

ALLOSTERIC REGULATION OF G_s ON AGONIST, ANTAGONIST AND INVERSE
AGONIST BINDING TO THE β₂AR

by

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To my family especially my mom and siblings, best friends: Grisel and Nelly, and to my boyfriend Osvaldo. Your constant love, support and eternal encouragement gave me the strength and motivation to accomplish my goals.

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TABLE OF CONTENTS

DEDICATION.....	ii
ACKNOWLEDGMENTS.....	iii
LIST OF FIGURES.....	viii
LIST OF ABBREVIATIONS.....	x
ABSTRACT.....	xiii

CHAPTER 1: INTRODUCTION

G Protein-Coupled Receptor Signaling.....	1
Orthosteric and Allosteric Regulation of GPCRs.....	5
GPCR-G Protein Complex: Formation, Stability and Structure.....	8
High Density Lipoprotein (HDL) in the Study of GPCRs.....	12

CHAPTER 2: THE EFFECT OF LIGAND EFFICACY ON THE FORMATION AND STABILITY OF A GPCR-G PROTEIN COMPLEX

Introduction.....	15
Results.....	17
Site-Specific Labeling of β_2 AR with Monobromobimane (mB), an Environmentally Sensitive Fluorophore.....	17
Agonist and G_s Induced Changes in mB- β_2 AR Reconstituted into Recombinant HDL Particles.....	21
The Effect of Ligand Efficacy on mB- β_2 AR- G_s Coupling.....	26

Agonist.....	27
Neutral Antagonist.....	27
Inverse Agonist.....	28
The Effect of Ligands on β_2 AR-G _s Complex Formation.....	29
Discussion.....	33
Structural Insights into the Active State Complex, Basal Activity, and Ligand Efficacy.....	33
Relevance to Cellular Signal Transduction.....	34
Materials and Methods.....	37

CHAPTER 3: ALLOSTERIC REGULATION OF AGONIST, INVERSE AGONIST AND ANTAGONIST BINDING TO THE β_2 AR BY G PROTEINS

Introduction.....	43
Results.....	46
Formation of the Nucleotide-Free β_2 AR-G _s Complex in rHDL.....	46
Nucleotide Sensitivity of Antagonist Binding to the β_2 AR-G _s Complex...48	
The Nucleotide-Free β_2 AR-G Protein Complex and High Affinity Agonist Binding.....	55
The Behavior of Inverse Agonists.....	57
Allosteric Regulation of [³ H]DHAP Binding by Antibodies Directed Against the Agonist-Bound β_2 AR.....	59
Discussion.....	61
Materials and Methods.....	66

CHAPTER 4: CONCLUSIONS

Summary and Discussion.....	72
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Implications for GPCR Research (and Drug Discovery).....	76
Implications for GPCR Theory.....	77
APPENDIX: FORMATION AND STABILITY OF A GPCR-ARRESTIN 2 COMPLEX IN RECONSTITUTED HIGH-DENSITY LIPOPROTEINS	
Introduction.....	82
Results.....	85
rHDL Particles as a Means to Study the β_2 AR-Arrestin 2 Complex.....	85
Arrestin Recruitment: Conformational Changes on the Receptor.....	86
Discussion.....	91
Materials and Methods.....	93
REFERENCES.....	98

LIST OF FIGURES

Figure	
1-1	The Heterotrimeric G protein Cycle.....3
1-2	High-density lipoproteins.....14
2-1	Site specific labeling of purified β_2 AR with monobromobimane.....20
2-2	Reconstitution of purified bimane-labeled β_2 AR (mB- β_2 AR) into rHDL particles.....22
2-3	Reconstitution of purified bimane-labeled β_2 AR (mB- β_2 AR) and G_s into rHDL particles.....23
2-4	rHDL- mB- β_2 AR- G_s coupling.....25
2-5	The effect of ligands having different efficacies on the stability of mB- β_2 AR- G_s29
2-6	The effect of ligand efficacy on G_s induced changes in mB- β_2 AR fluorescence.....32
2-7	Conceptual model depicting the dynamic behavior of β_2 ARs.....36
3-1	The β_2 AR- G_s complex is nucleotide-free.....47
3-2	Role of nucleotides on [3 H]DHAP binding to β_2 AR- G_s : Removal of nucleotides by apyrase treatment.....49
3-3	Role of nucleotides on [3 H]DHAP binding to β_2 AR- G_s : Dose response.....50
3-4	Addition of nucleotides increases the apparent Bmax for [3 H]DHAP but has no effect on its affinity.....52
3-5	The nucleotide-free β_2 AR- G_s complex may be observed in reconstituted phospholipid vesicles, native and over-expressing β_2 AR membrane preparations.....54

3-6	Failure to detect high affinity agonist (isoproterenol) inhibition of [³ H]DHAP binding to the nucleotide-free β_2 AR-G _s complex.....	56
3-7	Alprenolol fails to inhibit agonist ([³ H]epinephrine) binding to reconstituted and apyrase-treated β_2 AR-G _s complex.....	57
3-8	Inverse agonist ICI-118, 551 inhibits [³ H]DHAP binding with high affinity to the uncoupled β_2 AR.....	58
3-9	The active conformation of β_2 AR is incompatible with [³ H]DHAP binding	60
A-1	Regulation of GPCR trafficking by GRKs and arrestin.....	83
A-2	Arrestin 2 and rHDL do not interact.....	86
A-3	The effect of arrestin 2-382 on the mB- β_2 AR conformation.....	88
A-4	Phosphorylation of the β_2 AR in rHDL by GRK2.....	90

LIST OF ABBREVIATIONS

A _{2a}	Adenosine Receptor
Alp	Alprenolol
apoA-I	Apolipoprotein A-I
β ₁ AR	β ₁ Adrenergic Receptor
β ₂ AR	β ₂ Adrenergic Receptor
B _{max}	Maximum Receptor Binding
cAMP	Cyclic Adenosine Monophosphate
Cz	Carazolol
CFP	Cyan Fluorescent Protein
CHAPS	3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate
CMC	Critical Micelle Concentration
DDM	Dodecyl Maltoside
DHAP	Dihydroalprenolol
DOPC	Dioleoyl Phosphocholine
EC ₅₀	Half Maximal Effective Concentration
EDTA	Ethylenediaminetetraacetic
ERK	Extracellular Signal-Regulated Kinase
FRET	Fluorescence Resonance Energy Transfer
GDP	Guanosine Diphosphate

GPCR	G Protein-Coupled Receptor
GRK	G Protein-Coupled Receptor Kinase
GTP	Guanosine Triphosphate
GTP γ S	Guanosine 5'-O-(3-thiotriphosphate)
HDL	High Density Lipoprotein
ICI	ICI- 118, 551
ICL	Intracellular Loop
ISO	Isoproterenol
JNK	Janus Kinase
K _d	Dissociation Constant
K _i	Inhibitory Constant
K _{low}	Low Affinity K _i
MAP	Mitogen-Activated Protein
mB- β ₂ AR	Monobromobimane β ₂ Adrenergic Receptor
MWCO	Molecular Weight Cutoff
NABB	Nanoscale Apolipoprotein-Bound Bilayer
PBS	Phosphate Buffered Saline
PI	Protease Inhibitors
PKA	Protein Kinase A
PLC	Phospholipase C
POPC	Palmitoyl-oleoyl-Phosphatidylcholine
POPG	Palmitoyl-oleoyl-Phosphatidylglycerol
RGS	Regulators of G protein Signaling

rHDL	Reconstituted High Density Lipoprotein
RT	Room Temperature
SDS-PAGE	Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
SEC	Size Exclusion Chromatography
Sf9	<i>Spodoptera frugiperda</i>
Src	Sarcoma
TBS	Tris Buffered Saline
TCM	Ternary Complex Model
TM	Phase Transition Temperature
Tris	Trishydroxymethylaminomethane
UV	Ultraviolet
UV-Vis	Ultraviolet-Visible
WT	Wild Type

ABSTRACT

ALLOSTERIC REGULATION OF G_s ON AGONIST, ANTAGONIST AND INVERSE AGONIST BINDING TO THE β_2AR

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G protein-coupled receptors are seven transmembrane domain proteins that regulate a diverse array of cellular functions primarily through G protein-mediated signaling. GPCR function can be regulated by different ligands that are classified as agonists, partial agonists, antagonists or inverse agonists. However, many GPCRs exhibit a degree of basal or ligand-independent activity. Full and partial agonists stabilize a conformation of the receptor that can couple to G proteins. Neutral antagonists are believed to bind to all receptor conformations indiscriminately and block the binding of other ligands, thus having no effect on second messenger signal transduction pathways. In contrast, inverse agonists bind to and stabilize the inactive conformation causing a decrease in basal activity. However, the molecular mechanisms for basal activity, ligand binding and their activation and inhibition of G proteins are poorly understood, largely because receptors exist in a dynamic ensemble of conformations that are difficult to isolate. Here we investigate the activation of the G protein (G_s) by the β_2 Adrenergic

Receptor (β_2 AR) by using a purified receptor reconstituted into recombinant HDL particles with a stoichiometric excess of G_s . The β_2 AR was site-specifically labeled with a small, environmentally sensitive fluorophore allowing direct monitoring of ligand- and G_s induced conformational changes. In the absence of an agonist, the β_2 AR and G_s can be trapped in a complex that is nucleotide-free. Formation of this complex is enhanced by the agonist isoproterenol, and disrupted by the addition of saturating amounts of guanine nucleotides. The inverse agonist ICI prevents the formation of the complex but it is unable to disrupt the pre-formed complex. Saturation and ligand inhibition assays used to further characterize this complex revealed unexpectedly that the nucleotide-free β_2 AR- G_s complex does not bind to the antagonist alprenolol. Moreover, binding is recovered by addition of guanine nucleotides. These results provide insights into G protein-induced conformational changes in the β_2 AR and the structural basis for ligand efficacy. More importantly, we provide evidence that argues against the existence of “neutral” antagonists. Thus a re-evaluation of current GPCR binding theory and experimental design is required.

CHAPTER 1
INTRODUCTION

G Protein-Coupled Receptor Signaling

G protein-coupled receptors (GPCRs) are the largest family of integral membrane proteins localized at the cell surface. GPCRs contain seven α -helical transmembrane domains capable of binding a wide range of ligands. These ligands range from endogenous compounds such as adrenaline, dopamine and chemokines to exogenous ligands including morphine, as well as sensory stimuli like odors, taste and light. They constitute the third largest family of genes in the human genome with approximately 1000 genes (1). Their vast diversity and their importance in cellular signaling make them prime therapeutic targets constituting nearly 30 percent of available drugs (2)

GPCRs are divided into three main classes with no sequence homology: Class A, Class B and Class C. The largest class, Class A, is made up of ~85% of the GPCR encoding genes and recognizes a diverse array of ligands, including catecholamines, photons, nucleotides, phospholipids, and peptides. In all, they can be classified into six classes based on sequence homology and functional similarity: Class A (Rhodopsin like), Class B (Secretin like), Class C (Metabotropic like), Class D (Fungal pheromone), Class E (cAmp receptors), and the Frizzled/Smoothed class.

GPCRs regulate a diverse number of cellular functions primarily through heterotrimeric G protein-mediated signaling. Heterotrimeric G proteins are comprised of three subunits: $G\alpha$, $G\beta$, and $G\gamma$. The $G\alpha$ subunit differs among the members of the family, and defines the individual. Common $G\beta$ and $G\gamma$ subunits are shared among some α subunits to form the specific oligomers. There are at least 20 α , 5 β and 10 γ subtypes in humans (3). The α -subunits are divided into several families: α_s (stimulates adenylyl cyclase, AC), $\alpha_{i/o}$ (inhibits AC), α_q (stimulates phospholipase C β , PLC β), $\alpha_{12/13}$ (stimulates guanine nucleotide exchange factors for small G protein Rho, Rho-GEF); and the more specialized α_t (visual, regulates phosphodiesterase gamma, PDE γ) and α_{olf} (also stimulates AC). The G proteins are anchored to the membrane by lipid modifications in the α -subunits (usually by a myristoyl and/or palmitoyl moiety) and γ subunit (prenyl, farnesyl or geranylgeranyl acyl group (4)).

$G\alpha$ -subunits also contain guanine nucleotide binding sites that in the basal inactive state (trimeric form) is bound by GDP. Activation of the G protein through the exchange of GDP for GTP results promotes the functional dissociation of the α and $\beta\gamma$ subunits, allowing each to regulate their respective downstream effectors. $G\alpha$ subunits may regulate ACs, PLC β s, RhoGEFs or PDE, while $G\beta\gamma$ may modulate receptor kinases, ion channels, PLC β isoforms, and certain AC isoforms, to name a few. Together these effectors may regulate the production or gating of second messengers such as cAMP, phosphoinositides or cations that in turn may regulate downstream protein kinase cascades.

Signaling is eventually terminated by the hydrolysis of GTP to GDP by the α -subunits and the subsequent reassociation of the heterotrimer (**Fig. 1-1**). Hydrolysis is

achieved by the intrinsic GTPase activity of the α subunit and may be accelerated through an interaction with proteins such as Regulators of G protein Signaling (RGS) (5). However, the key step in the entire cycle is the process of exchange of GDP for GTP, a step that is catalyzed by hormone-bound receptor.

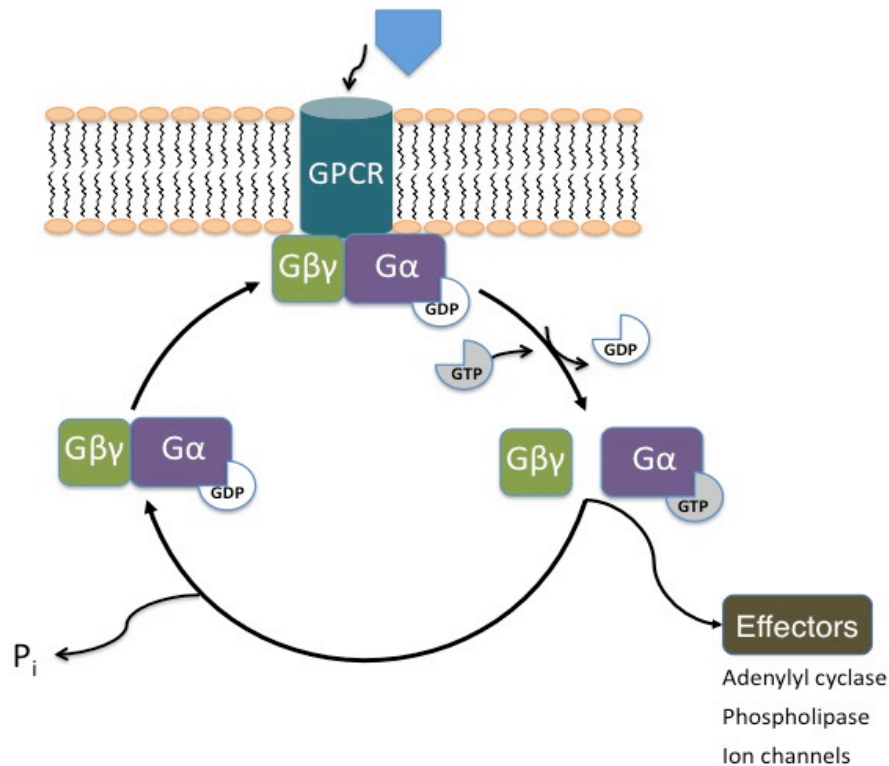


Figure 1-1. The Heterotrimeric G protein cycle. Representative overview of G protein activation and signaling. Ligand binding (blue) to the receptor (GPCR) induces a conformational change that promotes the exchange of GDP for GTP on the α -subunit of the heterotrimeric G protein ($G\alpha\beta\gamma$). Upon GTP binding the heterotrimeric G protein functionally dissociates into α -GTP and $\beta\gamma$ subunits that activate different effectors. Hydrolysis of GTP to GDP promotes the reassociation of the heterotrimer, which can be activated again by the receptor.

Receptors can also be desensitized through an event that is triggered through phosphorylation by G protein-coupled receptor kinases (GRKs). GRK family members are divided into three main groups based on sequence homology: rhodopsin kinase

(GRK1 and 7), the β_2 adrenergic receptor subfamily (GRK2/3) and the GRK4 subfamily (GRK4, 5 and 6) (6). Receptor phosphorylation leads to the recruitment and binding of arrestin, one function of which is to arrest signaling by preventing G protein signaling. In addition, arrestins can recruit clathrin adaptor protein (AP1) and clathrin, which mediate receptor endocytosis. There are four known genes encoding arrestins. Arrestin 1 and 4 have restricted expression patterns, localizing in the retina. Arrestin 2 and 3 are ubiquitously expressed and interact with the vast majority of GPCRs (7). Recently, arrestins 2/3 were discovered to stimulate G protein-independent signals, largely in pro-survival and anti-apoptotic pathways (8-11). Thus, arrestins are known to mediate three GPCR properties: desensitization, internalization and signaling.

This thesis focuses on a prototypic Class A receptor, the β_2 Adrenergic receptor (β_2 AR). The β_2 AR has several roles in the body, including the regulation of smooth muscle relaxation, glycogenolysis, and lipolysis. The β_2 AR is activated by epinephrine, which causes the activation of the stimulatory G protein, G_s . GTP-bound G_s binds and activates adenylyl cyclase causing an increase in cAMP levels and subsequent activation of protein kinase A (PKA). In contrast, the β_2 AR can also couple to G_i and lead to inhibition of adenylyl cyclase and the reduction of cAMP levels. However, the mechanisms by which the “G protein switch” occurs are still not well understood, but have implicated phosphorylation of the β_2 AR itself (12, 13).

Recent work has shown that the β_2 AR can signal through G protein-independent pathways, namely through arrestins (14). This new paradigm is an example of the concept of biased agonism and biased signaling. Ligands may serve as agonists for a G protein-dependent pathway but serve as antagonists for the arrestin pathway or vice versa

(15). An example of this is the case of ICI 118, 551, an inverse agonist for the G protein-dependent pathway but a full agonist for the arrestin stimulated MAPK pathway (16). The discovery of biased agonism has opened the doors for the development of new therapeutics that can rely on already established drugs to study multiple effectors and responses different from those stimulated/inhibited by G proteins.

