were examined in turn, by giving
each a value of 10 (positive cooperativity), whilst the remaining two constants
were given values of 1 (neutral cooperativity).

Fig. 5.11 demonstrates the effect of an allosteric ligand on the binding of an
agonist to the G-protein coupled and uncoupled states of a receptor. When β is
given a value > 1, positive cooperativity is observed at both the coupled and
uncoupled states of the receptor (Fig. 5.11a). The effect of β is therefore
equivalent to the cooperativity constant of 'classical' allosteric ligands which
modulate the affinity of an orthosteric ligand for a receptor. Allosteric ligands
with a value of β > 1 are predicted by the quaternary complex model to increase
the apparent affinity of an agonist for a receptor, in a manner which is independent
of the G-protein coupling characteristics of the receptor.

When δ was given a value >1 (Fig. 5.11b), the effect of an allosteric ligand was
predicted to be dependent on the extent of G-protein coupling of the receptor.
Figure 5.11. Simulation of the effects of an allosteric ligand using the Quaternary Complex Model: Binding.

The simulations were performed as described in Chapter 2 using the equations derived in Appendix 2. The following model parameters were used:

Agonist affinity ($K_A$): $1 \times 10^6$ M$^{-1}$

Agonist cooperativity constant ($\alpha$): 1000

Allosteric ligand affinity ($K_{PD}$): 1

<table>
<thead>
<tr>
<th>Concentration of PD (relative to $K_{PD}$)</th>
<th>0 (curves 1 and 4)</th>
<th>1 (curves 2 and 5)</th>
<th>10 (curves 3 and 6).</th>
</tr>
</thead>
</table>

The following values for the cooperativity constants $\beta$, $\gamma$, and $\delta$ were used:

Panel a: $\beta=10$, $\gamma=1$, $\delta=1$

Panel b: $\beta=1$, $\gamma=1$, $\delta=10$

Panel c: $\beta=1$, $\gamma=10$, $\delta=1$

The binding of the unlabelled orthosteric ligand to the G-protein coupled (curves 4, 5, and 6) and uncoupled (curves 1, 2, and 3) receptor was simulated by setting the value of $K_G$ to 10 and $1 \times 10^{-3}$ respectively. Using these values, the apparent affinity constants for the G-protein coupled and uncoupled receptor were close approximations to $\alpha K_A$ and $K_A$ respectively.

The radioligand was assumed to be a silent antagonist and present at a concentration (relative to its affinity) at which the Cheng-Prusoff correction was negligible. The model parameters used for the radioligand were as follows:

Radioligand affinity: 1

Radioligand concentration: 0.01

Cooperativity constants: 1

The total concentration of G-protein ($G_T$) and receptor ($R_T$) were 1 and 0.1 respectively (i.e. $G_T \gg R_T$). In all cases the inhibition curves were monophasic with Hill slopes of unity.

The simulated data were analysed by non-linear least squares regression as described in Chapter 2. Data were analysed by fitting to a one-site model for the estimation of the apparent ligand dissociation constant.
When \([G].K_G>1\) (G-protein coupling favoured), the magnitude of the increase in agonist affinity induced by the allosteric ligand was similar to the value of \(\delta\). The effect of \(\delta\) on the binding of an agonist to the uncoupled receptor was dependent on the value of \([G].K_G\). When \([G].K_G < 1\) (G-protein uncoupling favoured) and the agonist dissociation constant approached its lower limit of \(\frac{1+[PD]K_{PD}}{K_A(1+\beta[P]K_{PD})}\), smaller effects of the allosteric ligand were observed. By analogy with the ternary complex model, an allosteric ligand with a \(\delta\) value greater than one acts by increasing the efficacy of an orthosteric ligand, and the effects of ligand binding are dependent on the G-protein coupled state of the receptor.

The effect of an allosteric ligand with \(\gamma > 1\) was analogous to an increase in \(K_G\) in the ternary complex model. Hence, when the receptor was fully coupled to G-protein (\([G].K_G >1\)) and the apparent dissociation constant approached its lower limit of \(\frac{1+\gamma[P]K_{PD}}{\alpha.K_A(1+\beta.\gamma.\delta[P]K_{PD})}\), no effect of the allosteric ligand is observed. In contrast, as the apparent agonist dissociation constant approached, but did not reach, its upper limit of \(\frac{1+[PD]K_{PD}}{K_A(1+\beta[P]K_{PD})}\), \([G].K_G <1\), G-protein uncoupling favoured, an increase in the apparent affinity of the agonist was observed. Hence, allosteric ligands with a value of \(\gamma >1\) will increase the apparent affinity of an agonist in a manner which is dependent on the G-protein coupled state of the receptor. Under certain conditions, the increase in agonist affinity will be observed at the G-protein uncoupled state, but not at the G-protein coupled state.

A further requirement for this prediction is for a residual amount of receptor-G-protein coupling under conditions where G-protein coupling is unfavoured.
5.3.2.2. Function

The mid-point curve location for an agonist concentration-response curve, when \( R_T << G_T \) is described by the QCM as

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with the limiting values of \( 1 + \alpha[G]K_G + [PD]K_{PD} \) and \( \frac{1 + \gamma[PD]K_{PD}}{\alpha K_A(1 + \beta[PD]K_{PD})} \) (Appendix 2 and Fig. 10). As for the TCM, when \( R_T > G_T \), the EC_{50} is an underestimate of

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but would still be expected to lie between the limiting values.

The predictions of the QCM for the effects of an allosteric modulator on the functional properties of a receptor are presented in Fig. 5.12. Fig. 5.12a demonstrates that for an allosteric ligand with \( \beta > 1 \), i.e. a 'classical' allosteric enhancer of agonist affinity, an increase in agonist potency is observed, with no change in the basal response (in the absence of agonist) or the maximum response of the agonist.

For allosteric ligands where \( \delta > 1 \), an increase in the potency of full agonists, and an increase in both the potency and the maximum response of partial agonists is observed. However, allosteric ligands of this nature do not modulate the basal functional response (Fig. 5.12b).

The basal level of receptor activity is defined in the QCM as

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(Appendix 2 and Fig. 10). From this
Figure 5.12. Simulation of the effects of an allosteric ligand using the Quaternary Complex Model: Function.

The simulations were performed as described in Chapter 2 using the equations derived in Appendix 2. The following model parameters were used:

Agonist affinity ($K_A) = 1 \times 10^6 \text{ M}^{-1}$

Agonist cooperativity constant ($\alpha$): 1000 (high efficacy, curves 1, 2 and 3)
30 (low efficacy, curves 4, 5 and 6)

Allosteric ligand affinity ($K_{PD}$): 1

Concentration of PD (relative to $K_{PD}$): 0 (curves 1 and 4)
1 (curves 2 and 5)
10 (curves 3 and 6).

The following values for the cooperativity constants $\beta$, $\gamma$ and $\delta$ were used:

Panel a: $\beta=10$, $\gamma=1$, $\delta=1$

Panel b: $\beta=1$, $\gamma=1$, $\delta=10$

Panel c: $\beta=1$, $\gamma=10$, $\delta=1$

The concentration of $G_T$ and $R_T$ were 1 and 10 respectively and $K_G$ was given a value of $1 \times 10^{-3}$. Using these values, the simulated basal response was close to zero, but was able to be increased when by the allosteric ligand when $\gamma > 1$ (panel c). The simulated data were analysed by non-linear least squares regression as described in Chapter 2, by fitting to a sigmoidal dose-response curve for the estimation of agonist potency and maximum response. In all cases the curves were monophasic with Hill slopes of unity.
expression it can be seen that allosteric ligands with a value of γ > 1 will increase basal activity and therefore will possess intrinsic efficacy. Furthermore, the effect of an allosteric ligand of this type on orthosteric agonists will be an increase in the potency of full agonists, and an increase in the maximum response of partial agonists.