Orthosteric and Allosteric Regulation of GPCRs

All GPCRs have a distinct binding site for their respective endogenous ligand(s) known as the orthosteric site. Ligands that bind to this site are considered classical or traditional orthosteric ligands and have been typically characterized using functional or radioligand binding methods (17). This group includes small-molecule agonists, partial agonists, antagonists and inverse agonists. Although most GPCRs exhibit a degree of constitutively active basal activity, agonists can further stabilize the activated state and enhance G protein activation. Neutral antagonists bind to the orthosteric site and block the binding of the native agonist without altering the constitutive activity of the receptor. In contrast, inverse agonists occupy the binding site and decrease the constitutive activity (18).

In addition, GPCRs have allosteric binding sites that are commonly spatially and functionally distinct from the orthosteric site, displaying no overlap between them (19, 20). Allosteric modulators bind to the allosteric site where they stabilize a receptor conformation and equilibrium shift that increases (positive allosteric modulator, PAM) or decreases (negative allosteric modulatory, NAM) the affinity and/or efficacy of an orthosteric ligand.

The effects of allosteric and orthosteric ligands on receptor activity can be described by equations based on various mass-action models. The orthosteric models describe the binding between different ligands to a common site on the receptor. The earliest described model was the classical occupancy model that assumed that the binding of an agonist activated the receptor (21). In contrast, allosteric models take into consideration the ability of the receptor to present different binding sites to different ligands. Allostery was originally explained in the simple two state model, which stated that the bound-receptor existed in one of two states: active or inactive. This model was modified (full two state model) to incorporate basal activity in which the receptor can be active or inactive regardless of ligand binding (22).

The ternary complex model (TCM) was an extension of the previous models and takes into account not only the interaction of the receptor and ligand, but also the active receptor and G protein (23). This model predicts that in the presence of GDP, agonist binding promotes the formation of a long-lived ternary complex between agonist (H), GPCR (R), and heterotrimeric G protein (G) that exhibits high affinity agonist binding. In the absence of G protein, or when the presence of GTP allows for receptor-catalyzed G protein activation, the H-G-R complex is dissociated, and the receptor resides in a low affinity (H-R) state (24). The realization that some ligands (inverse agonists) have higher affinity for the inactive receptor and suppress basal receptor activity lead to the extension of the TCM, this expansion proposes that the intrinsic efficacy of a ligand reflects its ability to alter the equilibrium between active (R*) and inactive (R) (25).

According to the extended TCM, full agonists stabilize the R* conformation, pulling the equilibrium toward the active state to obtain a full response and maximal

activation. Partial agonists have lower intrinsic efficacy than full agonists, resulting in submaximal activation. Antagonists, theoretically bind R and R* indiscriminately, producing no physiological response but blocking the binding/response of agonists. In contrast, inverse agonists block the binding of other ligands but reduce receptor-mediated constitutive activity of GPCRs by binding to R and shifting the equilibrium to the inactive state. Although, this model takes all types of ligands into account, it is still limited in that it accommodates the existence of only two receptor conformations. However, in practice the TCM is the model with the minimal number of parameters that are required to define drug properties that can be accurately determined experimentally.

Current experimental data have led to the proposal of a new model that recapitulates the principles of its predecessors and expands the idea that GPCRs exist in multiple active states. The idea of differential active states arose from the observation that many GPCRs at physiological levels or when over-expressed displayed promiscuity (26-28). In this multistate model, certain agonists are predicted to induce distinct active conformations of the receptor by differentially exposing regions of the intracellular domains involved in coupling to different pools of G proteins. In fact, the β_2 -adrenergic receptor can exist in various active conformations that can be distinguished in the presence of guanine nucleotide analogs (29). These results can be extrapolated to analyze the conformational changes that GPCRs undergo upon ligand binding (agonists, antagonists and inverse agonists) and G protein coupling.

All these models have taken into account the contributions of ligand binding and G protein coupling to the conformational state of the receptor and describe the effects they impose on each other. However, allosteric regulation of the orthosteric site of

GPCRs is not limited to G proteins. Monovalent metal ions have been shown to be allosteric regulators of ligand binding for several receptors (30, 31). It is hypothesized that sodium affects ligand binding through an interaction with a conserved aspartate in transmembrane two of the receptor. Similar to G proteins, the binding of sodium to the allosteric site affects ligand dissociation at the orthosteric site.

Although effects of guanine nucleotides on the receptor conformation and ligand binding have been observed (just like the effects of ions) these have not been characterized in depth as nucleotides are seen as direct modulators of G proteins not the receptor. Interestingly, increases in antagonist binding due to the addition of guanine nucleotides have been observed (32-34). This observation is intriguing because according to the TCM, antagonists bind to all receptor conformations indiscriminately. This observation implies that there is a state of the receptor-G protein complex that cannot be detected by antagonists, suggesting that antagonists are not conformation-neutral. Such GPCR-G protein complex had not been isolated or characterized until recently by our lab.

GPCR-G Protein Complex: Formation, Stability and Structure

GPCR-G protein interactions represent the fundamental signaling interface underlying the majority of physiological responses due to hormones or neurotransmitters. Crystallography has served as an integral tool in the study and understanding of both G protein and GPCR function. To date, the structures of several active and inactive G proteins have been published (35-37), and the structures of several prototypical GPCRs have been also obtained (38-42). These structures, in combination with functional data, give insights into the molecular mechanisms that allow these receptors to exist in a

diverse array of conformations and highlight key regions critical in ligand and effector binding.

All of the published structures contain a ligand in their binding site and lack a prominent tilt of the cytoplasmic half of TM6 away from the transmembrane helical bundle which is considered to be mandatory for G protein activation (43-45). To date the only structure of a GPCR-G protein complex available is that of opsin bound to an 11-amino acid peptide derived from the C-terminal helix of the α -subunit of transducin (40). This model lacks structural elements that are thought to be involved in receptor and G protein interaction (*e.g.* $G\beta\gamma$). Although $G\beta\gamma$ is not mandatory for catalyzed nucleotide exchange, modulation of the $G\alpha$ - $G\beta\gamma$ interface in the holo G protein greatly facilitate GDP release (40, 46). However, most of the attempts to crystallize a full length GPCR-G protein complex have failed.

GPCRs require the presence of a membrane environment for maximal stability even though their intracellular loops are extremely dynamic. These factors and the rapid kinetics of the G protein-GPCR interaction (47) make the isolation and crystallization of a stable and functional complex in detergent micelles complicated. To circumvent some of these issues, a camelid antibody fragment (nanobody) was raised against the active (agonist-bound) conformation of the β_2 AR. This nanobody, NB80, shows G-protein-like properties upon binding to either wild-type β_2 AR and β_2 AR-T4L, the β_2 AR-T4 lysozyme fusion protein that was used to obtain the inactive state crystal structure (48). Comparison with the inactive β_2 AR structure reveals subtle changes in the ligand binding pocket. These small changes are associated with an outward movement of the cytoplasmic end of TM6, and rearrangements of TMs 5 and 7 that are similar to those

seen in the active form of rhodopsin (48, 49). This structure provides insights into the process of agonist binding and activation.

In addition, numerous studies involving chimeric substitutions, various mutational approaches, and synthetic peptides have provided insight into structural elements important for the interaction with G proteins. These studies have established critical roles for the second and third intracellular loop (ICL2 and ICL3, respectively), and at least in some receptors, the proximal part of the carboxy terminus in G protein coupling (50-52). ICL3 is the key determinant of coupling specificity among the different G protein α -subunits. Crucial residues for coupling have been localized to the amino-terminal part of ICL3 adjacent to TM5 (53) and in the carboxyl-terminal part of ICL3 close to TM6 (54). ICL2 is not as important for recognition as it is for activation efficiency. Random mutagenesis studies suggest that residues on ICL2 that are close to TM3 are responsible for maintaining the receptor in an inactive state, while residues on the opposite site compromised G protein coupling (55). These observations are consistent with a role of the E/DRY motif (amino-terminal of ICL2) in receptor activation (56-58) and movement of TM3 relative to TM6 predicted from spectroscopic analysis (43, 59).

Regardless of all these findings, the mechanisms and conformations by which the signal is transmitted from the active receptor to the G protein remains elusive. The points of contact between the two proteins and the relative orientation to each other are still greatly unknown. Using the data available, it has been proposed that the nucleotide of the α -subunit of the G protein is localized $\sim 30\text{\AA}$ away from the membrane (60). This would suggest that the nucleotide-binding domain of the α -subunit releases GDP as it interacts with the receptor.

GPCRs are thought to trigger nucleotide release by an allosteric mechanism. It has been proposed that the carboxyl-terminus of the α -subunit binds the receptor and this leads to changes in the conformation and/or orientation, which are propagated to $\alpha 5/\beta 6$ loop that is in direct contact with the nucleotide. The loss of binding interactions involving residues in this loop may trigger GDP release, leading to G protein dissociation and activation (61, 62).

We have defined the active-state GPCR-G protein complex as the physical association between a GPCR and a G protein that can be identified by the allosteric effects that they impose on each other (63). This effect is observed for agonist binding where the formation of a GPCR-G protein complex enhances the affinity of the receptor for the ligand. This enhanced agonist-affinity for the active-state complex is abolished by guanine nucleotides suggesting that the bound protein is nucleotide-free. Furthermore, it suggests that a fraction of G proteins may exist in a pre-coupled state that differs from the active-state. These distinct states have not been observed in cells since the nucleotide concentrations in the cytoplasm is too high, leading to rapid G protein activation. The *in vitro* characterization, both functional and structural, of these conformations has been limited by the lack of experimental systems that resemble their natural environment successfully.

In order to characterize and isolate discrete conformational states of GPCRs we have taken advantage of biophysical techniques that allow us to monitor specific regions of the receptor upon treatment with ligand and/or G protein binding. These studies allowed direct structural analysis and represent a first step toward a more deep understanding of GPCR function at the molecular level. To accomplish this, we relied on

an unique approach for isolating functional GPCR monomers in phospholipid bilayers of a discoidal high density lipoprotein (HDL) particle.

High Density Lipoprotein (HDL) in the Study of GPCRs

Over the past decade membrane protein research has escalated thanks to a diverse array of membrane modeling systems such as detergent micelles, bicelles and liposomes (64). These have allowed the functional and structural characterization of a great number of membrane proteins. However, in some instances it is unclear how these systems mimic the natural milieu. In most cases, the orientation and oligomerization state of the reconstituted proteins cannot be determined.

In the past couple of years, a new class of model membranes has been developed to study the function of isolated membrane proteins especially GPCRs (65-73). In this approach a purified membrane protein is reconstituted into the phospholipid bilayer of a high-density lipoprotein (HDL) particle. HDL is a protein-lipid complex that is part of the reverse cholesterol transport pathway in the body (74) that serves to sequester and transport excess cholesterol from the vasculature to the liver for excretion. It is composed of a 100 nm-diameter phospholipid bilayer that is stabilized and surrounded by a belt composed of a dimer of apolipoprotein A-I (apoA-I). The bilayer mimics the zwitterionic environment of the cell membrane and the whole complex can be reconstituted *in vitro* by adding lipids to purified apoA-I.

The reconstituted HDL (rHDL) particles are monodispersed, homogenous and preferentially incorporate monomeric GPCRs (70). Furthermore, they allow full access to both the N and C termini of the receptor (extracellular and cytoplasmic surfaces,

respectively), allowing the interaction of the receptors with orthosteric and allosteric regulators (**Fig. 1-2**). Several forms of rHDL particles have been described and have successfully been used to reconstitute membrane proteins, *e.g.* nanodiscs and NABBs (nanoscale apolipoprotein-bound bilayers) (72). A variety of GPCRs have now been reconstituted into rHDL particles: rhodopsin (71-73), β_2 -adrenergic receptor (70) and the μ -opioid receptor (75), each fully capable of activating its G protein when reconstituted as monomers in rHDL particles. Furthermore, reconstituted receptors display strong allosteric modulation by both G proteins and arrestin (73).

The advantages of the rHDL system allow us to investigate and characterize different conformational states of the β_2 adrenergic receptor (β_2 AR) induced by binding of allosteric and orthosteric regulators. In this thesis, I use fluorescence spectroscopy and radioligand binding assays to elucidate distinct conformations of the receptor induced by agonists, antagonist and inverse agonists in the presence or absence of its cognate partner, G_s . More importantly, we were able to isolate the high-affinity agonist state: the nucleotide-free β_2 AR- G_s complex. Interestingly, we found that the

“neutral” antagonist, alprenolol, preferentially binds to the β_2 AR alone or in complex with GDP- G_s but not to the β_2 AR- G_s nucleotide-free complex. These data suggest that alprenolol is not a neutral ligand as it does not recognize all receptor populations. Moreover, our studies have allowed us to establish a model to explain ligand binding that has significant implications for GPCR theory, because equilibrium binding assumes that all receptor conformations exist in equilibrium and are all recognized by neutral antagonists which is not the case.

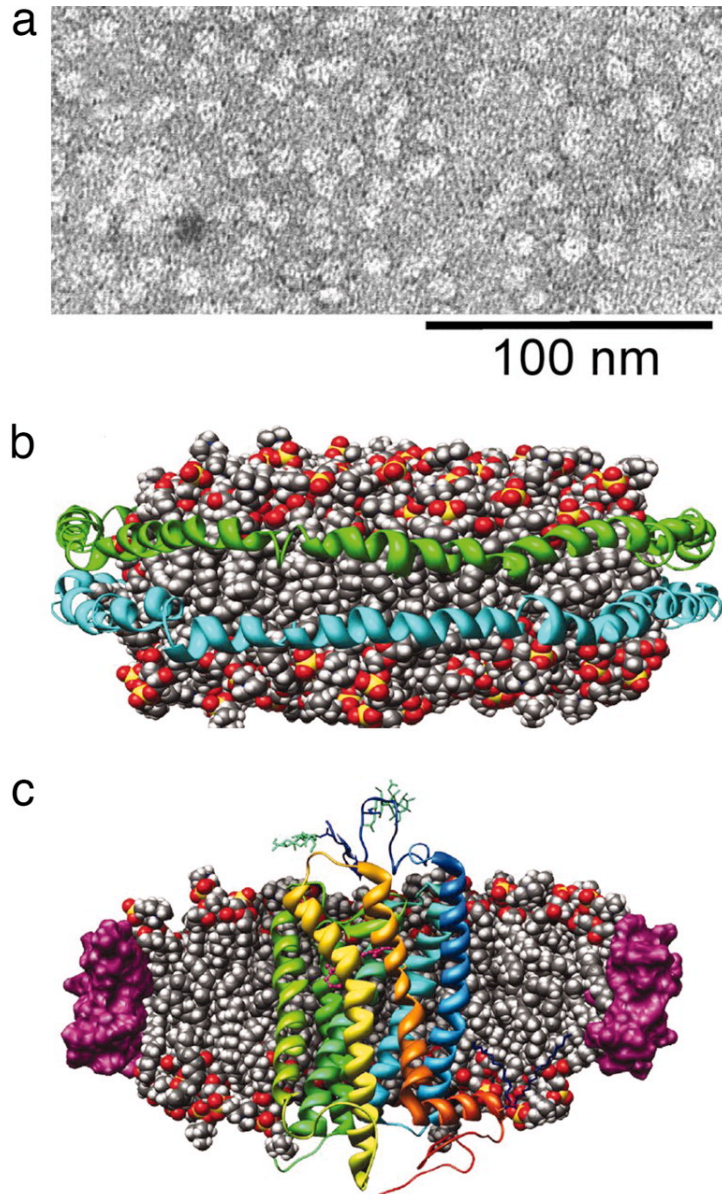


Figure 1-2. High-density lipoproteins. **(A)** Transmission electron micrograph of negatively stained rHDL. The 10 nm rHDL particles are homogenous. **(B)** Molecular model illustrating rHDL composed of a dimer of apoA-I wrapped around a phospholipid bilayer of ~160 lipids. **(C)** Molecular model of a GPCR (bovine rhodopsin, 1F88) reconstituted into rHDL. Image adapted from Whorton *et al.* (70)

CHAPTER 2

THE EFFECT OF LIGAND EFFICACY ON THE FORMATION AND STABILITY OF A GPCR-G PROTEIN COMPLEX

Introduction

G protein independent signaling pathways have been identified for a number of G protein-coupled receptors (8, 14, 76); however, GPCR-G protein interactions represent the fundamental signaling interface, underlying the physiologic response to the majority of hormones and many neurotransmitters. Crystal structures of G proteins have been obtained in both the active and inactive states (35-37), and structures of bovine rhodopsin (38, 39, 77-80), squid rhodopsin (81, 82), bovine opsin (40, 49), human β_2 AR (42, 83, 84), turkey β_1 AR (85), adenosine A2a receptor (86), chemokine CXCR4 (87) and dopamine D3 receptors (88) have been reported. However, relatively little is known about the active-state GPCR-G protein complex.

The active-state GPCR-G protein complex is herein defined as the physical association between a GPCR and a G protein that promotes GTP binding to the $G\alpha$ subunit. The specific allosteric effects that each protein imposes on the other can identify this complex. Agonist-bound GPCRs promote exchange of GDP for GTP on the $G\alpha$ subunit. G protein effects on receptor structure are more difficult to detect, but agonist

binding affinity for many GPCRs is enhanced when complexed with a G protein. This effect was originally demonstrated for the β_2 AR (89) and led to the ternary complex model of receptor activation (23). The enhanced agonist affinity observed for the active-state complex is abolished by both GTP and GDP (at higher concentrations) suggesting that the bound G protein is nucleotide-free (32).

The active-state complex should be distinguished from other types of physical association or co-localization of GPCRs and G proteins that may be observed in cells and may be important for signal transduction. Recently it has been possible to study GPCR-G protein interactions in cells by using FRET between components tagged with fluorescent and/or luminescent proteins (90-92). These studies provided evidence that a fraction of inactive receptors and G proteins may exist in a pre-coupled state or at least in close proximity. These associations may occur through direct receptor-G protein interactions, or through interactions with common scaffolding proteins. However, this state differs from the active-state or ternary complex as defined above, because G protein-dependent high-affinity agonist binding is not observed at GTP and GDP concentrations present in intact cells (93, 94).

For many GPCRs, the active-state complex can form in the absence of agonists leading to a certain level of basal, agonist-independent activity also called constitutive activity. There is a growing appreciation that drugs that inhibit basal activity, called inverse agonists, may be more effective therapeutics for some indications than neutral antagonists (95).

Herein we use site-specific labeling of the β_2 AR with a conformationally sensitive fluorescent probe together with recombinant HDL particles (70), to investigate the active-

state complex. This experimental system allows us to directly monitor ligand and G protein-induced conformational changes in the monomeric β_2 AR. We observed that the β_2 AR forms a stable complex with the G_s heterotrimer in the absence of guanine nucleotides. This complex can be detected by changes in the fluorescence of labeled β_2 AR. Conformational changes induced in the β_2 AR by G_s alone or by agonist alone result in similar changes in the fluorescence of labeled β_2 AR. The β_2 AR- G_s complex rapidly dissociates in the presence of both GTP and GDP. A saturating concentration of the inverse agonist ICI-118, 551 does not interrupt the preformed complex, but prevents complex formation. In contrast, the neutral antagonist alprenolol has little effect on the stability of the complex nor does it prevent complex formation. These results provide insights into G protein-induced changes in the β_2 AR.

Results

Site-Specific Labeling of β_2 AR with Monobromobimane, an Environmentally Sensitive Fluorophore

To detect agonist and G protein-induced conformational changes in the β_2 AR, we introduced a conformationally sensitive fluorophore adjacent to the G protein-coupling region of transmembrane segment 6. It has been previously shown that fluorophores covalently bound to C265 at the cytoplasmic end of TM6 (*Fig. 2-1A*) are capable of detecting agonist-induced conformational changes (96-98). Cysteine 265 is well positioned to detect conformational changes associated with G protein activation. The recent crystal structure of the human β_2 AR (83) predicts that monobromobimane bound

to C265 lies in a relatively nonpolar pocket formed at the interface between TMs 3, 5 and 6 (**Fig. 2-1C**). The recent structure of opsin bound to the carboxyl terminus of the $G\alpha$ -subunit of transducin (40) suggests that the G protein interacts with residues in the inner side of the cytoplasmic TM5 and TM6. A similar pattern of interactions between the β_2 AR and Gs would involve residues V222^{5.61}, A226^{5.65}, and Q229^{5.68} in TM5, and K263^{6.25}, F264^{6.26}, K267^{6.29}, and A271^{6.33} in TM6, in the vicinity of C265^{6.27} (**Fig. 2-1D**). Conformational changes associated with activation of the β_2 AR would be expected to displace bimane bound to C265 to a more polar environment.

C265 is highly reactive to labeling with polar, cysteine-reactive fluorophores (96, 99). The remaining reactive cysteines can be removed by mutagenesis (C77V, C327S, C378A, and C406A) without altering receptor function (99) (**Fig. 2-1A**). It was previously found that receptor palmitoylation site (C341) is not reactive (96). Stable palmitoylation of C341 was observed in the crystal structure of the β_2 AR where purified protein was alkylated with 4 mM iodoacetamide (100 fold molar excess) under the same conditions. Both palmitate bound to C341 and iodoacetamide bound to C265 were clearly ordered in the crystal structure.

The modified receptor was expressed in Sf9 insect cells using recombinant baculovirus technology and purified as previously described (99). The purified receptor was labeled with an equivalent amount of monobromobimane. This modified β_2 AR labeled at C265 with monobromobimane will be referred to as mB- β_2 AR.

Monobromobimane is an ideal fluorophore for my experiments because of its small size (about the size of tryptophan) and short linker, together with its high sensitivity to the polarity of its molecular environment (**Fig. 2-1B**), which allow it to be highly

sensitive to detect conformational changes without greatly interfering with receptor function. Any ligand or G protein-induced movement at the cytoplasmic end of TMs 3, 5, or 6 would be expected to change the molecular environment of the Cys265-bound fluorophore, and therefore its fluorescent properties.

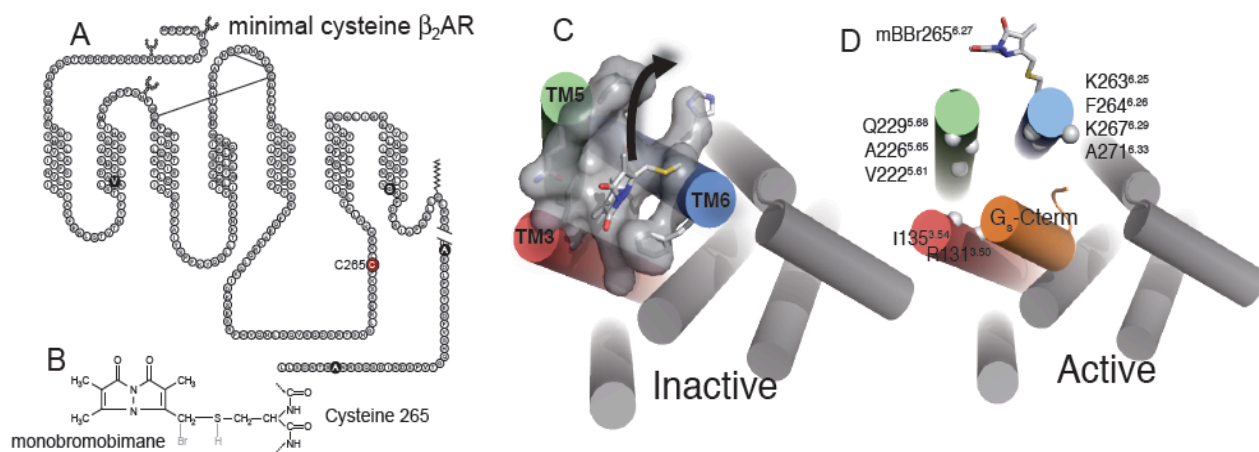


Figure 2-1. Site-specific labeling of purified β_2 AR with monobromobimane. **(A)** Sequence and secondary structure of the human β_2 AR showing sites where the reactive cysteines were mutated to the indicated amino acid (black circles with white letters) C265 is shown in red. **(B)** The structure of bimane covalently bound to C265 after reaction with monobromobimane. **(C)** In an inactive structure of the β_2 AR, bimane bound to C265 is predicted to occupy a cavity formed between TM3, TM5, and TM6. When TM6 adopts an active conformation (due to agonist binding or basal activity), bimane is displaced (black arrow) out of this cavity into a more polar environment, which is detected as change in fluorescence intensity and λ_{max} . **(D)** A model of the active state of the β_2 AR in complex with the carboxyl terminal peptide of $G\alpha_s$, based on the crystal structure of opsin in complex with transducin peptide (40). The amino acids at the positions marked by solid spheres are predicted to form interactions with $G\alpha_s$. The residues of the β_2 AR are numbered according to their position in the sequence followed by the Ballesteros general number in superscript (100). In this numbering scheme, the most conserved residue within each helix is designated x.50, where x is the number of the transmembrane helix. All other residues on that helix are numbered relative to the conserved position. The model of the active conformation of β_2 AR shown was built by homology modeling using the β_2 AR (2RH1) and opsin (3CAP) structure as templates. The $G\alpha_s$ fragment bound to the active-like β_2 AR was modeled by threading the 11 C-terminal residues of $G\alpha_s$ on the structure of the synthetic peptide derived from the carboxy terminus of $G\alpha_t$ bound to opsin. All the homology models and figures were made with pymol (DeLano WL, 2002).

Agonist and G_s-Induced Changes in mB-β₂AR Reconstituted into Recombinant HDL Particles

β₂AR requires a lipid bilayer to efficiently couple to G_s. Our lab recently showed that purified β₂AR can be reconstituted into recombinant HDL particles (rHDL) as monomers, and that monomeric β₂AR couples efficiently to G_s (70). Thus, mB-β₂AR was reconstituted into rHDL, and the response to the agonist isoproterenol (ISO) was determined. **Fig. 2-2A** shows the emission spectra of mB-β₂AR in the absence of G protein but in the presence of increasing concentrations of ISO. We observe a dose dependent decrease in fluorescence intensity and an increase in the maximal emission wavelength (λ_{\max}) of 15 nm (**Fig. 2-2A**). The concentration-dependent effects of ISO on intensity and λ_{\max} are shown in **Fig. 2-2B and 2-2C**. It should be noted that the EC₅₀ for the fluorescence dose response curve (**Fig. 2-2B**) is approximately 3-fold higher than the IC₅₀ for ISO in a conventional competition binding experiment (**Fig. 2-4A, black curve**). It is possible that an agonist-binding event is not always associated with a conformational change. Agonists such as ISO have relatively low affinity and very rapid on and off rates (101). ISO can occupy the binding pocket for a long enough time to compete with radiolabeled antagonist, but this event may not always be associated with an activating conformational change.

Purified G_s heterotrimer (G α_s β₁γ₂) was added to rHDL containing mB-β₂AR at a ratio of 10 G_s per β₂AR monomer to ensure that mB-β₂AR would have access to at least 1 G_s trimer (during the reconstitution, some G_s is lost because of aggregation due to detergent removal).

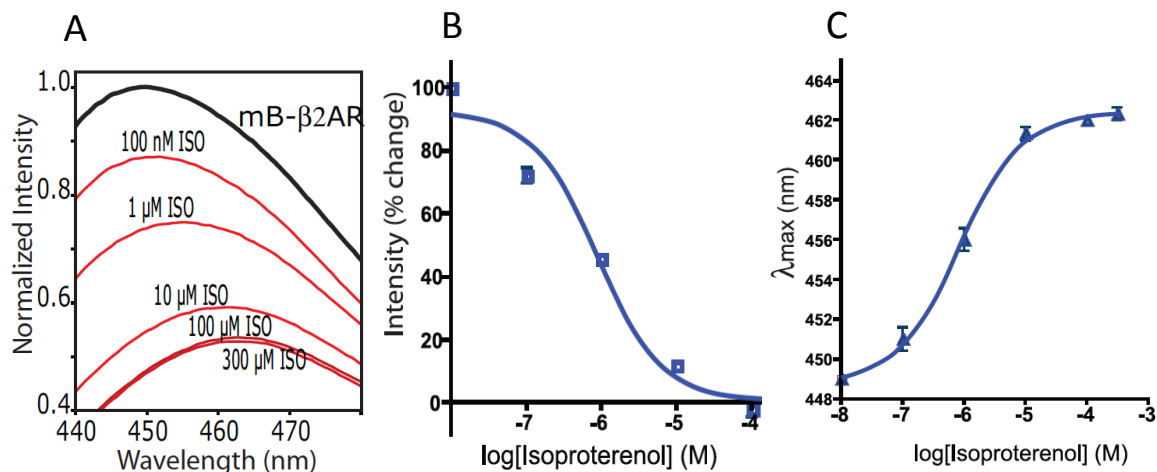


Figure 2-2. Reconstitution of purified bimane-labeled β_2 AR (mB- β_2 AR) into rHDL particles. **(A)** Bimane emission spectra of reconstituted mB- β_2 AR in the absence (black spectrum) and presence of increasing concentration of the agonist ISO (red spectra). Agonist-induced conformational changes lead to a decrease in fluorescence intensity of bimane and a shift in the λ_{\max} . **(B-C)** Effect of an agonist (isoproterenol) on mB- β_2 AR fluorescence. The normalized change in fluorescence intensity **(B)** and the λ_{\max} **(C)** for mB- β_2 AR in response to different concentrations of ISO. These data were obtained from the experiment in A.

Fig. 2-3A and *B* show the emission spectra of mB- β_2 AR after reconstitution with G_s . The λ_{\max} of mB- β_2 AR- G_s fluorescence is similar to that observed for mB- β_2 AR following the addition of isoproterenol (*Fig. 2-2A*). The effect of G_s on mB- β_2 AR fluorescence can be reversed by uncoupling the receptor from G_s by using either GTP γ S or GDP. The addition of GTP γ S or GDP results in an increase in fluorescence intensity and a 15 nm decrease in the λ_{\max} (*Fig. 2-3A-B*) to a value similar to that observed in

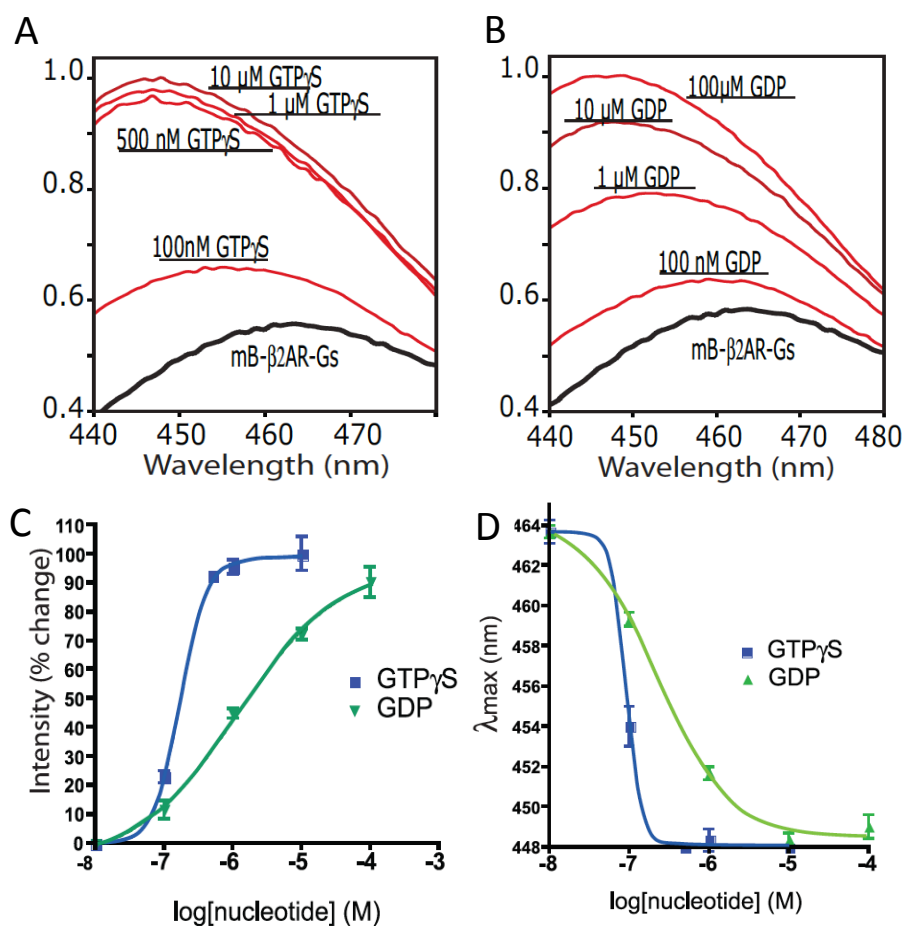


Figure 2-3. Reconstitution of purified bimane-labeled β_2 AR (mB- β_2 AR) and G_s into rHDL particles. (A-B) Bimane emission spectra of reconstituted mB- β_2 AR with G_s in the absence of agonist or nucleotides (black), and in the presence of increasing concentrations of GTP γ S (A) and GDP (B) (red spectra). All spectra were normalized to the unliganded, uncoupled state of mB- β_2 AR (value obtained upon addition of 10 μ M GTP γ S). (C-D) Normalized change in fluorescence intensity (C) and the λ_{\max} (D) for mB- β_2 AR- G_s in response to different amounts of guanine nucleotides.

unliganded receptor before reconstitution. GTP γ S is more potent at uncoupling mB- β_2 AR from G_s compared with GDP, consistent with previous studies indicating that G_s coupled to the β_2 AR has a higher affinity for GTP γ S than for GDP (32). The concentration dependent effects of GTP γ S and GDP on intensity and λ_{\max} of mB- β_2 AR are shown in **Fig. 2-3C** and **D**. The effect of GDP on mB- β_2 AR- G_s fluorescence may be unexpected

given the prevailing view that the receptor binds preferentially to G_s -GDP and induces GDP release. If mB- β_2 AR and G_s -GDP form a complex, this complex does not exhibit properties of the active state complex as defined above, because cytosolic concentrations of GDP can disrupt G_s dependent high-affinity agonist binding (32). An alternate explanation for these observations is that the β_2 AR binds to and stabilizes a small fraction of nucleotide-free G_s that is in equilibrium with G_s -GDP.

G_s induces a high affinity state for the agonist ISO (K_i 1.6 nM in the presence of G_s compared with 840 nM in the presence of G_s and GTP γ S, or mB- β_2 AR alone (**Fig. 2-4A**), comparable to what is observed with unlabeled receptor (70). Thus, modification of C265 with bimeans does not interfere with G protein coupling. Using these reconstitution conditions, we observed that more than 80% of mB- β_2 AR is in the G protein-dependent high-affinity agonist binding state.

Figure 2-4B shows a time course of the effect of 200 nM GTP, GTP γ S, and GDP on mB- β_2 AR fluorescence intensity measured at 450 nm. As expected, all guanine nucleotides induce a rapid increase in fluorescence consistent with the disruption of the active-state m- G_s complex. The effect at this concentration is larger for GTP and GTP γ S compared with GDP. After the initial increase in fluorescence, we observe a decrease in fluorescence for GTP, but not for GDP or GTP γ S. This decrease can be explained by the ability of G_s to hydrolyze GTP, but not GDP or GTP γ S. Thus, on the initial dissociation of the mB- β_2 AR- G_s complex, GTP is hydrolyzed to GDP and some of the mB- β_2 AR can reassociate with G_s .