In summary, the QCM describes the full interaction between an orthosteric and an allosteric ligand at a G-protein coupled receptor. An allosteric ligand with a value of the cooperativity constant γ >1 will behave as an allosteric agonist. This property alone is capable of describing the binding and functional properties of the PD at the adenosine A_1 receptor.

5.4 Discussion

The effects of the allosteric enhancer PD 81,723 on the binding and functional properties of the adenosine A_1 receptor were studied in order to investigate the mechanism of action of this compound. The aim of these studies was to attempt to incorporate the observed effects of PD within a model of drug-receptor interaction.

The classical allosteric ternary complex model is the simplest model which describes the interaction of an orthosteric and allosteric ligand with a protein (Fig. 5.7). The model postulates that an allosteric ligand modifies the affinity of an orthosteric ligand through a cooperative binding interaction and data from muscarinic acetylcholine (Lazareno & Birdsall, 1995) and α_2-adrenergic receptors (Leppik & Birdsall, 2000) are consistent with the predictions of this model. The
interaction between PD and four high efficacy agonists at the G-protein uncoupled state of the adenosine A₁ receptor has also been demonstrated to be consistent with the allosteric ternary complex model (Birdsall et al., 1994). Thus, PD demonstrates 5-10 fold positive cooperativity with either NECA, CHA, R-PIA or adenosine. This study has extended this work by examining the effects of PD on agonists of lower efficacy at two receptor expression levels.

Whilst PD increased the apparent affinity of agonists for the G-protein uncoupled receptor, there was little effect of PD on high affinity agonist binding in the absence of guanine nucleotides. The positive cooperative effects of PD on agonist binding therefore appeared to be sensitive to the G-protein coupled state of the A₁ receptor. This result is in contrast to the effects of 'classical' allosteric enhancers, such as analogues of brucine which bind to muscarinic receptors and enhance the affinity of acetylcholine in the absence or presence of GTP (Lazareno et al., 1999).

As the allosteric ternary complex model does not describe the interaction between receptor and G-protein, this model was rejected in favour of a ternary complex model (TCM, De Lean et al., 1980) which describes the cooperative effects of agonists and G-proteins at a receptor.

The binding and functional predictions of the TCM have been described in Chapters 3 and 4 respectively. The model predicts the existence of high and low affinity states of a receptor which represent G-protein coupled and uncoupled agonist-receptor complexes, respectively. High affinity agonist binding is predicted at high values of \([G].K_G\), whilst the actions of guanine nucleotides on agonist binding can be mimicked by assuming that guanine nucleotides decrease \([G].K_G\). As \(K_A\) contributes equally to the observed agonist affinity for both G-
protein coupled and uncoupled states of the receptor, an increase in agonist affinity for the uncoupled receptor will result in an increase of identical magnitude at the coupled receptor (Fig. 5.8). The TCM was therefore unable to predict the experimental data if PD was postulated to act as an enhancer of $K_A$.

Altering the values of either $\alpha$ or $K_G$ in the TCM predicted changes in agonist binding which were dependent on the G-protein coupled state of the receptor. However, if PD was postulated to increase agonist efficacy by a positive cooperative effect on $\alpha$, the model was also unable to predict the actions of PD. In this case, PD is predicted to enhance agonist binding under conditions where significant G-protein coupling is observed and have smaller effects when G-protein coupling is reduced (Fig. 5.8).

Fig. 5.8 demonstrates that the TCM is able to predict the equilibrium binding properties of PD if it is postulated that PD binds to its allosteric site on the $A_1$ receptor and increase the affinity for the receptor for its cognate G-protein i.e. PD behaves as an allosteric agonist. In order to understand the nature of the cooperativity of PD, the TCM was expanded to incorporate the allosteric ternary complex model, which allowed all of the components in the two models to be represented. The quaternary complex model (QCM) is the simplest model which describes the interactions of receptor, G-protein, orthosteric and allosteric ligands.

In addition to the cooperativity constant for the binding of PD and orthosteric agonists at the G-protein uncoupled state of the $A_1$ receptor ($\beta$), there are two other cooperativity constants which relate to the mechanism of action of PD: $\gamma$, which describes the ability of PD to modify the affinity of the agonist-unoccupied
receptor for the G-protein; and \( \delta \), which describes the change in cooperativity between PD and orthosteric agonist at the G-protein coupled receptor relative to what is observed at the uncoupled receptor. Both \( \gamma \) and \( \delta \) can contribute to the G-protein coupling properties of the \( \mathrm{A_1} \) receptor. \( \gamma \) can be considered as the cooperativity constant which determines the extent of agonism of PD, and \( \delta \) is the cooperativity term which describes how PD modulates the efficacy of the orthosteric agonist.

A value of \( \gamma > 1 \) in the QCM is equivalent to increasing the value of \( K_G \) in the TCM where \( R \) is replaced by \( R \cdot \text{PD} \). When \( \gamma \) is given a value of 10, all of the effects of PD on agonist binding can be predicted by the model without requiring cooperativity between the binding of any of the other components of the scheme. Hence, PD can increase the apparent affinity of high efficacy agonists for the G-protein uncoupled \( \mathrm{A_1} \) receptor exclusively through its actions as an agonist at the allosteric site, and without the need to invoke a direct positive cooperative action with the binding of the orthosteric ligand.

A requirement for the QCM to predict the binding properties of PD is a high degree of receptor-G-protein coupling in the absence of agonist and guanine nucleotides. This is to allow the apparent dissociation constant to approach its lower limit of \( 1/\alpha.K_A \). In the simulations presented, a value of \([G].K_G\) of 10 was used, at which 90% of receptors are precoupled to G-protein in the absence of agonist, and at which the effects of PD are likely to be beyond the detection limits of the experimental system i.e. less than two-fold. However, if \([G].K_G\) is given a value of 1 (50% precoupling), PD is still predicted to increase the apparent agonist
affinity for the G-protein coupled receptor by less than three fold, and less than that observed at the G-protein uncoupled state of the receptor. The assumption of \([G].K_G > 1\) in the absence of guanine nucleotides is supported by the data presented in Chapter 3. The simulations presented in Fig. 3.3 demonstrate that when \(K_G = 0.1\), the predicted data are poorly described by two-site binding models, and that both the apparent agonist affinity for the coupled \((K_H)\) and uncoupled \((K_L)\) receptor may vary with receptor density by two - three fold. This is not predicted when \(K_G\) is given values of 1 - 10. Furthermore, the respective values for \(K_L\) and \(K_H\) for CHA in high and low expressing membranes, determined in \[^3\text{H}]\text{DPCPX}\) competition experiments in the absence of guanine nucleotides, vary by less than two-fold (Table 3.1).