These fluorescence studies show that mB- β_2 AR and G_s form an active-state complex in the absence of agonist. The agonist ISO and G_s induce similar changes in the

fluorescence intensity and λ_{max} of the mB- β_2 AR. These changes are consistent with the movement of the fluorophore to a more polar environment that could be achieved by a clockwise rotation and/or outward movement of TM6 relative to TM3 and TM5 (**Fig. 2-1D**). This movement is in agreement with the changes observed in rhodopsin by double electron resonance (DEER)

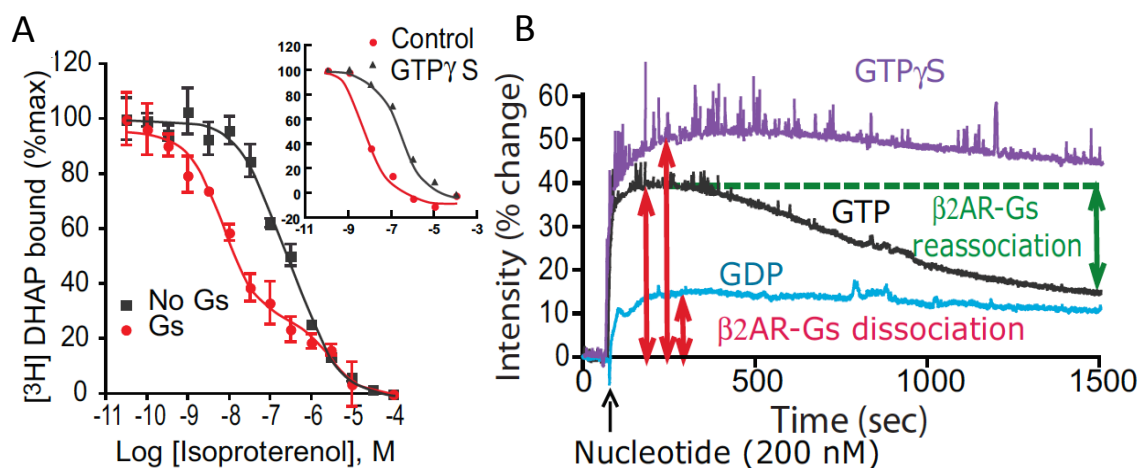


Figure 2-4. rHDL-mB- β_2 AR-G_s coupling. (A) Isoproterenol inhibition of [³H]DHAP binding to rHDL-mB- β_2 AR in the presence (red) or absence (black) of G_s. Inset shows the effect of 10 μ M GTP γ S on isoproterenol binding affinity for preformed rHDL-mB- β_2 AR-G_s. (B) Time-scan of fluorescence monitored at 450 nm. The effect of 200 nM GTP, GTP γ S, GDP on preformed rHDL-mB- β_2 AR-G_s are compared. GTP induced dissociation of mB- β_2 AR and G_s followed by reformation of the complex after GTP hydrolysis.

spectroscopy (44), as well as conformational differences between opsin and rhodopsin (49), and previous biophysical and mutagenesis studies on the β_2 AR (59, 102, 103). The fact that both G_s and the agonist ISO induce similar conformational changes in fluorescence is compatible with the hypothesis that they independently induce similar changes in receptor structure and is consistent with the allosteric effect of G on agonist

high affinity of agonists on G_s activation. Nevertheless, we cannot exclude the possibility that similar changes in mB- β_2 AR fluorescence could result from different conformational changes in the β_2 AR.

The Effect of Ligand Efficacy on mB- β_2 AR- G_s Coupling

The efficiency with which mB- β_2 AR couples to G_s in rHDL particles in the absence of agonist reflects the intrinsic basal activity of the receptor. The high effective local concentration of G_s and the capacity to trap mB- β_2 AR in this active G_s -coupled state in the absence of guanine nucleotides has allowed us to visualize this complex. In cells, however, GDP concentrations exceed 10 μ M (93, 94), levels that would destabilize this complex as shown by the capacity of GDP to reverse the effects of G_s on mB- β_2 AR (*Fig. 2-3A*). Nevertheless, our ability to trap this complex and monitor it through a conformationally sensitive fluorescent reporter allows us to examine the effects of ligand efficacy on the formation and stability of this otherwise transient complex. For the purpose of our discussion, we will assume that a decrease in intensity and increase in λ_{\max} of mB- β_2 AR- G_s is a reflection of the stability of the active-state complex. This assumption is based on the observation that conditions known to disrupt interactions between β_2 AR and G_s such as GTP γ S and detergents result in an increase in intensity and a decrease in λ_{\max} (*Fig. 2-3A*). However, we acknowledge that changes in λ_{\max} and intensity may not reflect a proportional change in the affinity of β_2 AR for G_s . Moreover, it is possible that β_2 AR and G_s remain associated in a manner that is not detected by bimane labeled C265.

Agonist

In *Fig. 2-5* we compare the effects of an agonist, a neutral antagonist, and an inverse agonist on mB- β_2 AR- G_s . Because there is a slight variability in receptor to G protein stoichiometry between reconstitutions, these comparisons were performed on the same preparation. *Fig. 2-5A* shows the baseline spectrum of the mB- β_2 AR- G_s complex before (black) and after (gray) the addition of 10 μ M GTP γ S, which completely dissociates the complex. The reconstitutions are typically contaminated with a low concentration of GDP needed to stabilize purified G_s . This residual GDP can be removed by the addition of apyrase, a non-selective nucleotide pyrophosphatase. As shown in *Fig. 2-5A*, a 40-minute treatment with apyrase reverses the uncoupling effect of residual GDP resulting in a small decrease in the intensity of mB- β_2 AR (blue spectrum). The addition of the agonist ISO after apyrase treatment causes a further decrease in intensity and a rightward shift in λ_{\max} (*Fig. 2-5A*-green spectrum). The fact that ISO induces a change in fluorescence intensity on top of that induced by G_s and apyrase suggests that a higher fraction of mB- β_2 AR couples to G_s in the presence of agonist and/or the conformation of the β_2 AR in the presence of agonist and G_s is different from that in the presence of G_s alone. The maximal effect of ISO on mB- β_2 AR- G_s fluorescence occurs at a lower agonist concentration than observed in the absence of G_s (*Fig. 2-4A*).

Neutral Antagonist

By definition, neutral antagonists inhibit binding of agonists, partial agonists, and inverse agonists at the orthosteric-binding site of GPCRs, but do not alter their basal receptor activity. It has been difficult to identify true neutral antagonists for the β_2 AR, as

most compounds display some partial agonist or inverse agonist activity when applied in sensitive signaling assays (104, 105). Nevertheless, alprenolol comes very close to exhibiting the properties of a neutral antagonist, having only very weak partial agonist activity or weak inverse agonist activity, depending on the assay. Alprenolol has no significant effect on the fluorescence of mB- β_2 AR alone. The effect of alprenolol on mB- β_2 AR- G_s was examined following the removal of residual GDP with apyrase. As shown in **Fig. 2-5B**, alprenolol (purple spectrum) induces a small decrease in the intensity and red shift in the λ_{max} of mB- β_2 AR- G_s that has been treated with apyrase. Thus, alprenolol does not induce dissociation of the mB- β_2 AR- G_s complex, and may enhance mB- β_2 AR- G_s coupling to a small extent, perhaps related to a low partial agonist activity (105).

Inverse Agonist

In contrast to neutral antagonists, inverse agonists inhibit basal, agonist-independent activation of G proteins. Although the precise mechanism for the action of inverse agonists is not completely understood, they may prevent receptor-G protein complex formation, destabilize preformed complexes, or both. Here we studied the effect of ICI-118, 551 (ICI), one of the most efficacious inverse agonists for the β_2 AR, on mB- β_2 AR fluorescence (**Fig. 2-5C, red**). mB- β_2 AR was first treated with apyrase to remove residual GDP. Following a 60-min incubation at room temperature, ICI induced only a small reversal of the fluorescence change induced by G_s and apyrase (compare blue and red spectra). These data show that ICI is much less effective at disrupting pre-formed receptor-G protein complexes than is GTP γ S (**Fig. 2-5A**).

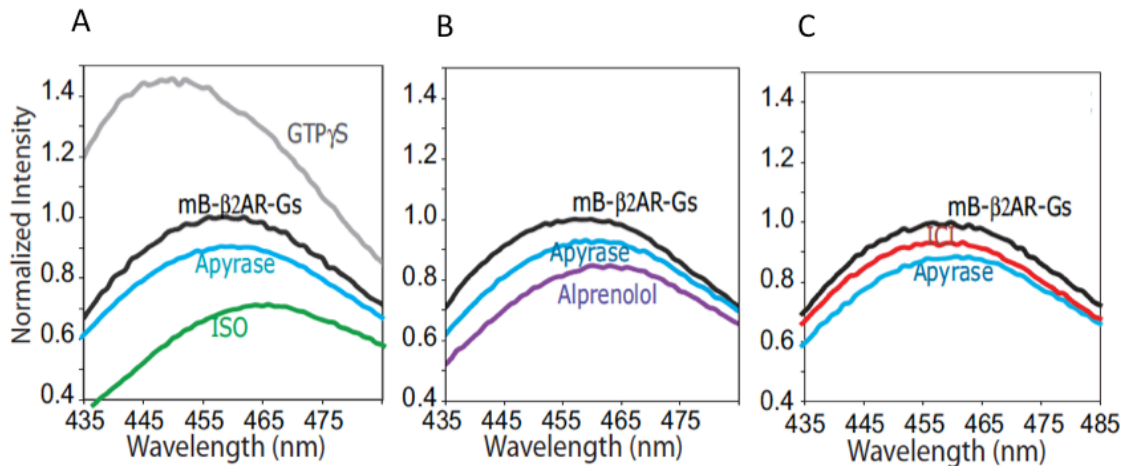


Figure 2-5. The effect of ligands having different efficacies on the stability of mB- β_2 AR-G_s. Emissions scans were performed on mB- β_2 AR-G_s complex obtained from a single reconstitution. For each treatment (apyrase, ISO, alprenolol, ICI or GTP γ S), a baseline scan was performed on mB- β_2 AR-G_s alone (black), and subsequent scans were taken 10 mins after the addition of 10 μ M GTP γ S to mB- β_2 AR-G_s (grey). This treatment uncouples G_s from mB- β_2 AR. The blue spectrum shows the effect of a 40-min incubation of mB- β_2 AR-G_s with apyrase to remove residual GDP. (A-C) The effect of ligands was determined after 40-min pretreatment with apyrase (blue) followed by a 60 min-incubation with the ligand. **A:** ISO, green; **B:** alprenolol, purple; **C:** ICI, red. This experiment is representative of 3 independent experiments.

The Effect of Ligands on β_2 AR-G_s Complex Formation

The relatively subtle effect of ICI on the stability of the receptor-G protein complex does not explain the inhibitory effect of ICI observed in signaling assays. An alternative mechanism for ICI efficacy is that the ICI-bound β_2 AR does not couple efficiently to G_s. To monitor the effect of ICI on the association of β_2 AR and G_s we incubated mB- β_2 AR reconstituted in rHDL in the presence or absence of ligands. We then added purified G_s and monitored changes in fluorescence. It should be noted that the

amount of G_s that can be added is limited by the detergent in the G_s preparation, consequently the G_s -induced changes in mB- β_2 AR are not as large as those observed before, where the reconstitution was done in the presence of a detergent binding resin. In the absence of ligand, G_s coupling to the β_2 AR results in an 18% decrease in intensity and a 5 nm red shift in λ_{\max} (**Fig. 2-6A**). The addition of 1 μ M ISO to mB- β_2 AR resulted in 25% decrease in intensity and a 7 nm red shift in λ_{\max} . The addition of G_s resulted in a further decrease in both intensity and λ_{\max} (**Fig. 2-6B**). In contrast, no change in fluorescence was observed following the addition of G_s to mB- β_2 AR that had been incubating for 15 min with 1 μ M ICI (**Fig. 2-6C**). Like ICI, the neutral antagonist alprenolol had no effect on mB- β_2 AR fluorescence. However, in contrast to ICI, alprenolol did not prevent G_s -induced changes in mB- β_2 AR intensity and λ_{\max} (**Fig. 2-6D**). In fact, these changes were larger than those observed for unliganded mB- β_2 AR, suggesting that alprenolol has partial agonist activity. We also examined carazolol, the less efficacious inverse agonist used to obtain the crystal structures of β_2 AR. We observed a small (approximately 3%) decrease in intensity, but no change in λ_{\max} following incubation with 1 μ M carazolol (**Fig. 2-6E**). G_s induced a further 5% decrease in intensity and a 2-nm shift in λ_{\max} . In comparison to unliganded receptor (**Fig. 2-6A**), carazolol appeared to partially inhibit G_s -induced changes, but it was not as effective as ICI. These results show that the inverse agonist ICI is most efficacious in preventing G_s -induced changes in mB- β_2 AR, underlying its effectiveness as an inverse agonist in signaling assays.

It should be noted that the lack of changes in mB- β_2 AR fluorescence on binding to ICI does not necessarily mean that ICI does not induce or stabilize a conformation distinct from unliganded receptor. It only suggests that conformational changes occurring on ICI binding may not result in a change in the environment around bimeane on C265. Previous studies using different fluorescent approaches observed conformational changes on binding ICI to the β_2 AR (106) and yohimbine for the α_{2A} adrenergic receptor (107). The fact that ICI prevents G_s -induced conformational changes observed in the absence of ligand is consistent with a distinct conformation that is incompatible with G protein binding.

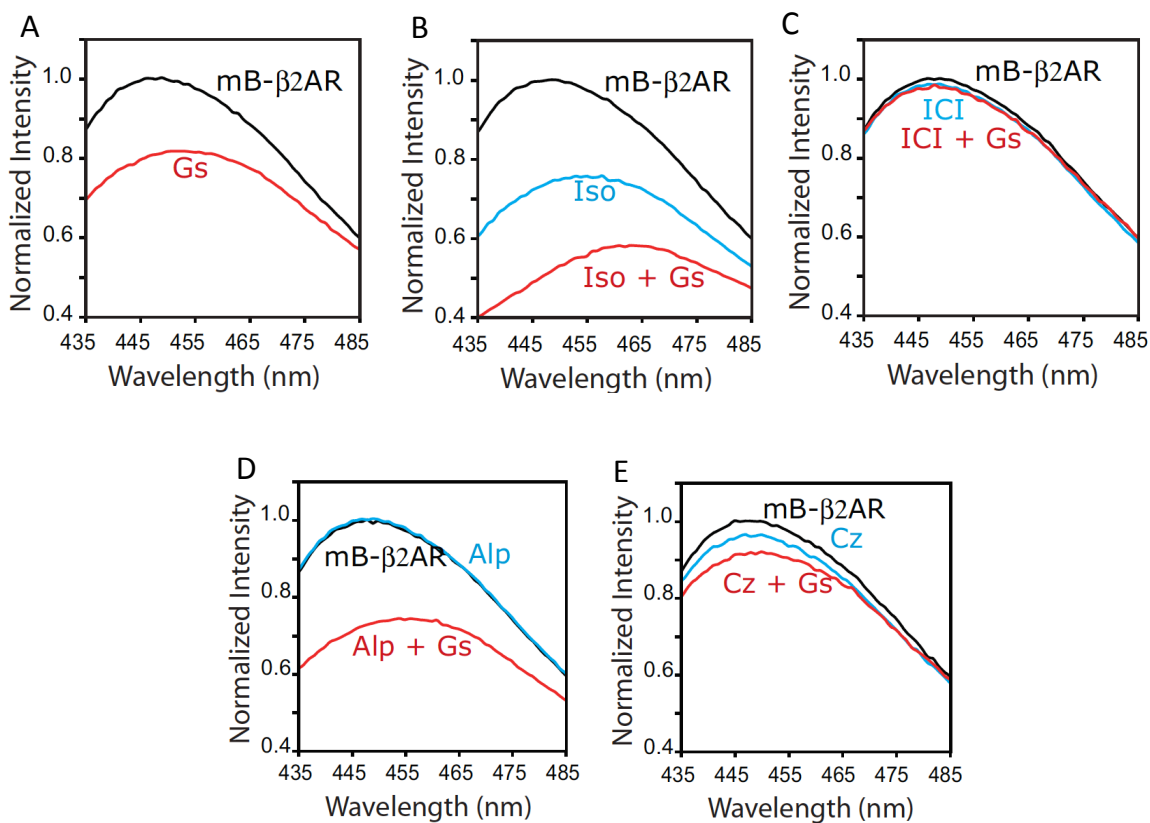


Figure 2-6. The effect of ligand efficacy on G_s induced changes in $mB-\beta_2R$ fluorescence. Initial emission scans (black spectra) of $mB-\beta_2R$ were obtained, and then scans were repeated after 15 min incubation with the indicated ligands (blue spectra). A concentrated solution of G_s was added (1:100 dilution of an 8 mg/ml solution), and emission scans were repeated after 8 min (red spectra). Preliminary studies showed that the effect of G_s was complete at 6 min. Ligands: **A**, no ligand; **B**, agonist-isoproterenol (ISO); **C**, inverse agonist-ICI 118, 551; **D**, antagonist-alprenolol (Alp); **E**, inverse agonist-carazolol (Cz). These scans are representative of 3 independent experiments.

Discussion

Structural Insights into the Active State Complex, Basal Activity, and Ligand Efficacy

The mechanism by which G_s couples to agonist-free receptor is not known. However, the recent structure of a complex between opsin and the carboxyl-terminal peptide of transducin, suggests that substantial conformational changes are needed in the inactive structure of the β_2 AR to permit similar docking of the carboxyl terminus of $G\alpha_s$ (**Fig. 2-1 C and D**). Evidence from fluorescence lifetime studies on purified β_2 AR shows that the unliganded receptor is structurally dynamic (108) and exists in an ensemble of basal conformational states (**Fig. 2-7**). Although an active state represents a minor fraction of this ensemble (**Fig. 2-7A**), this state would be stabilized by binding to either the agonist ISO (**Fig. 2-7B**) or the G protein G_s (**Fig. 2-7C**).