There is considerable evidence for the pre-coupling of adenosine A₁, and other 7TM receptors to G-proteins in the absence of agonist. Stiles (1988) postulated that adenosine receptors from rat cerebral cortex are ‘intimately associated’ with G-proteins even in the absence of an agonist, whilst Leung et al. (1990) found for A₁ receptors in bovine brain that a ‘sizeable percentage’ of receptors were coupled to G-proteins in the absence of agonist. Analysis of radioligand binding data with the TCM indicated that 40-50% of the dopamine D₂ receptor (Wreggett & De Lean, 1984) and 33% of the α₂A adrenergic receptor (Neubig et al., 1988) were precoupled to G-protein in the absence of agonist. Furthermore, purification of GPCRs, even using antagonist affinity columns, or antagonist elution from agonist affinity columns, frequently isolate receptor/G-protein complexes rather than the free receptor alone (Matsui et al., 1985; Senogles et al., 1985).
In these studies the effects of PD on agonist binding to the G-protein coupled receptor, whether measured directly with $[{^3}H]CHA$, or indirectly in $[{^3}H]CHA$-agonist competition experiments were extremely small. Small effects of this magnitude (7% increase in $[{^3}H]CHA$ binding, $<1.5$ fold increase in apparent affinity), for an allosteric enhancer where $\gamma>1$, are only predicted by the QCM when the apparent agonist dissociation constant ($K_h$) approaches its lower limit of $1/\alpha K_A$ (see section 5.3.2.1). Consistent with this prediction is the suggestion from the ligand binding studies presented in Chapter 3 (sections 3.2.1 - 3.2.3) that the value of $K_G$ is in the range of $1 - 10$. Furthermore, when $K_G$ was decreased by the addition of GTP, it became possible to observe a progressive increase in the % enhancement of $[{^3}H]CHA$ binding by PD (Fig. 5.4).

Interestingly, other laboratories are able to demonstrate effects of PD on high affinity $[{^3}H]$-agonist binding in the absence of guanine nucleotides of a greater magnitude than presented in this thesis (Bruns & Fergus, 1990; Kourounakis et al., 2000). These studies were performed in the absence of divalent metal cations, and the reported agonist affinity constants were generally found to be lower than those presented here. The experiments described in this thesis were performed in the presence of 10mM Mg$^{2+}$, which is known to facilitate receptor/G-protein coupling by increasing $K_G$, and thus promotes high affinity agonist binding (Williams et al., 1978). Based on these observations, and the predictions of the QCM, it would be expected that the effect of PD on the apparent affinity of agonists for the $A_1$ receptor in the presence of guanine nucleotides, would decrease as [Mg$^{2+}$] was increased. This prediction has not yet been tested.
A further requirement for the QCM to predict the binding properties of PD is for a residual amount of G-protein coupling in the presence of guanine nucleotides. In the simulations used in this study, the presence of nucleotides was simulated by using a 10,000 fold lower value of $K_q$ of $1 \times 10^{-3}$ ($[G]K_g = 1 \times 10^{-3}$), which would indicate that 0.1% of receptors are coupled to G-proteins in the presence of guanine nucleotides. This implies that in order to observe an effect of PD on agonist affinity for the G-protein 'uncoupled' $A_1$ receptor in the presence of guanine nucleotides, the apparent affinity of an agonist is not identical to, but may be experimentally indistinguishable from, the $K_A$ of the agonist for the receptor. In these simulations, a value of $K_g$ of $1 \times 10^{-3}$ is sufficient to generate this condition.

$K_G$ is the constant describing the affinity of $R$ for $G$ in the absence of agonist and guanine nucleotide although guanine nucleotides are not explicitly stated in QCM. However, the binding of guanine nucleotides to G-proteins would be expected to be a negative cooperative interaction with respect to the binding of $R$, and would therefore decrease $K_G$ by a factor that will approach, but not exceed, the magnitude of its cooperativity constant. Hence, it is predicted that there will be a lower limit to the affinity of receptor for G-protein in the presence of guanine nucleotides which can never have a value of zero.

A number of experimental predictions of the QCM can be made which are based on this requirement of residual G-protein coupling in the presence of guanine nucleotides. Firstly, the effects of PD on low affinity agonist binding should be abolished following treatments with agents that are likely to decrease $K_G$ further than that observed with GDP, such as pertussis toxin. Adenosine $A_1$ receptors...
which have had the threonine residue in position 277 (TM7) mutated to alanine, are without effect on forskolin stimulated cAMP at high concentrations of agonist, and agonist inhibition of radiolabelled antagonist binding is of low affinity and insensitive to GTP (Townsend-Nicholson & Schofield, 1994). This suggests that the receptor is capable of binding agonists, but is not able to couple to G-proteins. Interestingly, PD is without effect on agonist binding in this mutant (Kourounakis et al., 2001), which supports the requirement of residual G-protein coupling in order to observe the effects of PD on agonist binding. In contrast, in wild type A1 receptors, an effect of PD on agonist binding is still observed in the presence of suramin and N-ethylmaleimide, which would be expected to have profound effects on the ability of the A1 receptor to couple to G-protein (Kourounakis et al., 2000). An additional but as yet untested prediction of the requirement for residual G-protein coupling is that the magnitude of the PD shift may decrease as the concentration of guanine nucleotides is increased.

In addition to increasing the binding of radiolabelled agonist, PD also decreased the potency of guanine nucleotides to inhibit the binding of [3H]CHA to the adenosine A1 receptor (Fig. 5.4). This observation indicates negative cooperativity between the binding of PD and guanine nucleotides, consistent with PD being an allosteric agonist. Similar observations in previous studies have led several authors to suggest that PD mediates its effects through 'the stabilisation of receptor/G-protein interactions' (Bhattacharya & Linden, 1995; Kollias-Baker et al., 1994). The QCM supports this hypothesis and provides a molecular basis for this action, i.e. positive cooperativity between the binding of PD and G-protein, or co-agonism.
The observation that PD increases the affinity of agonists for the low affinity state of the receptor in the absence of guanine nucleotides (Fig. 5.3) is more difficult to reconcile with PD acting as an allosteric agonist. Within the context of both the TCM and the QCM, low affinity agonist binding in the absence of guanine nucleotides results from an excess of receptors ($R_T \gg G_T$), which are unable to couple to G-protein. Under this condition, an increase in the affinity of the receptor for the G-protein by PD ($\gamma=1$) will have no effect on the apparent agonist affinity for the uncoupled receptor (Fig. 5.8 and 5.11) and can only be explained by a positive cooperative effect on agonist binding ($\beta>1$). As discussed above, this can not occur without equivalent effects at the coupled receptor. One possible explanation for this discrepancy is that low affinity agonist binding is not a result of surplus receptor, but represents a dynamic component of the system. That is, the uncoupled receptor spends a proportion of its time coupled to the G-protein during the time scale of the experiment. Data in Chapter 3 suggests this is the case as both increasing concentrations of GDP, and agonists of varying efficacy appear to modulate the proportion of receptors in the G-protein coupled and uncoupled states.

A feature of the simple, allosteric ternary complex model is that the magnitude and direction of allosteric cooperativity is unique for each allosteric-orthosteric ligand pair, and independent of the ligand affinity or efficacy (Lazareno & Birdsall, 1995). In contrast, the QCM predicts that for an allosteric enhancer of G-protein binding, a correlation will exist between ligand efficacy and the magnitude of the increase in apparent affinity for the uncoupled receptor induced by PD (Fig. 5.13). The QCM therefore provides a mechanism for linking the intrinsic efficacy
Figure 5.13. Further predictions of the Quaternary Complex Model: Simulation of the effect of a co-agonist on the apparent affinity, for the G-protein uncoupled receptor, of agonists of varying efficacy.

The simulations were performed as described in Chapter 2 using the equations derived in Appendix 2. The following model parameters were used:

- Agonist affinity ($K_A$): $1 \times 10^6 \text{ M}^{-1}$
- Agonist cooperativity constant ($\alpha$): 100 (panel a) 300 (panel b) 1000 (panel c)
- PD affinity ($K_{PD}$): 1
- Concentration of PD (relative to $K_{PD}$): 0 (solid symbols) 10 (open symbols)
- PD cooperativity constant ($\gamma$): 10

The binding of the unlabelled orthosteric ligand to the G-protein uncoupled receptor was simulated by setting the value of $K_G$ to $1 \times 10^{-5}$. At this value of $K_G$ the apparent affinity constant of the orthosteric ligand for the uncoupled receptor was a close approximation to $K_A$. The cooperativity constants $\beta$ and $\delta$ were given values of 1.

The radioligand was assumed to be a silent antagonist and present at a concentration (relative to its affinity) at which the Cheng-Prusoff correction was negligible. The model parameters used for the radioligand were as follows:

- Radioligand affinity: 1
- Radioligand concentration: 0.01
- Cooperativity constants: 1

The total concentration of G-protein ($G_T$) and receptor ($R_T$) were 1 and 0.1 respectively (i.e. $G_T >> R_T$). In all cases the inhibition curves were monophasic with Hill slopes of unity.

The simulated data were analysed by non-linear least squares regression as described in Chapter 2. Data were analysed by fitting to a one-site model for the estimation of the apparent ligand dissociation constant.

Panel d: The relationship between agonist efficacy ($\alpha$) and the increase in the apparent orthosteric agonist affinity induced by the co-agonist (PD shift). The relationship becomes curved at high values of $\alpha$ as the PD shift approaches a maximum defined by the cooperativity constant $\gamma$. 

Radioligand occupancy

log PD-shift

Radioligand occupancy
of PD with the increases in the affinity of high efficacy agonists, and the increase in intrinsic activity of partial agonists.

This study used a number of ligands with relative efficacies (as determined by their $K_L/K_H$ ratios) of the range <1 (DPCPX), 1-3 (GR162900), 10 (GR161144), 100 (GR190178) and 1000-3000 (CHA, NECA). The increase in affinity of these ligands for the uncoupled receptor, in the presence of PD (determined by the ratio $K_{L-PD}/K_{L+PD}$) can be separated into three groups (CHA, NECA: ~10; GR162900, GR161144 GR190178: ~1; DPCPX: <1; Table 5.1, Fig. 5.2), which suggests a possible association between efficacy and PD shift. Interestingly, the QCM predicts that the range of efficacies of ligands used in this study, although large, is probably not sufficient to demonstrate a correlation between efficacy and PD shift (Fig. 5.13). The model predicts that for a value of $[G].K_G$ of $1 \times 10^3$, PD will induce a five-fold increase in agonist affinity when $\alpha=1000$ (high efficacy agonist), but less than a three-fold increase in affinity when $\alpha=100$. Hence the effect of PD, on the apparent affinity for the G-protein uncoupled $A_1$ receptor, on agonists with $K_L/K_H$ values $\leq 100$ may be difficult to detect experimentally. Furthermore, the model predicts that agonists with intermediate efficacy between CHA and GR190178, and agonists with 10-fold greater efficacy than CHA will be required to test this hypothesis (Fig. 5.13).

Parallels can be drawn between the PD occupied adenosine $A_1$ receptor and the binding and functional properties of constitutively active, mutant GPCRs (CAMs). CAMs have been extensively studied for a number of 7TM receptors and the general properties of CAMs are an increase in agonist affinity and potency, and an increase in functional activity in the absence of agonists (Lefkowitz et al., 1993).
It is therefore tempting to speculate that the conformation of the adenosine A₁ receptor, when occupied by PD in the allosteric binding site, is similar to a constitutively active A₁ receptor. It will be interesting to investigate the effects of PD on CAM A₁ receptors when these mutant receptors become available.

In the [³⁵S]GTPγS functional assay, PD increased the basal level of [³⁵S]GTPγS. The extent of the response was relatively small when compared to the levels of response to PD observed in the inhibition of forskolin stimulated cAMP, another measure of the function of the adenosine A₁ receptor (Bhattacharya & Linden, 1995; Bruns & Fergus, 1990; Kollias-Baker et al., 1997; Musser et al., 1999). These studies reported a response to PD of 50-75% of the response to full agonists. This magnitude of these responses to PD may be due to the inherent sensitivity of the adenylate cyclase system to agonists of Gαi linked 7TM receptors. It may also be explained by a direct effect of PD on adenylate cyclase, as PD has been shown to inhibit cAMP in non-transfected CHO cells and to inhibit [³H]forskolin binding to rat liver membranes (Musser et al., 1999). The lack of effect of PD on [³⁵S]GTPγS binding to low-expressing A₁ membranes indicates that the increase in basal [³⁵S]GTPγS binding in high expressing membranes is mediated by the adenosine A₁ receptor.

The increase in basal [³⁵S]GTPγS binding in the presence of PD is consistent with a co-agonist action of PD and is predicted by the QCM. However, an alternative explanation for the effects of PD on basal [³⁵S]GTPγS binding is an enhancement of the actions of endogenous adenosine. The ubiquitous presence of endogenous adenosine has the potential to confound the interpretation of pharmacological studies involving adenosine receptors and requires the presence of adenosine...
deaminase to remove potential binding and functional artefacts (Linden, 1989). The effect of PD on basal $[^{35}\text{S}]\text{GTPyS}$ binding in CHO A$_1$ high expressing membranes persisted at concentrations of ADA 10-100 fold higher than that required to degrade endogenous adenosine (Fig. 5.6). This data would suggest that if endogenous adenosine is responsible for the intrinsic efficacy of PD, it is present in cryptic pools which are inaccessible to ADA (Prater et al., 1992). Furthermore, given the lack of effect of PD in low expressing membranes, the concentration of endogenous adenosine is likely to be at the threshold of the adenosine concentration-response curve in high expressing membranes.

PD increased the maximum $[^{35}\text{S}]\text{GTPyS}$ binding response to partial agonists in CHO A$_1$ low expressing receptors, and the maximum response to low efficacy agonists of the inhibitory component of $[^{35}\text{S}]\text{GTPyS}$ binding in CHO A$_1$ high expressing membranes. As demonstrated in the simulations of Fig. 5.12, this effect is not consistent with an enhancement of either the affinity of agonists for the uncoupled A$_1$ receptor, nor an increase in the ability of agonists to stimulate G-protein binding, but can be explained by the co-agonist effects of PD at the allosteric site.

The concept of receptor activation via different binding sites is well described for the pharmacology of the ionotropic glutamate receptor. This ligand-gated ion channel is modulated by a number of molecules, including Zn$^{2+}$, H$, \text{Mg}^{2+}$ ions and glycine, each binding to a unique site (Corsi et al., 1996). Radioligand binding studies have demonstrated weak positive cooperativity for the binding of glycine and glutamate to NMDA receptors in rat brain synaptic membranes (Fadda et al., 1988; Kessler et al., 1989) whilst the absolute requirement of the presence
of glycine for a functional response to glutamate (Johnson & Ascher, 1987) has led to the use of the term co-agonism to describe this interaction.

Conformational changes in response to glutamate and glycine can be measured experimentally by the binding of \(^{3}\text{H}\)MK-801, which binds to the open state of the ion channel in a use-dependent manner, and can be considered analogous to the interaction of a 7TM receptor with its cognate G-protein. A model has been proposed which is analogous to the QCM, and describes the cooperative interactions between glutamate, glycine and MK-801 at the NMDA receptor in rat brain (Marvizon & Baudry, 1993). This study concluded that whilst a small degree of positive cooperativity is required between the binding of glutamate and glycine, the action of glutamate and glycine to cooperate at the level of the conformational change that produces channel opening is far more relevant.