As shown in **Fig. 2-5A**, the agonist ISO (green spectrum) induces a further decrease in the fluorescence intensity and increase in the λ_{\max} of the mB- β_2 AR- G_s complex. This result could be because of a change in the equilibrium favoring the formation of the complex (**Fig. 2-7D**), or to a further change in the structure of the mB- β_2 AR- G_s complex. Nevertheless; these observations have implications for our efforts to obtain a high-resolution structure of the active state of the β_2 AR and possibly other GPCRs. Saturating concentrations of an agonist alone (**Fig. 2-7B**) cannot induce the same change in intensity and λ_{\max} as that stabilized by both G_s and ISO (**Fig. 2-7D**). The results are in agreement with earlier fluorescence lifetime studies on the β_2 AR showing that the agonist ISO does not stabilize a single active conformation (108).

Analogous to agonists, the inverse agonist ICI binds to and stabilizes a minor fraction of the ensemble of basal conformational states, in this case corresponding to an inactive conformation (**Fig. 2-7E**). The capacity of ICI to prevent formation of the mB- β_2 AR- G_s complex (**Fig. 2-6C**) suggests that the conformation stabilized by the inverse agonist cannot couple to G_s (**Fig. 2-7F**) or it could couple but just not cause a change in bimane fluorescence. Similarly, the fact that 1 μ M ICI (100-fold greater than the K_d) has little effect on the pre-formed mB- β_2 AR- G_s complex (**Fig. 2-5C**) suggests that it cannot bind to G_s coupled to β_2 AR (**Fig. 2-7F**). This result is in agreement with predictions of the extended ternary complex model (109, 110) and previous studies showing that binding site for [3 H]-ICI-118, 551 were reduced in cells expressing high levels of G_s (~49% less) (111). These effects can also be rationalized in light of the crystal structure of the inactive, inverse agonist-bound β_2 AR. ISO is ~40% smaller in volume than the inverse agonist carazolol present in the β_2 AR crystal structure. Thus, the binding site of the ICI-bound receptor will have to readjust to satisfy all of the binding interactions predicted for the agonist ISO (42).

Relevance to Cellular Signal Transduction

We were able to characterize the properties of the mB- β_2 AR- G_s complex by reconstituting purified β_2 AR and G_s under conditions that would not be found in living cells. Reconstitutions were performed at low GDP concentrations, and GDP was further reduced using apyrase. In the presence of apyrase, virtually all G_s will be in the nucleotide-free state (**Fig. 2-7H**). Thus, it is possible to trap both unliganded and agonist-bound mB- β_2 AR- G_s complexes. Under these conditions, the inverse agonist is unable to

disrupt the complex (**Fig. 2-7G**), most likely because of allosteric effects of the G protein on the ligand-binding pocket. However, in the context of a cell, where concentrations of GDP may exceed 10 μM , the formation of an active state $\beta_2\text{AR-G}_s$ complex will be a relatively rare event governed by 2 equilibria: 1 for the conformational transition to an active state of the receptor (**Fig. 2-7A**), and 1 for the formation of the nucleotide free G_s (**Fig. 2-7H**). Once the nucleotide-free $\beta_2\text{AR-G}_s$ complex is formed, it would be rapidly disrupted on binding of either GDP or GTP. Thus, although ICI may not be able to disrupt the $\beta_2\text{AR-G}_s$ complex, these complexes are relatively rare and short-lived. ICI efficacy is due primarily to binding to the receptor and stabilizing a conformation that is unable to couple to G_s (**Fig. 2-7E**). In contrast, agonists facilitate the formation of the complex by increasing the fraction of $\beta_2\text{AR}$ in an active conformation (**Fig. 2-7B**), and possibly by stabilizing a conformation in the $\beta_2\text{AR}$ that allosterically reduces the affinity of G_s for GDP.

In conclusion, our studies examine the allosteric interaction between ligand binding and G_s coupling by using a conformational reporter on the $\beta_2\text{AR}$, and they provide a structural framework for understanding the concept of basal activity and ligand efficacy. Agonists and G_s induce similar changes in the fluorescence intensity and λ_{max} of mB- $\beta_2\text{AR-G}_s$, suggesting they may induce a similar conformational change involving TM6. The complex formed between the $\beta_2\text{AR}$ and G_s in the absence of agonist is stable in the absence of guanine nucleotides. A neutral antagonist has little effect on the formation of the $\beta_2\text{AR-G}_s$ complex, whereas an inverse agonist prevents complex formation. Neither agonist nor inverse agonist promotes complex dissociation, whereas both GDP and GTP rapidly reverse G_s induced changes in mB- $\beta_2\text{AR}$ fluorescence. These findings

provide insights into the structural basis of drug efficacy, that is, how different chemical structures are ultimately translated into divergent behaviors through the modulation of the interaction between the receptor and the G protein.

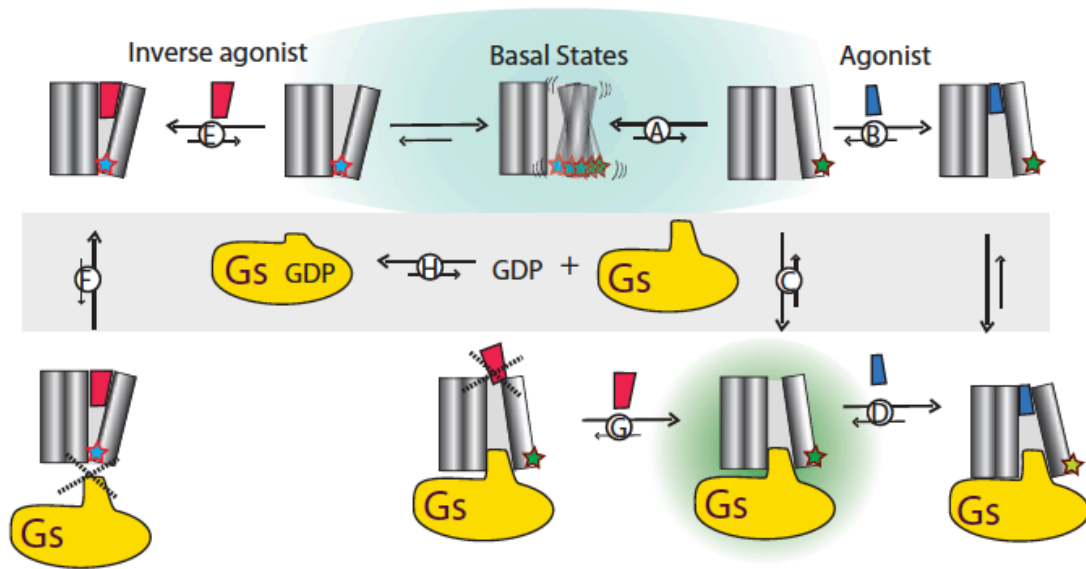


Figure 2-7. Conceptual model depicting the dynamic behavior of β_2 ARs. In the absence of a ligand, the β_2 AR exists in an ensemble of basal states in dynamic equilibrium (cyan background). Agonists and inverse agonists bind to and stabilize distinct substates. The nucleotide-free form of the G protein G_s can also bind to and stabilize an active state of the β_2 AR (green background). The different equilibrium processes between the receptor and its ligands and the receptor and the G protein G_s are displayed (A–H) and are described in detail in the text. Note that for each of these equilibria, the relative size of the arrows indicates the displacement of the reaction.

Materials and Methods

For this thesis I purified all preparations of Apo A-I and CFP- β_2 AR. I also performed all vesicle and rHDL reconstitutions, radioligand binding assay (initially with the assistance of Matthew Whorton), and some fluorescence spectroscopy studies. Purified heterotrimeric G_s were generously provided by Diane Calinski and Brian DeVree from the University of Michigan. Drs Xiao-Ji Yao and Soren Rasmussen from Stanford University purified and biotinyl-labeled the β_2 AR. They also performed some fluorescence spectroscopy assays. Xavier Deupi from the Universitat Autònoma de Barcelona generated a model for the activated β_2 AR, based on the opsin structure.

Materials

G protein baculoviruses encoding $G\alpha_s$, His-6- $G\beta_1$ and $G\gamma_2$ were provided by Dr. Alfred G. Gilman (UT Southwestern, Dallas, TX). All lipids were purchased from Avanti Polar Lipids (Alabaster, AL). Dodecylmaltoside (DDM) was obtained from Dojindo Molecular Technologies (Gaithersburg, MD). Sodium cholate was purchased from Sigma. \pm -alprenolol, (-)-isoproterenol, and ICI-118 551, were obtained from Sigma (St. Louis, MO). [3 H] Dihydroalprenolol and [35 S]GTP γ S were obtained from Perkin Elmer (Foster City, CA). Monobiotinylated biotinyl was purchased from Invitrogen (Carlsbad, CA). All other reagents of analytical grade were obtained from Fisher Scientific (Pittsburg, PA).

Expression, Purification and Labeling of β_2 AR

A modified version of the β_2 AR where 4 reactive cysteines were mutated (C77V, C327S, C378A, and C406A) was made. The modified β_2 AR was expressed in Sf9 insect cells by using recombinant baculovirus and solubilized using methods previously described (99). The DDM solubilized receptor was purified by sequential M1-Flag antibody affinity and alprenolol affinity chromatography as described (99). Briefly, CaCl_2 was added to the DDM solubilized extract to a final concentration of 1 mM and loaded to the M1-Flag column. The receptor was eluted from the flag resin with 20 mM HEPES pH 7.5, 100 mM NaCl, 0.1% dodecylmaltoside (Buffer A) with 1 mM EDTA. The concentration of functional, purified receptor was determined using a saturating concentration (10 nM) of [^3H] DHAP as previously described (112). Flag purified receptor was then purified by alprenolol-Sepharose chromatography as described (112). The receptor was eluted from alprenolol-Sepharose with Buffer A with 300 μM alprenolol and 1 mM CaCl_2 and loaded directly onto M1-Flag resin. The M1-Flag resin was washed with Buffer A to remove free alprenolol and eluted with Buffer A plus 1 mM EDTA. Two liters of sf9 cells typically yield 500 μl of a 5 μM solution of β_2 AR.

The purified β_2 AR and monobromobimane were mixed at the same molarity and incubated overnight on ice in the dark. The fluorophore-labeled receptor was purified immediately before use by gel filtration.

Purification of Recombinant ApoA-I

Wild type human apoA-I was purified from expired serum as previously described (70). A recombinant apoA-I with an N-terminal 43 amino acid deletion and a

hexa-histidine tag ($\Delta(1-43)$ -His₆-apoA-I) was expressed using a pET15b vector to transform competent *Escherichia coli* cells (BL21). Cells were resuspended and lysed by gently vortexing in 10 mM Tris-HCl pH, 8.0, 100 mM NaH₂PO₄, 6 M guanidine hydrochloride (GuHCl), 1 % Triton X-100. Lysate was fractionated by centrifugation at 10, 000xG and the supernatant was loaded onto a Ni-NTA column by gravity flow. The column was washed with 10 mM Tris-HCl, pH 7.0, 100 mM NaH₂PO₄, 6 M guanidine hydrochloride, 1 % Triton X-100 and then with 50 mM NaH₂PO₄, pH 8.0, 300 mM NaCl and 1 % Triton X-100. Bound $\Delta(1-43)$ -his₆-apoA-I was eluted with 50 mM NaH₂PO₄, pH 8.0, 300 mM NaCl, 250 mM imidazole, 1 % Triton X-100. Peak fractions were further purified on a Superdex 75 gel filtration chromatography column in 20 mM HEPES, pH 8.0, 100 mM NaCl, 1 mM EDTA, 20 mM sodium cholate. Pooled apoA-I was then dialyzed against 20 mM HEPES, pH 8.0, 100 mM NaCl, 1 mM EDTA, 5 mM sodium cholate. Purified apoA-I was concentrated to ~10 mg/mL and stored at -80°C until use.

In vitro Reconstitution of β_2 AR into rHDL

High-density lipoproteins were reconstituted *in vitro* according to a protocol adapted from Jonas (113). Briefly, a mixture of POPC and POP (3:2 molar ratio) in combination was used to mimic the zwitterionic environment of a cell membrane (114). A typical rHDL reconstitution consisted of the following components: 24 mM detergent (cholate or DDM), 8 mM lipids, and 100 μ M apoA-I. Lipids were solubilized with a solution of 20 mM HEPES, pH 8.0, 100 mM NaCl, 1 mM EDTA and 50 mM detergent. To reconstitute the bimeane labeled β_2 AR (mB- β_2 AR), purified apo A-I was added to at least 10-fold excess ([apoA-I]:[β_2 AR]) to receptor preparations diluted in solubilized

lipids. Following an incubation of 1-2 hrs at the TM of the lipid combination, samples were added to an equal volume of hydrated BioBeads (BioRad) for an additional 3 hrs to remove detergents, resulting in the formation of rHDL particles. Samples were stored on ice until used. If necessary, β_2 AR-rHDL particles were separated from receptor-free rHDL by M1-anti-Flag immunoaffinity chromatography. Purified β_2 AR-rHDL particles were eluted with EDTA (10 mM) and stored on ice until further use.

Saturation Radioligand-Binding Assays

Binding reactions were prepared in 100 μ l volumes in 96 well plates. Samples were incubated with various concentrations of β_2 AR antagonist [3 H] dihydroalprenolol ([3 H]DHAP) (0.1-20nM) in 50 mM Tris-HCl, pH 8.0, 150 mM NaCl (TBS) [or TBS with 1 % DDM for detergent solubilized binding). Nonspecific binding was determined in the presence of 10 μ M propranolol. Receptor particles were incubated for 90 minutes at RT for saturation isotherms. For separating free [3 H]DHAP from bound, the samples were filtered on glass fiber plates.

For glass filtering, GF/B 96 well filter plates (Whatman) were used in conjunction with a vacuum manifold. Wells were prewetted with 0.3 % polyethyleneimine (PEI) for 30 minutes. Samples were applied and washed 3X with 200 μ l of TBS. Scintillation mixture cocktail was added (Microscint0, Packard) and plates were counted on a TopCount scintillation counter (Packard). Specific binding was determined by subtracting nonspecific binding from total binding.

G protein Reconstitution

Purified G_s heterotrimer (115) (stored in 0.02% DDM) was reconstituted into preformed impure β₂AR-rHDL particles (containing excess empty rHDL particles) at an initial R:G ratio of 1:10. Concentrated G_s stocks were added such that the DDM was diluted at least 200-fold to reduce the DDM concentration to well below the CMC (0.12-0.18 mM or 0.006% in NaCl containing buffer). This had no effect on the integrity of the particles, as assessed by size exclusion chromatography (not shown). Treatment of G_s-reconstituted samples with BioBeads, to remove trace amounts of DDM, before gel filtration chromatography had no effects on the results. Nucleotide-free β₂AR-G_s complex was prepared by incubating with 1 unit of apyrase (NEB, Ipswich, MA) in the presence of 1 mM MgCl₂ (final concentration) for 30 minutes at room temperature.

Agonist Competition Assays

Agonist competition assays were performed on G protein-reconstituted samples under similar conditions as used in the saturation binding assays except that a fixed concentration of [³H] DHAP (2 nM) was competed with various concentrations of isoproterenol (1 X 10⁻¹² – 1 X 10⁻³ M) with or without the addition of 10 μM GTPγS. Binding reactions contained 0.02% ascorbic acid to prevent oxidation of the isoproterenol. Samples were incubated for 90 minutes at RT and then filtered on glass fiber plates as above. Normalized data were fitted to a two-site competition-binding model using Prism (GraphPad).

Fluorescence Spectroscopy

Fluorescence spectroscopy experiments were performed on a Spex FluoroMax-3 spectrofluorometer (Jobin Yvon, Inc) with photon counting mode by using an excitation and emission bandpass of 4 nm. For each scan, the final concentration of receptor ranges from 50-100 nM. For emission scans, excitation was set at 370 nm and emission was measured from 435-485 nm with an integration time of 0.5 s/nm. To determine the effects of ligands, the spectra were taken after 15 minutes incubation with the drugs. For time course experiments, excitation was set at 370 nm, and emission was monitored at 450 nm. All experiments were performed at 25°C (RT), and the sample underwent constant stirring. Fluorescence intensity was corrected for dilution by ligands in all experiments and normalized to the initial value. Fluorescence intensity was corrected for background fluorescence from buffer and ligands in all experiments. All of the compounds tested had an absorbance of <0.01 at wavelengths between 370 nm and 485 nm at the used concentrations, excluding any inner filter effect in the fluorescence experiments.

CHAPTER 3

ALLOSTERIC REGULATION OF AGONIST, INVERSE AGONIST AND ANTAGONIST BINDING TO THE β_2 AR BY G PROTEINS

Introduction

The recognition of hormones by G protein-coupled receptors and the occupation of solvent accessible surfaces within the binding pocket by ligands serve as the basis for their affinity, specificity and efficacy. The diverse binding pockets offered by the complex superfamily of GPCRs make these cell-surface receptors prime therapeutic targets. Recent crystallographic evidence highlights the network of van der Waals contacts and ionic interactions between ligands and residues within the receptor core containing the binding pocket. A comparison of the structures of the inactive photoreceptor, rhodopsin and ligand-bound forms of the three receptors (all inverse agonists) β_2 -adrenergic receptor (β_2 AR), β_1 -adrenergic receptor (β_1 AR) and adenosine A₂ receptor (A₂AR) (84-86) with that of the 'post' photo-activated form of rhodopsin, opsin (116) reveals striking differences within the receptor core. Alteration of the structure of the receptor core leads to dramatic rearrangement of the intracellular face of the receptor and facilitates binding of the C-terminal region of the G protein α -

subunit. These data suggest that the inactive state of the photoreceptor, like the state stabilized by an inverse agonist bound to hormone receptors, is incompatible with productive interactions with G proteins. Indeed, photoactivation of rhodopsin induces or stabilizes an active conformation of the receptor and therefore promotes G protein interactions, where G proteins modulate agonist binding in a thermodynamic-cooperative manner.

The behavior of neutral antagonists, however, is quite puzzling, as it is hypothesized that they maintain high affinity binding properties without displaying intrinsic effects on G protein activation. Most neutral antagonists, like inverse agonists, take the advantage of the large surface area within the binding site to enhance binding affinity and do not stabilize conformations that allow G protein coupling.

One property that many agonists display upon binding is their capacity to inhibit radio-labeled antagonist probes in a multiphasic manner, displaying a nucleotide-sensitive high affinity state and a nucleotide-insensitive low affinity state. The biphasic agonist inhibition curve and the nucleotide-dependent shift of the high affinity site to the low affinity site serve as the basis for the Ternary Complex Model (TCM) of GPCR activation (23, 117). The TCM is based on the assumption that radiolabeled antagonists bind independently of the G protein-coupling state of the receptor and that binding may be competitively inhibited by agonists with high affinity. However, increases in antagonist and inverse agonist binding due to the addition of guanine nucleotides are present in the literature (32-34, 118-122). Indeed, observed increases in radiolabeled antagonist binding are significant enough to force investigators to normalize their data in

order to compare the affinities of competing ligands in the presence or absence of nucleotides.

We have previously demonstrated that the nucleotide-free form of the G protein-receptor complex represents the high affinity agonist state, consistent with the notion that G proteins allosterically enhance agonist binding (63). In addition, we demonstrated that the efficacy of the inverse agonist to decrease G protein activation is based on the capacity of the inverse agonist to stabilize an inactive conformation of the receptor. Productive G protein-receptor interactions would therefore be prevented by the stabilized inactive receptor conformation. These data would imply that inverse agonist binding, as with agonist binding, must be modulated by G protein coupling and therefore display biphasic binding in competition assays with radiolabeled antagonists. However, ICI-118,551, the most efficacious inverse agonist for the β_2 AR, potently prevents G protein-induced formation of the active formation of β_2 AR, but displays single-phase inhibition of [3 H] dihydroalprenolol binding (123, 124).

Taken together these data prompted the question as to whether radiolabeled antagonist probes for GPCRs truly evaluate the multiple conformations receptors may adopt. Here we investigate the central dogma of GPCR binding theory and determine whether antagonist binding, like that of agonists and inverse agonists, are altered by interactions with G proteins. We demonstrate that the neutral antagonists for the β_2 AR, alprenolol binds with high affinity but in a conformational-dependent manner, preferring the native receptor. In its coupled state, i.e. when bound to a G protein in the G protein's nucleotide-free state, alprenolol is incapable of binding to the β_2 AR.

Results

Formation of the Nucleotide-Free β_2 AR-G_s Complex in rHDL

We have previously reported the identification of a nucleotide-free form of the β_2 AR-G_s complex in rHDL particles by fluorescence spectroscopy using the conformational sensor bimane-labeled β_2 AR (63). Next we established that the non-selective nucleotide phosphatase, apyrase, facilitates the removal of high amounts of nucleotide (100 μ M GDP) in conditions that resemble those from reconstituted β_2 AR-G_s complex in rHDL. Analysis of GDP hydrolysis to GMP was completed within 10 min as demonstrated by an ion exchange chromatography (Mono Q) separation (**Fig. 3-1A black vs. blue trace**). Addition of a 10-fold excess of pyrophosphate (PPO₄) that is thought to bind the active site of apyrase inhibited nucleotide hydrolysis (**Fig. 3-1A red trace**). However, PPO₄ was not used in subsequent experiments due to its interaction with G_s that could affect our results.

Apyrase-treated β_2 AR-G_s-rHDL was subjected to anti-Flag affinity chromatography to remove apyrase and the products of the GDP degradation (GMP and PO₄⁻). Nucleotide-bound states of the G protein were assessed through monitoring both the kinetics and degree of [³⁵S]GTP γ S binding.

In the absence of ligand, [³⁵S]GTP γ S binding to β_2 AR-G_s appeared to be complete within the first time point measured accurately (10 s), suggesting that the half time of association of [³⁵S]GTP γ S is less than 5 seconds. The kinetics of GTP γ S binding appear to superimpose on top of increases in bimane fluorescence in response to the addition of GTP γ S to β_2 AR-G_s in rHDL, the rate of which appears to be diffusion limited (**Fig. 3-1B**). Thus, to a first approximation the rapid binding event suggests that the complex is

devoid of nucleotide, based on the limited temporal resolution of the technique employed that consists of series of mixing and filtration steps. [³⁵S]GTP γ S binding to the reconstituted complex yields a final R:G ratio of 1:0.95 (50 fmol:59 fmol), suggesting that up to 95 % of the β_2 AR-rHDL particles contain a single-functional G protein. This suggests that only those G proteins associated with the β_2 AR will bind [³⁵S]GTP γ S within this time frame in the absence of ligands (agonists). The total G protein content was determined following particle disruption and solubilization with detergent C₁₂E₁₀. The preparations used in these series of experiments have a R:G ratio of ~1:3.

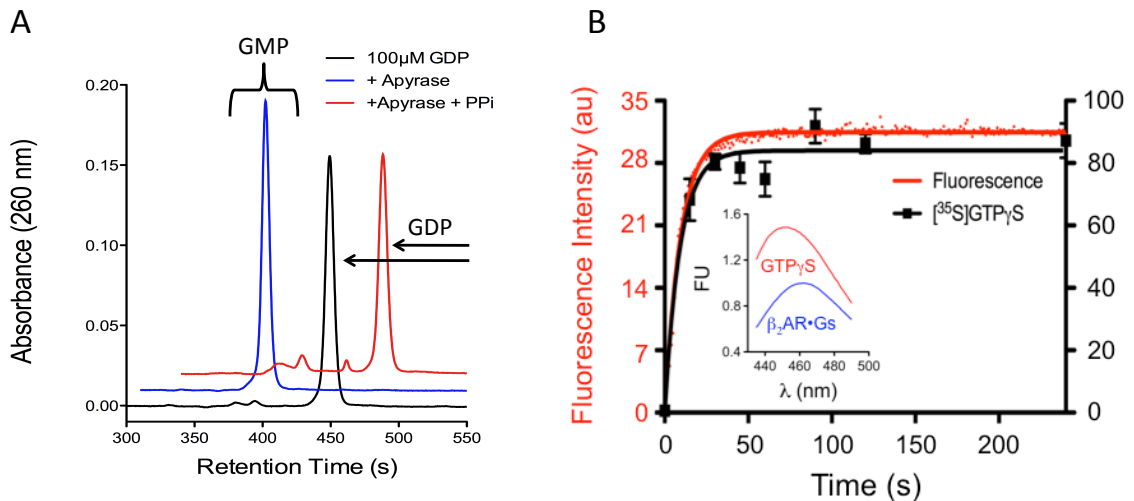


Figure 3-1. The β_2 AR-G_s complex is nucleotide-free. Removal of nucleotides by apyrase treatment. **A)** Ion exchange chromatogram (Mono Q) after hydrolysis of 100 μ M GDP to GMP by apyrase in 10 mins at RT. Pyrophosphate inhibits apyrase-mediated GDP hydrolysis. **B)** Nucleotide uptake kinetics. Uncoupling of G_s from bimane- β_2 AR (mB- β_2 AR) with 10 μ M GTP γ S induces an increase in bimane fluorescence as describe previously (*inset*, (63)). The rapid GTP γ S-induced increase in bimane fluorescence (red tracing) is superimposable onto the rapid binding of [³⁵S]GTP γ S measured by filter binding. The rapid binding strongly suggests that the G protein in the β_2 AR-G_s complex is nucleotide-free.

Nucleotide Sensitivity of Antagonist Binding to the β_2 AR-G_s Complex

After isolation of the nucleotide-free β_2 AR-G_s complex, we investigated how removal of nucleotide influences [³H]dihydroalprenolol ([³H]DHAP) binding to the reconstituted complex in rHDL. A series of experiments were devised where a β_2 AR preparation experiences G protein coupling, removal of free GDP (through the addition of apyrase), followed by the re-addition of high concentrations of GDP. The capacity of [³H]DHAP to bind to the β_2 AR was assessed at each step as illustrated in Figure 3-2 and 3-3. Figure 3-2 clearly illustrates that the addition of as little as 100 nM GDP to reconstituted β_2 AR-G_s complex in rHDL results in increased [³H]DHAP binding. Removal of free GDP appears to decrease antagonist binding where the decrease occurs in a time-dependent manner. The release of nucleotide appears to be rate-limiting since apyrase is capable of completely hydrolyzing 100 μ M GDP within 10 mins under identical assay conditions (*Fig. 3-1A*).

The release of nucleotide, as a result of the formation of the G protein-receptor complex is due to the basal receptor activity. Only through the subsequent addition of high concentrations of GDP (100 μ M) can [³H]DHAP binding be restored, albeit not completely. Apyrase will likely continue to degrade GDP during the entirety of the equilibrium of the binding assay. To date there is no known apyrase inhibitor that can effectively inactivate the enzyme without having adverse effects on receptor activity and/or ligand binding capabilities.

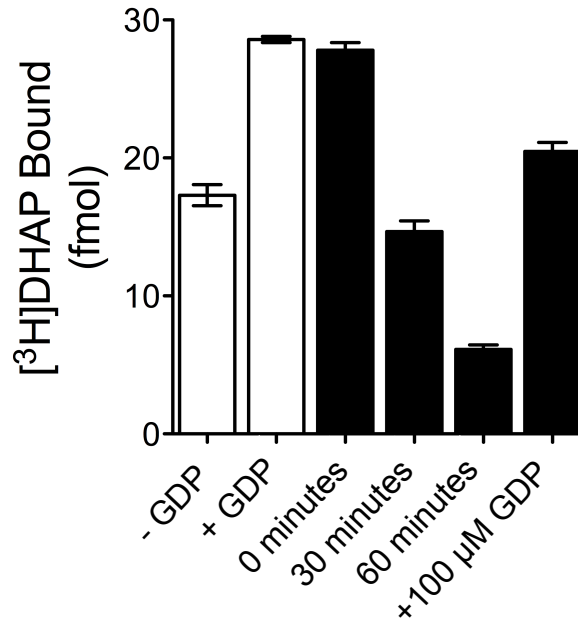


Figure 3-2. Role of nucleotides on [³H]DHAP binding to β_2 AR- G_s : Removal of nucleotides by apyrase treatment. Purified G_s was added to reconstituted β_2 AR and allowed to incubate as described in the Materials and Methods section. Samples were incubated with 100 nM GDP then subjected to apyrase treatment for various times (as indicated). Following apyrase treatment 100 μ M GDP was added to the sample. All the samples were assayed for their capacity to bind 2 nM [³H]DHAP after each sequential step.

The ability to observe a nucleotide effect on [³H]DHAP binding appears largely dependent on the basal activity of the β_2 AR and propensity to induce GDP dissociation from G_s and on the availability of GDP. Since no exogenous GDP is added to our assays the effective GDP concentration is equal to the concentration of G protein added to the mixture (~1-3 nM). As GDP is being released through basal receptor activation, it is therefore diluted to levels below its affinity leaving the G protein nucleotide-free. This nucleotide-free form of the G protein is stabilized by the active conformation of the β_2 AR, and vice-versa. It is this nucleotide-free form of the β_2 AR- G_s complex that represents the high affinity agonist state of the β_2 AR (63).

It is well established that high-affinity agonist binding may be disrupted by guanine nucleotides binding to G protein α -subunits. Heterotrimeric G protein α -subunits bind several forms of guanine nucleotides but display a higher affinity for the triphosphate forms. Here we demonstrate that GTP γ S binds with a higher apparent affinity than GDP ($EC_{50} \sim 35 \pm 14$ nM and $\sim 384 \pm 131$ nM, respectively) to the nucleotide-free forms of G_s , as indicated by the capacity of the guanine nucleotides to increase [3 H]DHAP binding (**Fig. 3-3**). Although the affinity difference between GDP and GTP γ S for G_s is documented (32), the affinities for the nucleotide-free form (in the lower nanomolar range as reported here) have not. The difficulty in generating affinities for the nucleotide-free $G\alpha$ -subunits is likely related to the inability to isolate a nucleotide-free form of the G protein.

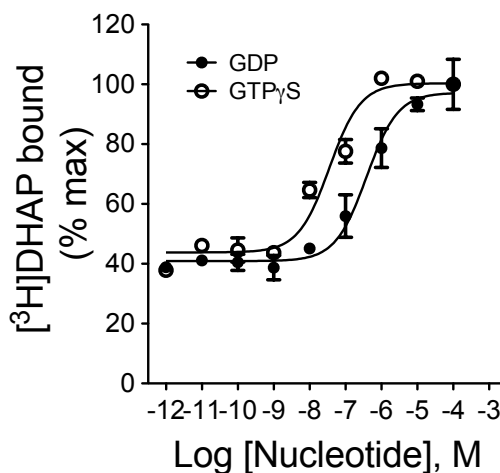


Figure 3-3. Role of nucleotides on [3 H]DHAP binding to β_2 AR- G_s : Dose response. Concentration-dependent effect of nucleotides on [3 H]DHAP binding to the β_2 AR- G_s complex. Apyrase-treated β_2 AR- G_s in rHDL was assayed for its capacity to bind 2 nM [3 H]DHAP at increasing concentrations of either GDP or GTP γ S. [3 H]DHAP binding is expressed as a percent of maximal binding defined by GTP γ S binding (maximal binding observed).

Saturation isotherms on apyrase-treated and purified $\beta_2\text{AR-G}_s$ complexes in rHDL suggest that [^3H]DHAP binds with comparable affinities in the absence or presence of 10 μM GTP γ S: 0.41 ± 0.10 nM and 0.66 ± 0.77 nM, respectively (**Fig. 3-4A-B**). However, uncoupling G_s from the $\beta_2\text{AR}$, through the addition of guanine nucleotides (GTP γ S), results in a dramatic increase in the maximal number of [^3H]DHAP binding sites ($B_{\text{max}} \sim 16.6 \pm 1.9$ fmol), compared to control ($B_{\text{max}} \sim 5.5 \pm 0.52$ fmol). Non-apyrase treated and freshly reconstituted $\beta_2\text{AR}$ particles containing G_s , or purified $\beta_2\text{AR-G}_s$ complexes in rHDL assayed in the presence of 100 nM GDP reveals significantly diminished effects of GTP γ S on [^3H]DHAP binding (**Fig. 3-4C & D**).

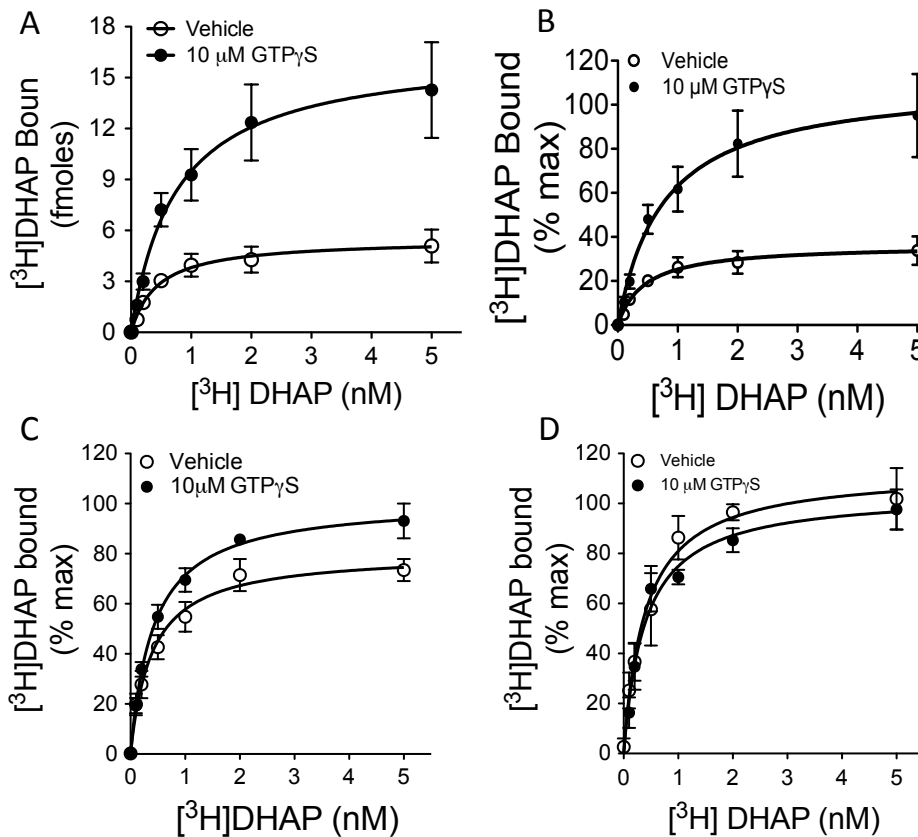


Figure 3-4. Addition of nucleotides increases the apparent B_{max} for [3 H]DHAP but has no effect on its affinity. **A-B)** Purified $G_s\alpha\beta_1\gamma_2$ (G_s) was added to β_2 AR reconstituted in rHDL and later treated with apyrase, as described in the Materials and Methods section. Empty rHDL particles were separated from β_2 AR-containing particles by anti-FLAG immunoaffinity column. Mixtures of reconstituted β_2 AR- G_s and β_2 AR alone were incubated with increasing concentrations of [3 H]DHAP as indicated. Non-specific binding was determined using 10 μ M propranolol. [3 H]DHAP binding is expressed in fmoles **B)** Data presented as percent of maximal binding defined by the total binding observed in the presence of GTP γ S (highest binding observed). **C)** [3 H]DHAP saturation analysis of mixtures of reconstituted β_2 AR- G_s and β_2 AR as in **A)** but in the presence of 100 nM GDP. **D)** [3 H]DHAP saturation analysis of β_2 AR reconstituted in HDL with G_s were performed immediately after reconstitution (without apyrase).