The effects of glutamate and glycine at the NMDA receptor supports the hypothesis that apparent cooperativity in ligand binding studies can be produced by a mechanism that is distinct from a classical orthosteric-allosteric cooperative interaction. The NMDA receptor differs from the adenosine A\(_1\) receptor in that both glycine and glutamate are very low efficacy agonists alone, but demonstrate marked increases in efficacy when applied together. In contrast, at the A\(_1\) receptor, CHA is a high efficacy agonist and PD demonstrates measurable, but very low, efficacy. The QCM predicts that the mechanism underlying co-agonism for both the NMDA receptor and the A\(_1\) receptor are identical, and that the quantitative differences between the two systems arise from differences in the magnitude of the cooperativity constants.
Studies of the effects of allosteric ligands at 7TM receptors have been largely restricted to consideration of the nature of cooperativity between the binding of allosteric and orthosteric ligands. Few studies suggest a functional receptor-modulating role for allosteric ligands. At the α₁-adrenergic receptor, benzodiazepines have been demonstrated to increase the maximum functional response to the partial agonist clonidine, and have little effect on the responses of the full agonist phenylephrine, which have been claimed to be mediated by an allosteric binding effect (Waugh et al., 1999). As classical allosteric ligands are not predicted to alter the maximum response of partial agonists, a model of co-agonism may be more appropriate to describe the interactions between adrenergic agonists and benzodiazepines.

Several groups have described the mechanism of action of PD using cubic variants of the allosteric two state model of receptor activation (Fig. 5.14). These models postulate that receptors exist in an equilibrium between R and R*, and that agonists and allosteric enhancers have a higher affinity for R* than for R. Agonists can activate G-proteins either by increasing the affinity of R for G, or by altering the equilibrium between R and R* in favour of the R* conformation. Bruns and Fergus (1990, Fig. 5.14a) have proposed that PD increases the proportion of receptors in the R* conformation. The predictions of this model have been examined extensively and demonstrated to explain many of the pharmacological observations of PD (Hall, 2000). However, this model does not include a G-protein and will not predict biphasic agonist binding curves and could therefore be invoked should the effects of PD on agonist binding be demonstrated to persist in the absence of receptor-G-protein coupling.
Figure 5.14. Alternative models used to describe the actions of PD at the adenosine A₁ receptor.

Mathematical models of drug receptor interactions proposed by Bruns & Fergus (1990, panel A) and Kourounakis (2000, panel B). These models are identical to the cubic ternary complex model proposed by Weiss et al. (1996) and postulate the receptor to exist in an inactive (R) and active (R*) states, the relative proportions of which are governed by the isomerisation constant J. Both models are incomplete as descriptions of the behaviour of PD, as neither includes agonist, receptor, G-protein and PD together in a single scheme. The model of Bruns & Fergus does not include the presence of G-protein and will therefore not predict heterogeneous binding curves. The model of Kourounakis does not incorporate PD.
A

\[ PD \rightarrow PD.R \rightarrow A.R.PD \]

\[ R \rightarrow K_A \rightarrow AR \]

\[ PD.R^* \rightarrow A.R^*.PD \]

\[ R^* \rightarrow A.R^* \]

\[ K_{PD} \quad \gamma J \quad \beta K_{PD} \quad \alpha \gamma \delta J \]

\[ \alpha J \quad \alpha \beta \delta K_A \quad \beta \gamma \delta K_{PD} \]

B

\[ R.G \rightarrow A.R.G \]

\[ R \rightarrow K_A \rightarrow A.R \]

\[ R^*G \rightarrow A.R^*.PD \]

\[ R^* \rightarrow A.R^* \]

\[ K_G \quad \gamma J \quad \alpha K_G \quad \beta \gamma \delta J \]

\[ \gamma K_G \quad \beta J \quad \alpha \beta \delta K_A \quad \alpha \gamma \delta K_G \]

\[ \beta K_A \]
Kourounakis et al (2000, Fig. 5.14 b) also proposed that PD increases the proportion of receptors in the R* state, capable of interacting with G-protein, but did not consider the effect of PD on ligand binding.

The models proposed by Bruns and Fergus (1990), and Kourounakis et al (2000), are simplifications of more complex models, which require a number of additional cooperativity constants to describe the interactions of R and G, and R and PD, respectively. This added level of complexity invoked by postulating a two-state cubic allosteric model is at present not justified, as the QCM is the simplest scheme to include receptor, G-protein, agonist and PD and which is capable of describing the experimental data. In addition, the predictions of the QCM are experimentally verifiable, and the scheme is simple enough to potentially allow direct fitting to experimental data for estimation of the model parameters (Marvizon & Baudry, 1993).

This study has attempted to explain the mechanism of action of the allosteric modulator PD 81,723 at the adenosine A₁ receptor using measurements of ligand binding and receptor activation. The use of PD as a tool compound to test the predictions of mathematical models are restricted by its poor solubility at concentrations above 10μM, together with a relatively low affinity for the A₁ receptor of the order 1×10^5 M⁻¹ (this study and Cohen, 1995). This would suggest that at the highest concentrations used in this study PD is only occupying ~50% of the total population of receptors ([PD].K_PD=1). Furthermore, in addition to its poor solubility, the potential but unproven competitive effects of PD at the orthosteric binding site preclude its use at these higher concentrations. Further
studies to test the quantitative predictions of the QCM will be greatly aided by the availability of high affinity PD analogues with improved solubility.

In summary, this study has demonstrated that the allosteric enhancing effects of PD can be explained by PD acting as a co-agonist to increase the affinity of the $A_1$ receptor for its cognate G-protein. The binding and functional effects of PD can be accommodated within a quaternary complex model of agonism, which postulates the formation of the quaternary complex A.R.G.PD. This model links the intrinsic efficacy of PD with the effects of PD on the binding and functional properties of orthosteric agonists. Indeed, the effects of PD on orthosteric agonists are predicted to be a direct consequence of the co-agonism inherent in PD. Furthermore, the QCM has a number of predictions which are amenable to experimental verification.
Chapter 6. Summary, criticism and future directions

6.1 Summary

There were three principal aims of this research project. Firstly, the binding properties of the adenosine A₁ receptor were characterised in membranes from cells expressing a twenty-fold difference in receptor levels. The results from these binding studies were compared and contrasted with the predictions of the ternary complex model. Secondly, the functional properties of membranes from these cells were also characterised. It was clear from these studies that an inhibitory response was present which had not previously been described. A detailed pharmacological characterisation of this response was undertaken. Finally, a mathematical model of drug receptor interactions was developed to accommodate the findings from studies on the effects of PD 81,723 on the binding and functional properties of the A₁ receptor expressed at different levels.

6.2 The radioligand binding properties of the adenosine A₁ receptor.

The binding studies in this thesis investigated the effects of receptor density, guanine nucleotides and agonist efficacy on agonist affinity for the G-protein coupled and uncoupled states of the A₁ receptor, and the fraction of high affinity agonist binding sites. The results were largely consistent with the predictions of the ternary complex model. Thus, increasing receptor density altered the fraction of high affinity binding sites with no effect on agonist or antagonist affinity, whilst
guanine nucleotide decreased the affinity of agonists for the G-protein coupled state of the A1 receptor. Furthermore, the ratio of agonist affinity for the G-protein uncoupled and coupled states of the receptor were demonstrated to correlate with agonist efficacy.

The results from these binding studies did not appear to be consistent with the predictions of the ternary complex model with regards to the fraction of high affinity binding sites. The extent of the decrease in frH in high expressing membranes was not consistent with a twenty-fold increase in receptor expression unless it was postulated that an increase in the concentration of G-protein had also occurred. In addition, treatment with guanine nucleotides, or agonists of varying efficacy also demonstrated that frH was not constant as predicted by the TCM, which does not provide a mechanistic basis for frH to vary.

This study has used cell lines expressing only two different levels of receptor expression. Further understanding of the relationship between receptor density and frH could come either from studies using a range of expression levels generated using an inducible promoter or by selecting and screening a number of cell line clones. The expression of the A1 receptor fused with a fluorescent marker such as green fluorescent protein would facilitate this process as individual cells could be rapidly sorted on the basis of expression levels using a flow cytometric method.

During this project, attempts were made to visualise adenosine receptor expression in LE and HE CHO cells using confocal microscopy, which were unsuccessful due to the lack of suitable antibodies for the A1 receptor (data not shown). The aims
of these studies were to examine the regulation of the localisation of the A₁ receptor with respect to possible interactions with lipid rafts and caveolae, and to investigate whether G-protein expression varied with receptor localisation. The use of a tandem protein of the A₁ receptor and GFP would be invaluable for studies of this kind as receptor expression could be directly visualised without the need for specific antibodies (Bevan et al., 1999). The use of cell lines expressing the A₁ receptor fused with a pertussis toxin resistant G-protein, which constrains the receptor-G-protein stoichiometry, would also be valuable in understanding the relationship between Rₜ;Gₜ and fᵣₜ (Wise et al., 1999).

Radioligand dissociation studies demonstrated distinct differences between the dissociation of radiolabelled agonist ([³H]CHA) and antagonist ([³H]DPCPX). Whilst the dissociation of [³H]DPCPX was consistent with a simple, reversible bimolecular interaction, the dissociation of [³H]CHA was minimal and in HE membranes was facilitated by the presence of an excess concentration of agonist but not antagonist. The mechanisms underlying the phenomena of agonist locking and agonist induced agonist release are unclear. The experiments presented in this thesis suggest that agonist locking is dependent on both receptor expression level and the receptor-G-protein interaction. Agonist induced agonist release appears to be dependent on receptor activation and could be considered to be a functional response, rather than a simple binding site interaction. This hypothesis could be further investigated by initiating radolabelled agonist dissociation using agonists of varying efficacy. Detailed kinetic studies to determine whether the phenomenon of agonist induced agonist dissociation is generated slowly and the concentrations of agonist required to induce dissociation could be informative.
Agonist induced agonist release requires information to pass from unlabelled agonist occupied receptor to radiolabelled agonist occupied receptor, and one mechanism for this to occur is the presence of receptor dimers or higher order oligomers. This could be further investigated by co-expressing the A₁ receptor with another GPCR at high densities and investigating the ability of an agonist at one receptor to induce radiolabelled agonist dissociation from the second receptor.

It may also be possible to investigate receptor-receptor interactions in equilibrium studies using strategies similar to Armstrong & Strange (2001) in their investigations of Na⁺ regulation of antagonist binding to D₂ receptor dimers.

6.3 The functional properties of the adenosine A₁ receptor.

The functional properties of the A₁ receptor expressed at different levels were evaluated using [³⁵S]GTPγS binding. Both the basal and agonist stimulated levels of [³⁵S]GTPγS binding were consistent with an increase in the apparent concentration of G-protein in HE membranes which confirmed the findings of the radioligand binding studies. In HE membranes a novel inhibitory [³⁵S]GTPγS binding response was observed and characterised. The inhibitory response shared the same pharmacological properties as the stimulatory response, indicating that it is mediated by the A₁ receptor, and it was demonstrated that the response was dependent on agonist efficacy. The nature of the inhibitory response remains unclear although the gel-filtration and DPCPX ‘rescue’ experiments suggest that it is not due to the translocation of G-protein α subunits from the membranes.
The inhibitory $[^{35}\text{S}]\text{GTP}_{\gamma}\text{S}$ binding response was only observed in HE membranes. It would be useful to identify and characterise inhibitory $[^{35}\text{S}]\text{GTP}_{\gamma}\text{S}$ binding responses in membranes from cells expressing levels of the $A_1$ receptor lower and higher than those in HE cells, in order to establish the relationship between receptor expression and the inhibitory response. The methods suggested in section 6.2 for generating cell lines with varying levels of receptor expression would therefore be useful.

The observation that the inhibitory response only occurs in HE membranes, is greatest for high efficacy agonists, and is dependent on G-protein activation makes it tempting to draw a link between the inhibitory $[^{35}\text{S}]\text{GTP}_{\gamma}\text{S}$ binding response and agonist locking and agonist induced agonist release. The possibility therefore remains that the inhibitory response is a consequence of receptor dimerisation or oligomerisation. The continued identification of novel protein–protein interactions, such as the interactions between GPCRs and scaffold proteins, raises the possibility that the inhibitory component is a result of an interaction between the $A_1$ receptor and an as yet unidentified membrane protein. This could be further explored using yeast two-hybrid techniques which have been successful in identifying novel interactions such as the GABA$_{\beta}$R1 and GABA$_{\beta}$R2 (White et al., 1998). Potential candidates could then be overexpressed with the $A_1$ receptor to determine whether the interaction leads to a decrease in the ability of the $A_1$ receptor to stimulate $[^{35}\text{S}]\text{GTP}_{\gamma}\text{S}$ binding.
6.3 The mechanism of action of the allosteric enhancer PD 81,723.

In radioligand binding studies, PD 81,723 increases the apparent affinity of high efficacy agonists such as CHA for the G-protein uncoupled state of the receptor but has no effect at the G-protein coupled state. Furthermore, PD has no detectable effect on the binding of lower efficacy agonists for either the G-protein coupled or uncoupled state of the receptor. In functional studies PD increases the basal level of \([^{35}\text{S}]\text{GTP}_{\gamma}\text{S}\) binding in a manner which appears to be independent of endogenous adenosine. PD also increases agonist potency and the intrinsic activity of partial agonists. The simulations with the ternary complex model indicated that the binding and functional properties of PD are not consistent with a simple increase in agonist affinity, but were related to an increase in \(K_G\), the affinity of the receptor for G-protein. The development of a quaternary complex model (QCM) established that the effects of PD can be explained in terms of a single cooperativity constant, \(\gamma\), which describes the interaction between PD and G-protein. Thus, the binding and functional properties of PD can be explained by PD acting as a coagonist.

A number of predictions of the QCM have yet to be tested. The hypothesis developed in this thesis is that PD influences the binding and function of agonists at the primary binding site by an allosteric agonist action. The QCM predicts that the effects of PD on agonist binding to the uncoupled receptor requires a residual level of G-protein coupling in the presence of guanine nucleotides. Whether the effect of PD only requires a conformational change in the receptor consistent with
receptor activation, or that the binding of G-protein to activated receptor is required, remains to be established. Experiments using membranes from pertussis toxin treated cells, or mutant A1 receptors which bind agonists with low affinity and fail to functionally couple to G-proteins may help to address this issue. Experiments using purified A1 receptor expressed in membranes in the absence of G-proteins should definitively establish whether the actions of PD require the presence of G-protein.

One area which has not been addressed in this thesis is how models such as the quaternary complex model can be used to quantify parameters relating to the action of allosteric agonists, such as the affinity for the allosteric site, and some of the key cooperativity constants. This is important if a rational approach is to be used to identify novel allosteric ligands with improved affinity and 'efficacy'. PD itself is of limited use because of its low affinity and poor solubility. The identification of compounds with high affinity for the allosteric site will open the possibility of developing a radioligand for the allosteric binding site, as has been achieved for the muscarinic receptor (Trankle et al., 1999), which would greatly enhance our understanding of the allosteric regulation of adenosine receptors.