These data suggest that the nucleotide-free G protein stabilizes a form of the receptor that is less capable of binding [³H]DHAP. Only upon uncoupling of the G protein, through the addition of guanine nucleotides can [³H]DHAP binding be fully restored.

The conformational selectivity that G_s imparts on [³H]DHAP binding is not an artifact of the rHDL reconstitution system since similar effects of nucleotides can be observed in vesicle reconstitutions with model bilayers, native lung membranes and in Sf9 cells co-expressing β₂AR, and G_s heterotrimer (**Fig. 3-5A-C**). Freshly reconstituted β₂AR in lipid vesicles revealed no qualitative difference in the number of receptor sites or any differences in K_d for [³H]DHAP (not shown). Enriching the nucleotide-free form of the complex through pre-incubation of receptor and G protein, or by accelerating the process with the addition of apyrase, decreases the B_{max} for [³H]DHAP by approximately ~47%, consistent with observations in rHDL particles. The change in B_{max} observed in vesicles is not as dramatic as in reconstituted β₂AR and G_s in rHDL particles. This discrepancy may be accounted for the inaccessibility of G proteins to the luminal side of the vesicle that contains the intracellular face of the receptor or perhaps the larger surface area of the vesicles. The inner diameter of the membrane portion of an rHDL particles is ~ 85 Å (125) which is similar to the G_sαβ₁γ₂ heterotrimer (> 80 Å from tip to tip). By spatially restricting the trimer to a β₂AR-containing rHDL particle we maintain a high β₂AR:G_s stoichiometry, increasing the probability that the receptor may productively collide with G_s and lead to GDP release from the α-subunit, as in the presence of apyrase, a stable complex between the nucleotide-free G_s and β₂AR may be isolated.

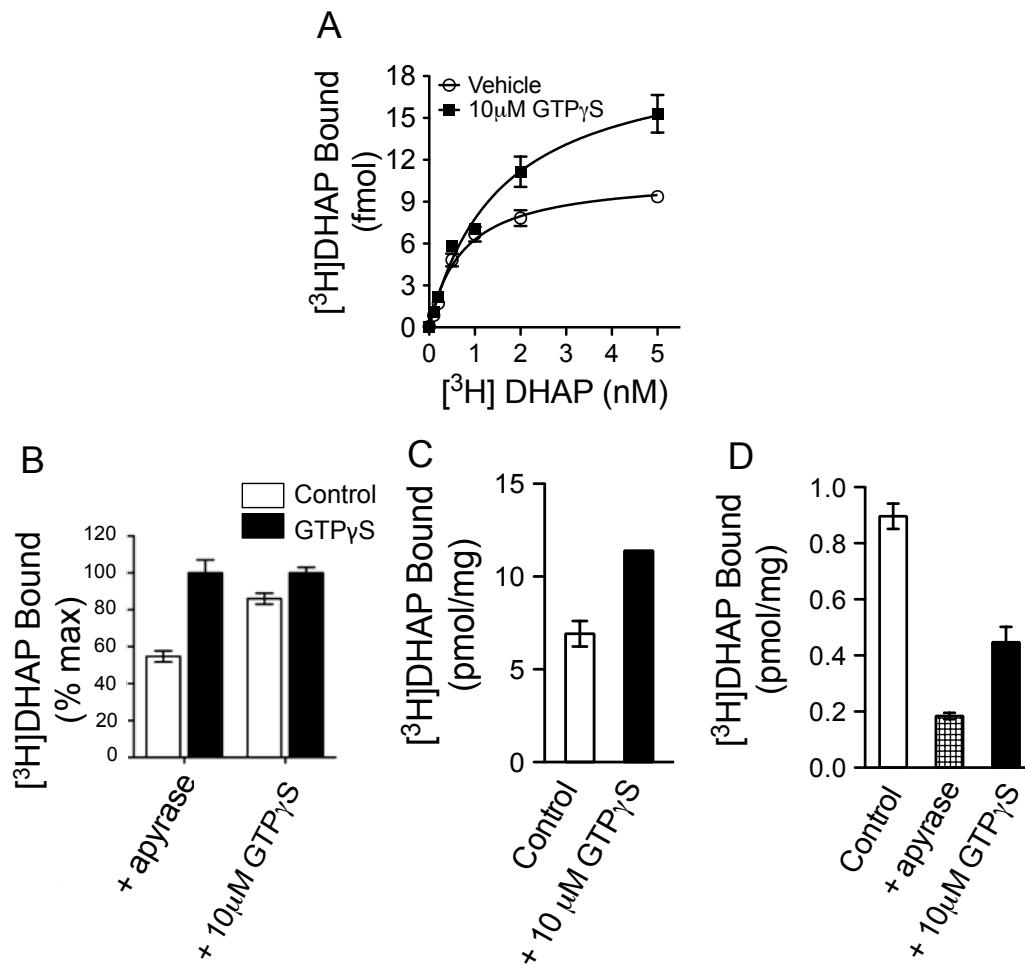


Figure 3-5. The nucleotide-free $\beta_2\text{AR-G}_s$ complex may be observed in reconstituted phospholipid vesicles, native and over-expressing $\beta_2\text{AR}$ membrane preparations. **A)** Saturation isotherm of $\beta_2\text{AR}$ and G_s heterotrimer reconstituted in phospholipid vesicles. The sample was treated with apyrase and analyzed by radioligand binding with [^3H]DHAP. There was an ~ 47 % increase in B_{max} upon addition of $\text{GTP}\gamma\text{S}$. **B)** as in **A**. After apyrase treatment the $\beta_2\text{AR-G}_s$ complex was treated with 100 nM GDP to restore antagonist binding. [^3H]DHAP binding presented as percent of maximal binding defined by the total binding observed in the presence of $\text{GTP}\gamma\text{S}$ (highest binding observed). **C)** Membranes prepared from baculovirus-infected *Sf9* cells expressing $\beta_2\text{AR}$ and $\text{G}_s\alpha\beta\gamma$ were washed several times and assessed for their capacity to bind 2 nM [^3H]DHAP. Uncoupling of G_s from $\beta_2\text{AR}$ with 10 μM $\text{GTP}\gamma\text{S}$ resulted in an increase in [^3H]DHAP binding consistent with the $\beta_2\text{AR-G}_s$ complex in rHDL particles. **D)** Homogenized lung tissue was treated with apyrase and antagonist binding was assessed as the other samples.

The Nucleotide-Free β_2 AR-G Protein Complex and High Affinity Agonist Binding

The determination of agonist affinity for binding GPCRs is often derived from their capacity to competitively inhibit [3 H]antagonist binding. The dependence of G proteins on agonist binding is often inferred from their sensitivities to guanine nucleotides. In the absence of additional nucleotide agonists inhibit antagonist binding in a biphasic manner, with a K_{high} and K_{low} that are typically separated by two or more orders of magnitude. The high affinity agonist site (K_{high}) is profoundly sensitive to the addition of nucleotides, and therefore reinforces the notion that G proteins stabilize agonist binding.

Analysis of isoproterenol inhibition of [3 H]DHAP binding to apyrase-treated nucleotide-free preparations yielded a monophasic inhibition curve with a K_i 175 ± 120 nM, resembling that obtained after the addition of GTP γ S, K_i of 140 ± 118 nM (**Fig. 3-6A**). Upon addition of nucleotide (GTP γ S) the maximal number of [3 H]DHAP binding sites increased by $\sim 45\%$, consistent with the previous effects observed (**Fig. 3-6A inset**). This effect is also observed in (diluted) untreated membrane preparations from *Sf9* cells co-expressing β_2 AR and G_s (**Fig. 3-6B inset**) where the addition of nucleotide resulted in a $\sim 45\%$ increase in binding sites. The natural loss of bound GDP (and antagonist) in these preparations is a result of the release of nucleotide from the β_2 AR- G_s •GDP complex in order to reach equilibrium with its surrounding environment.

Interestingly, addition of a low concentration of GDP (10 nM) to the nucleotide-free β_2 AR- G_s complex restored the biphasic nature of the curve with an observed K_{high} of 1.5 ± 1.8 nM and a K_{low} of 190.5 ± 130.9 nM (**Fig. 3-6A**), in good agreement with reported values (126). Addition of GTP γ S to the sample resulted in a monophasic

inhibition curve (K_i of 108.4 ± 117.1 nM). Furthermore, restoring the nucleotide pool increased the percentage of high affinity sites present. The fact that no high affinity agonist binding was observed in the nucleotide-free preparations is consistent with the hypothesis that [3 H]DHAP is incapable of binding the same conformation of β_2 AR that agonists bind to. This suggests that [3 H]DHAP can only compete with agonists when the receptor population is in the low affinity-uncoupled state.

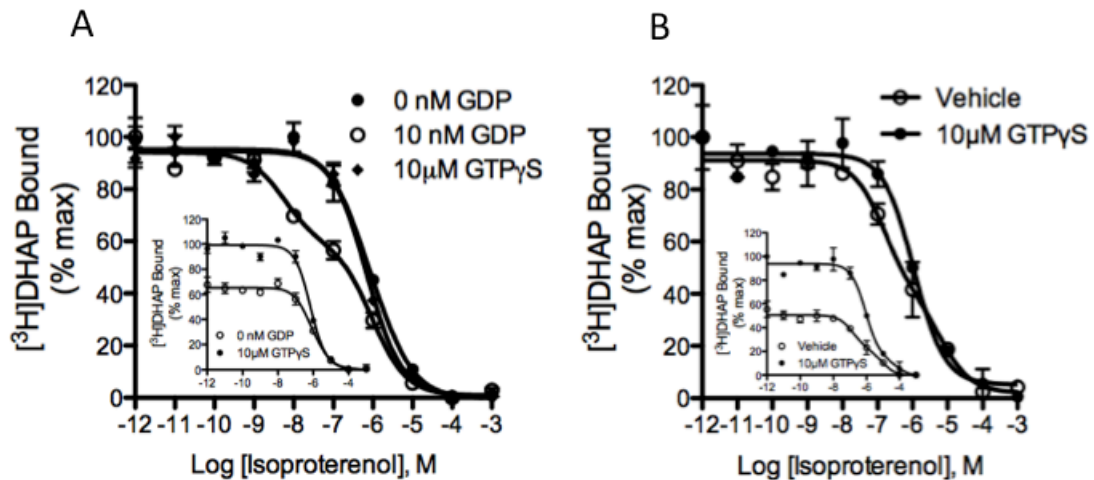


Figure 3-6. Failure to detect high affinity agonist (isoproterenol) inhibition of [3 H]DHAP binding to the nucleotide-free β_2 AR- G_s complex. **A)** Inhibition of [3 H]DHAP binding to the nucleotide-free β_2 AR- G_s complex in rHDL by increasing concentrations of isoproterenol (ISO) in the absence (open symbols) or presence of 10 μ M GTP γ S (closed symbols). The addition of GTP γ S exposed ~ 45 % more [3 H]DHAP binding sites. **A (inset)** The data are expressed as percent maximal binding in the absence of GTP γ S to illustrate that the remaining [3 H]DHAP binding following nucleotide loss can only be inhibited by isoproterenol with low affinity ($K_i \sim 175.1 \pm 120.1$ nM), identical to the GTP γ S-uncoupled β_2 AR ($K_i \sim 140.1 \pm 118.1$ nM). Addition of 10 nM GDP restores the biphasic nature of the curve and allows detection of maximal binding sites. **B)** The nucleotide effect is observed in Sf9 membranes that have not been treated with apyrase demonstrating that this phenomenon occurs in different systems. **Inset B)** Data normalized to absence of GTP γ S to illustrate difference in Bmax. Data were fit to a single site using GraphPad.

Consistent with these observations, non-isotopically labeled alprenolol (1 mM) failed to inhibit binding of tritiated agonist, [³H]epinephrine (30 nM), to the nucleotide-free β_2 AR-G_s complex, in contrast to agonists epinephrine (1 mM) and isoproterenol (1 mM) (**Fig. 3-7**).

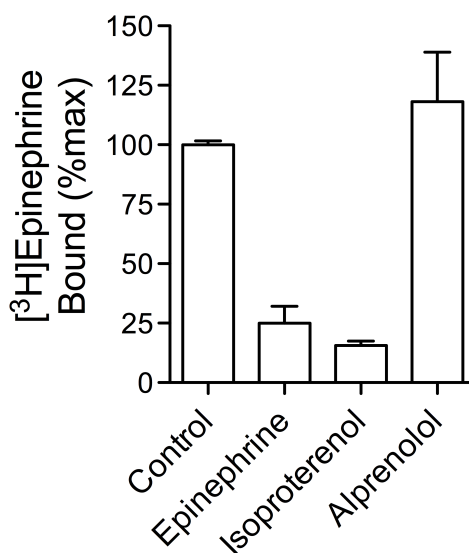


Figure 3-7. Alprenolol fails to inhibit [³H]epinephrine binding to reconstituted and apyrase-treated β_2 AR-G_s complex. Inhibition of [³H]epinephrine (30 nM) by unlabeled epinephrine (EPI, 1 mM), isoproterenol (ISO, 1 mM) or alprenolol (ALP, 1 mM). Data are expressed as percent of maximal binding in the absence of inhibitor.

The Behavior of Inverse Agonists

We previously demonstrated that the efficacy of inverse agonists to decrease basal receptor activity is dependent on their capacity to prevent G protein-dependent changes in receptor conformation. Moreover, we demonstrated that inverse agonists have little ability to disrupt a pre-formed nucleotide-free G protein-receptor complex (63). These data suggest that in preparations where mixtures of coupled and uncoupled receptors may be present (i.e. as a nucleotide-free R:G complex), multiple affinity states for inverse

agonists should be observed. Inverse agonist, ICI-118, 551 was assessed for its capacity to inhibit [³H]DHAP binding to apyrase-treated β₂AR-G_s in rHDL. ICI-118, 551 inhibits [³H]DHAP with a high affinity (K_i of 7.8 ± 0.2 nM) whether coupled to a G protein (nucleotide-free) or in its uncoupled state in the presence of GTPγS (K_i of 3.4 ± 0.1 nM) and fails to display multiple affinity states (**Fig. 3-8**). The fact that ICI inhibits [³H]DHAP binding at a single site whether the receptor is coupled or not suggests that both drugs compete for similar inactive conformations of the receptor. These data are in good agreement with our previous findings that ICI-118, 551 and alprenolol cannot disrupt the nucleotide-free G protein-receptor complex.

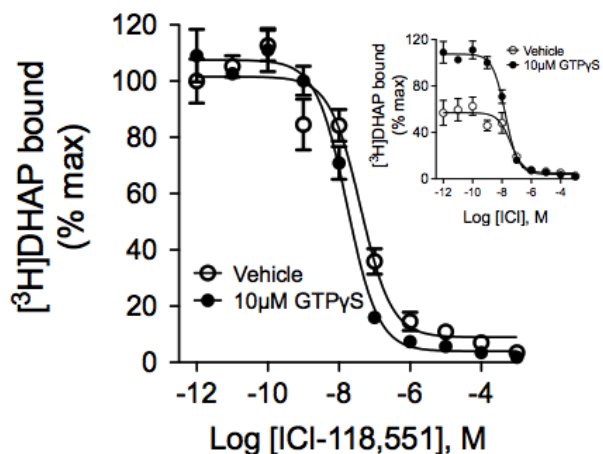


Figure 3-8. Inverse agonist ICI-118,551 inhibits [³H]DHAP binding with high affinity to the uncoupled β₂AR. Inhibition of [³H]DHAP binding to reconstituted and apyrase-treated β₂AR-G_s complex by increasing concentrations of ICI-118,551 in the absence (closed circles) or presence of 10 μM GTPγS (closed circles). Data are normalized and expressed as a percent of maximal binding in the absence (7.8 ± 0.2 nM) or presence of GTPγS (3.4 ± 0.1 nM). (**Inset**) The data are expressed as a percent of maximal binding in the presence of GTPγS to emphasize the inability of [³H]DHAP to bind to the nucleotide-free β₂AR-G_s complex. Data were fit to a single site using GraphPad (n=3).

Allosteric Regulation of [³H]DHAP Binding by Antibodies Directed Against the Agonist-Bound β_2 AR

Camelid antibodies (nanobodies) raised against an agonist-bound, active conformation of the β_2 AR (NB80), behave much like G proteins and support high affinity agonist binding (48). The crystal structure of the β_2 AR bound to NB80 reveals an overall transmembrane domain structure resembling the opsin-transducin C-terminus structure (48). We tested the capacity of NB80 to alter [³H]DHAP binding to the β_2 AR reconstituted in rHDL. **Figure 3-9** illustrates the capacity of NB80 to inhibit [³H]DHAP binding to the β_2 AR with an apparent K_i 2.9 ± 0.48 μ M. Primarily for solubility reasons NB80 can completely inhibit [³H]DHAP binding, in contrast to G protein. The high detergent concentration that accompany high G protein concentrations make G protein titrations significantly more challenging since the detergent can perturb the rHDL particles itself and alter receptor stability. NB80 and antibodies in general are extremely soluble and do not introduce deleterious detergent effects. NB80 appears to induce a similar conformation of the β_2 AR as nucleotide-free G protein bound in complex with the β_2 AR, both of which are incompatible with [³H]DHAP binding.