Little is known regarding the location of the allosteric binding site on the A1 receptor. Studies using chimaeric A1/A2A receptors indicate that the allosteric interaction may involve residues in TM7 and the C-terminus (Cohen, 1995). Site directed mutagenesis of the A1 receptor will facilitate in the identification of the allosteric binding site. Towards the end of this research project, a preliminary characterisation of a number of point mutants was performed. Two mutations (H251N, H184A) appeared to have the same effect as PD. They enhanced agonist
binding to the G-protein uncoupled receptor, increased basal $[^{35}\text{S}]\text{GTP} \gamma \text{S}$ binding and increased agonist potency and the intrinsic activity of partial agonists (data not shown). Further work with these mutant receptors may provide information regarding the location of the allosteric binding site, and the mechanism of action of PD.

The data presented in this thesis should lead to an increased understanding of receptor G-protein interactions and the utility of models of drug-receptor interactions for predicting and quantifying drug actions. This thesis has also clarified and progressed our understanding of how allosteric ligands such as PD differ from ‘classical’ allosteric ligands such as those acting at muscarinic cholinergic receptors, which appear to act solely by increasing agonist affinity. This knowledge may help in the identification of novel compounds which may be useful in treating diseases associated with neurotransmitter deficits, such as Parkinson’s and Alzheimer’s disease, together with diseases associated with oxygen deprivation such as myocardial ischaemia, stroke and seizures.
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**Abstracts arising from this thesis**


Appendix I: Derivation of the equations of the Ternary Complex Model.

The ternary complex model, as presented in figure 5.7, describes the allostERIC interaction between a ligand A and G-protein G, at receptor R. $K_A$ and $K_G$ are affinity constants describing the binding of A and G to unoccupied R, respectively, and $\alpha$ is the cooperativity constant. For clarity only the derivation for a single ligand is presented. The TCM simulations presented in Chapters 3 and 5 were generated using a model of two competitive orthosteric ligands, the derivation of which utilises exactly the same principles.

Expressions for the concentration of each of the free species are as follows:

$[AR] = [A].[R_f].K_A$

$[RG] = [G_f].[R_f].K_G$

$[ARG] = [A].[R_f].K_A.\alpha. [G_f].K_G$

where $[A]$, $[G_f]$ and $[R_f]$ are the free concentrations of ligand, G-protein and receptor respectively.

The total concentration of bound ligand is $[AR] + [ARG]$

The total receptor concentration $(R_T)$ is $[R_f] + [AR] + [RG] + [ARG]$

**Ligand binding**

The total concentration of bound ligand is $[AR] + [ARG]$ and relative receptor occupancy is given by the following expression:

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substituting the expressions for each of the free species gives:

\[
\frac{\text{Bound}}{R_T} = \frac{[\text{A}].K_A.(1 + \alpha.[G_F].K_G)}{1+[G_F].K_G + [\text{A}].K_A.(1 + \alpha.[G_F].K_G)}
\]  \hspace{1cm} \text{Equation 1}

When \([G_F] = \text{zero}\), occupancy is described by the following familiar binding isotherm:

\[
\frac{\text{Bound}}{R_T} = \frac{[\text{A}].K_A}{1 + [\text{A}].K_A}
\]

The apparent dissociation constant is:

\[
K_{\text{app}} = \frac{1+[G_F].K_G}{K_A.(1 + \alpha.[G_F].K_G)}
\]

\(K_{\text{app}}\) has the limiting values of \(\frac{1}{K_A}\) when \([G_F]\) approaches zero, and \(\frac{1}{\alpha.K_A}\) when \([G_F]\) approaches infinity.

**Receptor function**

Receptor function is assumed to be directly proportional to the total concentration of RG species ([RG]+[ARG]) and is given by the following expression:

\[
\frac{[\text{RG}]+[\text{ARG}]}{R_T} = \frac{[\text{RG}]+[\text{ARG}]}{[R_F]+[\text{AR}]+[\text{RG}]+[\text{ARG}]}
\]

Substituting the expressions for each of the free species gives
\[ [\text{RG}] + [\text{ARG}] = \frac{R_T \cdot [G_F] \cdot [K_G](1 + \alpha [A] \cdot K_A)}{1 + [A] \cdot K_A + [G_F] \cdot [K_G](1 + \alpha [A] \cdot K_A)} \]  
Equation 2

the EC\textsubscript{50} is given by the following expression:

\[ \text{EC}_{50} = \frac{1 + [G_F] \cdot K_G}{K_A \cdot (1 + \alpha [G_F] \cdot K_G)} \]

the EC\textsubscript{50} has identical limiting values to those described above for the apparent ligand binding dissociation constant.

In the absence of agonist ([A] = 0), basal receptor activity is given by the term

\[ \frac{[G_F] \cdot K_G}{1 + [G_F] \cdot K_G} \]

and the maximum agonist response, which occurs as [A] approaches infinity, is given by the term

\[ \frac{R_T \cdot \alpha [G_F] \cdot K_G}{1 + \alpha [G_F] \cdot K_G} \]

The above expressions are appropriate when the free concentration of G-protein ([G\textsubscript{F}]) approximates to the total concentration of G-protein ([G\textsubscript{T}]), which occurs when [G\textsubscript{T}] >> [R\textsubscript{T}]. When the concentrations of G\textsubscript{T} and R\textsubscript{T} are similar, or when [G\textsubscript{T}] < [R\textsubscript{T}], the free concentration of G-protein is not constant, but will vary with different concentrations of A and R. The mass of G must therefore be conserved as follows:

\[ [G_T] = [G_F] + [\text{RG}] + [\text{ARG}] \]

\[ [G_F] = \frac{G_T}{1 + [R_F] \cdot K_G \cdot (1 + \alpha [A] \cdot K_A)} \]

let \( y = K_G \cdot (1 + \alpha [A] \cdot K_A) \)

\[ [G_F] = \frac{G_T}{1 + [R_F] \cdot y} \]
This value of \([G_P]\) can now be used to find the corresponding value of \([R_F]\) with respect to \([R_T]\) and \([G_T]\):

\[
R_T = [R_F] + [AR] + [RG] + [ARG]
\]

\[
\]

Let \(x = 1 + [A].K_A\)

\[
R_T = [R_F].(x + y[.G_P])
\]

Substituting the expression for \([G_P]\) gives:

\[
R_T = [R_F].(x + \frac{[G_T]}{1+[R_F].y}).y
\]

\[
[R_F].x + [R_F].\frac{[G_T]}{1+[R_F].y}.y - [R_T] = 0
\]

\[
(1+[R_F].y)([R_F].x - [R_T]) + [R_F].[G_T].y = 0
\]

\[
[R_F]^2.y.x + (x - y([R_T] - [G_T])).y - [R_T] = 0
\]

If \(x.y = a, x - y([R_T] - [G_T]) = b\) and \(-[R_T] = c\)

\[
[R_F] = \frac{-b + \sqrt{b^2 + 4.a.[R_T]}}{2.a}
\]

this value of \([R_F]\) can now be substituted to find the value of \([G_P]\) in terms of \([R_T]\) and \([G_T]\)

\[
[G_P] = \frac{[G_T]}{1 + \frac{-b + \sqrt{b^2 + 4.a.[R_T]}}{2.a}.y}
\]
The theoretical values of \([R_F]\) and \([G_F]\) can now be determined for any value of \([A]\) and substituted into equations 1 and 2 to determine the predicted values for receptor binding and function respectively.
Appendix II: Derivation of the equations of the Quaternary Complex Model.

The quaternary complex model, as presented in figure 5.10, describes the allosteric interaction between an orthosteric ligand A, allosteric ligand PD, and G-protein G, at receptor R. $K_A, K_{PD}$ and $K_G$ are affinity constants describing the binding of A, PD and G to unoccupied R, respectively. $\alpha, \beta$ and $\gamma$ are the cooperativity constants for the binding between A and G, A and PD, and PD and G respectively. $\delta$ describes the change in cooperativity between PD and A at the G-protein coupled receptor relative to what is observed at the uncoupled receptor. For clarity only the derivation for a single ligand is presented. The QCM simulations presented in Chapters 3 and 5 were generated using a model of two competitive orthosteric ligands, the derivation of which utilises exactly the same principles.

Expressions for the concentration of each of the free species are as follows:

$$[AR] = [A][R_f].K_A$$

$$[PDR] = [PD][R_f].K_{PD}$$

$$[RG] = [G_f][R_f].K_G$$

$$[ARG] = \alpha.[A_f].K_A.[R_f].[G_f].K_G$$

$$[PDRG] = \gamma.[PD].K_{PD}.[R_f].[G_f].K_G$$

$$[ARPD] = \beta.[A].K_A.[PD].[R_f].K_{PD}$$

$$[APDRG] = \alpha.\beta.\gamma.\delta.[A].K_A.[R_f].[G_f].K_G.[PD].K_{PD}$$
where \([A], [PD], [G_F] \) and \([R_p]\) are the free concentrations of A, PD, G-protein and receptor respectively.

The total concentration of bound ligand is \([AR]+[ARG]+[APRD]+[APDRG]\)

The total receptor concentration \((R_T)\) is \([R]+[AR]+[PDR]+[RG]+[ARG]+[PDRG]+[ARPD]+[APDRG]\)

**Ligand binding**

The total concentration of bound ligand is \([AR]+[ARG]+[APDR]\) and relative receptor occupancy is given by the following expression:

\[
\frac{\text{Bound}}{R_T} = \frac{[AR]+[ARG]+[APDR]+[APDRG]}{[R_F]+[AR]+[RG]+[ARG]+[PDR]+[PDRG]+[ARPD]+[APDRG]}
\]

substituting the expressions for each of the free species gives:

\[
\frac{\text{Bound}}{R_T} = \frac{[A].K_A.(1+\gamma_\beta.[PD].K_{PD}+\alpha.[G_F].K_G(1+\beta_\gamma\delta.[PD].K_{PD}))-\alpha_\gamma_\delta.[PD].K_{PD})]}{1+[A].K_A.(1+\gamma_\beta.[PD].K_{PD}+\alpha.[G_F].K_G(1+\beta_\gamma\delta.[PD].K_{PD}))-\alpha_\gamma_\delta.[PD].K_{PD})]+[G_F].K_G+[PD].K_{PD}.(1+\gamma_\beta.[PD].K_{PD})]
\]

When \([PD] = 0\), occupancy is described by the expression identical for ligand binding described by the TCM in Appendix I:

\[
\frac{\text{Bound}}{R_T} = \frac{[A].K_A.(1+\alpha.[G_F].K_G)}{1+[G_F].K_G+[A].K_A.(1+\alpha.[G_F].K_G)}
\]

When \([G] = [PD] = 0\), occupancy is described by the following familiar binding isotherm:

\[
\frac{\text{Bound}}{R_T} = \frac{[A].K_A}{1+[A].K_A}
\]

The apparent ligand binding dissociation constant described by the QCM is:
Receptor function

Receptor function is assumed to be directly proportional to the total concentration of RG species \([\text{RG}_{\text{TOT}}] = [\text{RG}] + [\text{ARG}] + [\text{PDRG}] + [\text{APDRG}]\) and is given by the following expression:

\[
\frac{[\text{RG}] + [\text{ARG}] + [\text{PDRG}] + [\text{APDRG}]}{[\text{RG}] + [\text{ARG}] + [\text{PDRG}] + [\text{APDRG}]} = \frac{[\text{RG}] + [\text{ARG}] + [\text{PDRG}] + [\text{APDRG}]}{[\text{RG}] + [\text{ARG}] + [\text{PDRG}] + [\text{APDRG}]} \quad \text{RT}
\]

Substituting the expressions for each of the free species gives

\[
\text{RT} = \frac{[\text{RG}].K_{GA}.(l + \alpha [\text{A}].K_{A} + \gamma [\text{PD}].K_{PD} + \alpha \beta \gamma \delta [\text{A}].K_{A} [\text{PD}].K_{PD})}{1 + [\text{A}].K_{A} .(l + \beta [\text{PD}].K_{PD} + \alpha [\text{RG}].K_{G} (l + \beta \gamma \delta [\text{PD}].K_{PD}) + [\text{RG}].K_{G} + [\text{PD}].K_{PD}.(l + \gamma [\text{RG}].K_{G})}
\]

the EC\(_{50}\) is given by the following expression:

\[
\text{EC}_{50} = \frac{1 + [\text{RG}] .K_{G} + [\text{PD}].K_{PD}.(l + \gamma [\text{RG}].K_{G})}{K_{A} .(l + \beta [\text{PD}].K_{PD} + \alpha [\text{RG}].K_{G} (l + \beta \gamma \delta [\text{PD}].K_{PD}))}
\]

the EC\(_{50}\) has identical limiting values to those described above for the apparent ligand binding dissociation constant.

In the absence of agonist ([A] = 0), basal receptor activity is given by the term

\[
\frac{[\text{RG}].K_{G} (l + \gamma [\text{PD}].K_{PD})}{1 + [\text{PD}].K_{PD} + [\text{RG}].K_{G} (l + \gamma [\text{PD}].K_{PD})}
\]
and the maximum agonist response, which occurs as [A] approaches infinity, is given by the term \( \frac{R_T \cdot \alpha \cdot [G_F] \cdot K_G}{1 + \alpha \cdot [G_F] \cdot K_G} \).

The above expressions are appropriate when the free concentration of G-protein ([G_F]) approximates to the total concentration of G-protein ([G_T]), which occurs when [G_T] >> [R_T]. When the concentrations of G_T and R_T are similar, or when [G_T] < [R_T], the free concentration of G-protein is not constant, but will vary with different concentrations of A and R. The mass of G must therefore be conserved as follows:

\[
[G_T] = [G_F] + [RG] + [ARG] + [PDRG] + [APDRG]
\]

\[
[G_F] = \frac{G_T}{1 + [R_F] \cdot K_G \cdot (1 + \alpha \cdot A \cdot K_A + \gamma \cdot [PD] \cdot K_{PD} + \alpha \beta \gamma \delta \cdot A \cdot K_A \cdot [PD] \cdot K_{PD})}
\]

let \( y = K_G \cdot (1 + \alpha \cdot A \cdot K_A + \gamma \cdot [PD] \cdot K_{PD} + \alpha \beta \gamma \delta \cdot A \cdot K_A \cdot [PD] \cdot K_{PD}) \)

\[
[G_F] = \frac{G_T}{1 + [R_F] \cdot y}
\]

This value of [G_F] can now be used to find the corresponding value of [R_F] with respect to [R_T] and [G_T]:

\[
R_T = [R_F] + [AR] + [PDR] + [RG] + [ARG] + [PDRG] + [APDR] + [APDRG]
\]

\[
R_T = [R_F] \cdot (1 + [A] \cdot K_A + [PD] \cdot K_{PD} + \beta [A] \cdot K_A \cdot [PD] \cdot K_{PD} + y \cdot [G_F])
\]

Let \( x = 1 + [A] \cdot K_A + [PD] \cdot K_{PD} + \beta [A] \cdot K_A \cdot [PD] \cdot K_{PD} \)

The theoretical values of [R_F] and [G_F] can now be calculated for any value of [A] using the values of x, y, [R_T] and [G_T] to determined the predicted values of receptor binding and function as described in Appendix I.
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