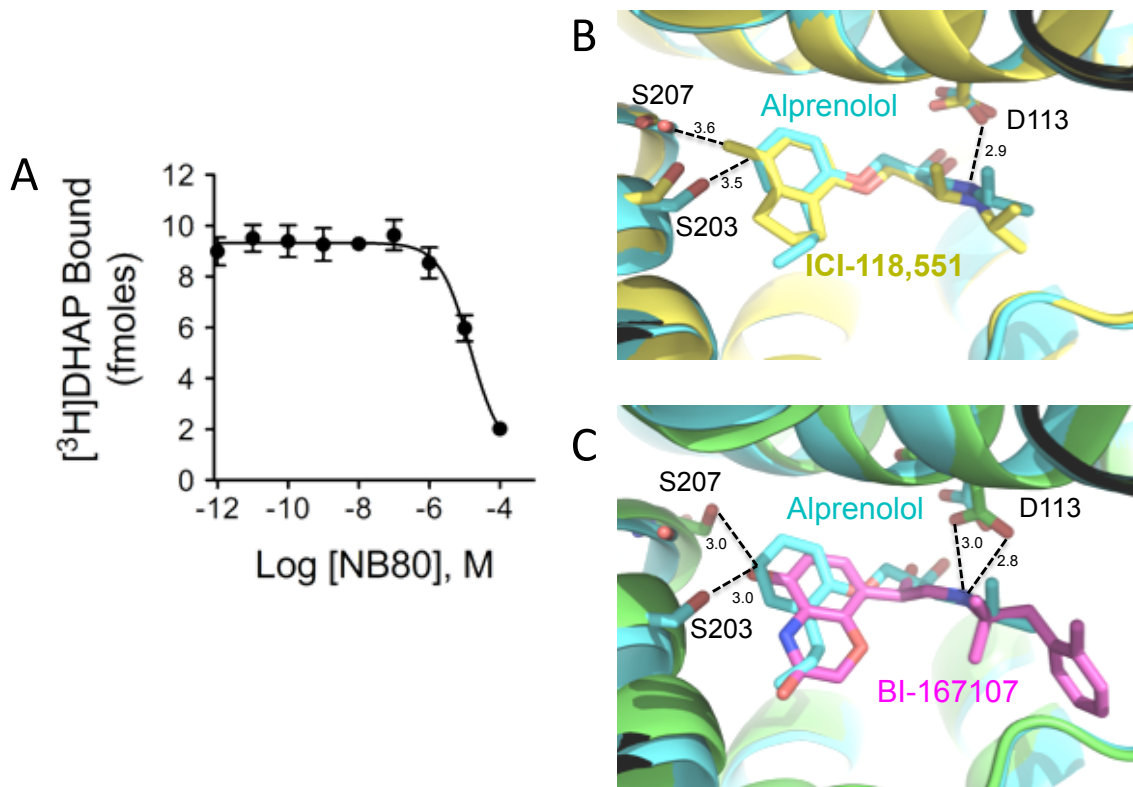


Figure 3-9. The active conformation of β_2 AR is incompatible with [3 H]DHAP binding. **A**) Nanobody (NB80) allosterically inhibits [3 H]DHAP binding to the β_2 AR similar to nucleotide-free G proteins. Reconstituted β_2 AR was incubated with 2 nM [3 H]DHAP in the presence of varying concentrations of a nanobody directed against the activated β_2 AR (NB80, (48)) NB80 inhibits [3 H]DHAP binding with a K_i $2.9 \pm 0.48 \mu\text{M}$. **B**) & **C**) Crystallographic evidence suggests that alprenolol is unable to bind the activated conformation of the β_2 AR. **B**) Crystallographic evidence recently reported by Wacker *et al.* (127) suggests that alprenolol binds to the inactive conformation of the β_2 AR, similar to ICI-118,551. Demarcated are sidechains of residues Ser203 and Ser207 located approximately 3.5 Å away from the phenyl ring of alprenolol. **C**) Recently defined crystal structure of the active conformation of the β_2 AR bound to agonist (BI-167107) and a camelid antibody (nanobody, NB80) raised against the agonist-bound β_2 AR (48). Superimposition of the alprenolol-bound structure reveals the collapse of the agonist site including the movement of TM5 and hence Ser203 and Ser207 by approximately 0.5 Å.

Discussion

Classical pharmacology has provided the essential tools by which quantitative studies of ligand affinity and efficacy can be measured using indirect means (128, 129). Among the most widely used functional assay platforms for GPCRs is the radioligand binding assay (130). Here, quantitative analysis of the receptor's capacity to selectively bind radiolabeled ligands, usually antagonists, and the capacity of drugs to inhibit the radiolabeled probe binding serve as the basis for defining the receptor type. Accurate estimations of affinities of unlabeled competing drugs may also be ascertained, an extremely valuable parameter when considering novel therapeutic drugs. For GPCRs the intrinsic activity of some ligands, particularly agonists, may be assumed by their pattern of inhibition of the radiolabeled antagonist probe.

GPCRs are dynamic membrane proteins that oscillate between an ensemble of inactive and active states, many of which may be sampled and stabilized by hormones and ligands (108, 131, 132). In addition, GPCRs can be allosterically modulated by protein-protein interactions such as those with G proteins and arrestins. The allosteric role that G proteins impart on hormone binding to GPCRs has long been described pharmacologically. Indeed, agonist binding stabilizes a receptor conformation that facilitates G protein binding and subsequent release of GDP from the G protein α -subunit. Likewise, G protein association appears to stabilize a receptor conformation that supports high affinity agonist binding, an event that may be relieved upon uncoupling via the addition of GTP or non-hydrolyzable analogues of GTP. The high affinity agonist binding site, usually gleaned by high affinity inhibition of radiolabeled antagonist binding

helped to form the basis for the “ternary complex” model, originally proposed by De Lean *et al.*, to describe G protein activation (23).

Biophysical analysis of the G protein-receptor complex, using a conformational sensor on the β_2 AR, reveals that the nucleotide-free form of the bound G protein stabilizes the high affinity agonist site (63). Logically one should therefore observe that once achieving this state, agonists should competitively inhibit radiolabeled antagonist binding with high affinity. Indeed, high affinity agonist binding observed through inhibition of radiolabeled antagonist is a common feature of G protein coupling of many receptors, as it is usually guanine nucleotide-sensitive.

However, a common feature observed with many GPCRs is a guanine nucleotide-dependent increase in radiolabeled antagonist or inverse agonist binding (33, 120-122). Moreover, without an explanation for the enhanced [3 H]antagonist binding observed with the addition of non-hydrolyzable GTP analogues in agonist competition assays many investigators express their data as a percent of maximal binding (*i.e.* with GTP analogues). Normalizing the data provides a facile comparison of the high and low states and a prediction of the G protein coupling efficiency. However, the fact that [3 H]antagonists poorly bind to the nucleotide-free complex suggests that the true fraction of the high affinity agonist-bound form is not even sampled by the radiolabeled antagonist and is therefore largely underestimated. Although high affinity agonist binding is occurring it is just not revealed by the [3 H]antagonist probe. In the extreme case where removal of all free GDP is aided by apyrase treatment, virtually all high affinity agonist binding is eliminated when it's assessed using the [3 H]DHAP probe (**Fig. 3-6**).

Under conditions where GDP concentrations are above its apparent affinity for G_s, [³H]DHAP can exist in equilibrium with the receptor and G protein. The G alpha subunit in this case can cycle between GDP and empty states and allow the heterotrimer to exist in equilibrium between the receptor-coupled and –uncoupled forms. Here, agonists, receptors, and G proteins should participate in equilibrium more amenable to allow [³H]antagonists to reveal many conformational states of the receptor.

The fact that nucleotides play such a strong role in agonist and antagonist binding suggests that ligand binding should take into account free nucleotide concentration. Typical membrane fractions prepared in a laboratory setting remove free nucleotide simply by cell lysis, sedimentation of particulate fractions (containing membranes) and by dilution in assays. The concentration of nucleotide is likely to be several orders lower than intracellular concentration of nucleotide (20 μM for GDP and 200 μM for GTP (93, 94)) and perhaps closer to the concentration of GTP-binding proteins themselves. Moreover, conditions for radioligand binding assays often require extended incubations in order to achieve “equilibrium”. The propensity of GPCRs to couple to G proteins as a result of basal receptor activity will accumulate nucleotide-free G protein-receptor complexes in such membrane preparations.

Recent crystallographic evidence may provide a structural rationale for the poor capacity of [³H]DHAP to bind to the G protein bound β₂AR. Alprenolol appears to bind in a remarkably similar overall manner to the inverse agonist-bound forms (ICI-118, 551 or carazolol) (42, 83, 127) (**Fig. 3-9B**). Both ligands appear to induce a closed conformation at the intracellular face of the receptor, prohibiting productive G protein interactions. In collaboration with Dr. Brian Kobilka (Stanford University) we have

recently elucidated the crystal structure of an agonist-bound β_2 AR in a complex with a camelid antibody (nanobody, NB80), specifically raised against an active, agonist-bound conformation of β_2 AR (48). Interestingly, the conformation-specific NB80 supports high affinity agonist binding much like G proteins. The NB80-bound β_2 AR, like the nucleotide-free G protein, stabilized the active conformation and likewise explains why [3 H]DHAP binds so poorly.

The overall structure of the agonist-and NB80-bound β_2 AR is reminiscent of the opsin-transducin C-terminus complex, where TM5 and TM6 undergo dramatic spatial alterations including rotations and translations to permit G protein C-terminus binding. Analysis of the agonist-binding site stabilized by NB80 reveals a significant collapse in comparison to the alprenolol-bound conformation ((48, 127) and **Fig.3-9C**). With D113 anchoring the amine of agonist BI167107 (and alprenolol) the inward translation of TM6 by 1-1.5 Å in the active conformation would likely not support alprenolol binding. S203 and S207 would be less than 3 Å from the phenyl ring of alprenolol, which in the alprenolol-bound β_2 AR structure is greater than 3.5 Å away. The proximity of the serine residues may thus confer unfavorable steric clashes with alprenolol and thus diminish the affinity of alprenolol for the β_2 AR- G_s bound.

In light of the existence of a receptor-G protein complex that is both nucleotide-free and unable to bind antagonist, revisiting some of the basic assumptions about GPCR allostery and ligand binding are required. Specifically, the nature of the G protein-induced agonist high affinity state must be elaborated. The fact that a GPCR has (at least) two conformational states is undisputable (43, 63, 96, 133, 134). Current theory postulates that the inactive 'R' state, binds agonists with relatively low affinity whereas

the ' R^* ' state binds agonist with high affinity. Our previous assumption relied on the capacity of antagonists to bind both states with approximately equal affinity and not alter the distribution of R and R^* states. Agonists may therefore freely compete with [^3H]antagonists for both the R and R^* states, which we have now shown not to be the case. Moreover, the proportion of the R^* state may be influenced by basal receptor activity. In either case, progression to R^* catalyzes guanine nucleotide exchange (GDP release) on the G proteins. For agonist binding, formation of the nucleotide-free G protein-receptor complex stabilizes the R^* state and cooperatively favors agonist binding, slowing the off-rate and hence increasing agonist's affinity. In contrast, inverse agonists bind with high affinity to- and stabilize the ' R ' or similar states that are incapable of catalyzing nucleotide exchange on G proteins. The data presented here, however, implies that antagonists such as alprenolol do not sample all conformational states equally and therefore cannot under equilibrium conditions accurately depict the multiple binding states of agonists. Of course these problems may be taken into account through factoring in free nucleotide concentration, either by including their concentration in the thermodynamic equations or by assuming that they are in moderate excess.

In summary these observations have significant implications in GPCR research reinforcing the concept that most ligands, if not all, have varying degrees of intrinsic activity. The intrinsic activity of antagonists is extremely relevant as most ligand probes were designed to take advantage of their "neutral" properties. The fact that these probes selectively bind and stabilize specific receptor conformations (usually inactive) suggests that they may not sample active conformations, the state that promotes high affinity agonist binding. Finally, the nucleotide state of the G protein, and hence the

concentration of free GDP, is an extremely important component that influences this active conformation.

Materials and Methods

For this thesis I purified all preparations of Apo A-I and CFP- β_2 AR. I also performed all vesicle and rHDL reconstitutions, radioligand binding assay (initially with the assistance of Matthew Whorton), and some fluorescence spectroscopy studies. Purified heterotrimeric G_s were generously provided by Diane Calinski and Brian DeVree from the University of Michigan. Drs Xiao-Ji Yao and Soren Rasmussen from Stanford University purified and biotinyl-labeled the β_2 AR. Dr. Xiao-Ji Yao performed one fluorescence spectroscopy assay. Purified NB80 was generously provided by Dr. J.J Fung, a fellow in the Kobilka laboratory.

Materials

Recombinant AcMNPV baculoviruses encoding $G\alpha_s$, his-6- $G\beta_1$ and $G\gamma_2$ were provided by Dr. Alfred G. Gilman (UT Southwestern, Dallas, TX). All lipids were purchased from Avanti Polar Lipids (Alabaster, AL). Dodecylmaltoside (DDM) was obtained from Dojindo Molecular Technologies (Gaithersburg, MD). Sodium cholate was purchased from Sigma. \pm -Alprenolol, (-)-isoproterenol, \pm -propranolol and ICI-118 551, were obtained from Sigma (St. Louis, MO). [3 H]Dihydroalprenolol, [3 H]epinephrine and [35 S]GTP γ S were obtained from Perkin Elmer (Foster City, CA). Recombinant potato apyrase was purchased from New England Biolabs (Ipswich, MA). All other reagents of analytical grade were of obtained from Fisher Scientific (Pittsburg, PA).

Expression, Purification and Labeling of β_2 AR

A modified version of the β_2 AR where 4 reactive cysteines were mutated (C77V, C327S, C378A, and C406A) was made. The modified β_2 AR was expressed in *Sf9* insect cells by using recombinant baculovirus and solubilized using methods previously described (99). The DDM solubilized receptor was purified by sequential M1-Flag antibody affinity and alprenolol affinity chromatography as described (99). Briefly, CaCl_2 was added to the DDM solubilized extract to a final concentration of 1 mM and loaded to the M1-Flag column. The receptor was eluted from the flag resin with 20 mM HEPES, pH 7.5, 100 mM NaCl, 0.1% dodecylmaltoside (Buffer A) with 1 mM EDTA. The concentration of functional, purified receptor was determined using a saturating concentration (10 nM) of [^3H] Dihydroalprenolol as previously described (112). Flag purified receptor was then purified by alprenolol-Sepharose chromatography as described (112). The receptor was eluted from alprenolol-Sepharose with Buffer A with 300 μM alprenolol and 1 mM CaCl_2 and loaded directly onto M1-Flag resin. The M1-Flag resin was washed with Buffer A to remove free alprenolol and eluted with Buffer A plus 1 mM EDTA. Two liters of *Sf9* cells typically yield 500 μl of a 5 μM solution of β_2 AR.

The purified β_2 AR and monobromobimane were mixed at the same molarity and incubated overnight on ice in the dark. The fluorophore-labeled receptor was purified right before use by gel filtration.

Purification of Recombinant ApoA-I

Wild type human apoA-I was purified from expired serum as previously described (70). A recombinant apoA-I with an N-terminal 43 amino acid deletion and a

hexa-histidine tag ($\Delta(1-43)$ -his₆-apoA-I) was expressed using a pET15b vector to transform competent *Escherichia coli* cells (BL21). Cells were resuspended and lysed by gently vortexing in 10 mM Tris-HCl pH 8.0, 100 mM NaH₂PO₄, 6 M guanidine hydrochloride (GuHCl), 1 % Triton X-100. Lysate was fractionated by centrifugation at 10, 000 G and the supernatant was loaded onto a Ni-NTA column by gravity flow. The column was washed with 10 mM Tris-HCl, pH 7.0, 100 mM NaH₂PO₄, 6 M guanidine hydrochloride, 1 % Triton X-100 and then with 50 mM NaH₂PO₄, pH 8.0, 300 mM NaCl and 1 % Triton X-100. Bound $\Delta(1-43)$ -his₆-apoA-I was eluted with 50 mM NaH₂PO₄, pH 8.0, 300 mM NaCl, 250 mM imidazole, 1 % Triton X-100. Peak fractions were further purified on a Superdex 75 gel filtration chromatography column in 20 mM Hepes, pH 8.0, 100 mM NaCl, 1 mM EDTA, 20 mM sodium cholate. Pooled apoA-I was then dialyzed against 20 mM Hepes, pH 8.0, 100 mM NaCl, 1 mM EDTA, 5 mM sodium cholate. Purified apoA-I was concentrated to ~10 mg/mL and stored at -80°C until use.

In Vitro Reconstitution of β_2 AR into rHDL

High-density lipoproteins were reconstituted *in vitro* according to a protocol adapted from Jonas (113). Briefly, a mixture of POPC and POP (3:2 molar ratio) in combination was used to mimic the zwitterionic environment of a cell membrane (114). A typical rHDL reconstitution consisted of the following components: 24 mM detergent (cholate or DDM), 8 mM lipids, and 100 μ M apoA-I. Lipids were solubilized with a solution of 20 mM Hepes pH 8.0, 100 mM NaCl, 1 mM EDTA and 50 mM detergent. To reconstitute the bimane labeled β_2 AR (mB- β_2 AR), purified β_2 AR was added to at least 10-fold excess ([apoA-I]:[β_2 AR]) to receptor preparations diluted in solubilized lipids.

Following an incubation of 1-2 hrs at the TM of the lipid combination, samples were added to an equal volume of hydrated BioBeads (BioRad) for an additional 3 hrs to remove detergents, resulting in the formation of rHDL particles. Samples were stored on ice until used. If necessary, β_2 AR-rHDL particles were separated from receptor-free rHDL by M1-anti-Flag immunoaffinity chromatography. Purified β_2 AR-rHDL particles were eluted with EDTA (10 mM) and stored on ice until further use.

G Protein Reconstitution in rHDL

Purified G_s heterotrimer (*I15*) (stored in 0.02% DDM) was reconstituted into preformed impure β_2 AR-rHDL particles (containing excess empty rHDL particles) at an initial R:G ratio of 1:10. Concentrated G_s stocks were added such that the DDM was diluted at least 200-fold to reduce the DDM concentration to well below the CMC (0.12-0.18 mM or 0.006% in NaCl containing buffer). This had no effect on the integrity of the particles, as assessed by size exclusion chromatography (not shown). Treatment of G_s -reconstituted samples with BioBeads, to remove trace amounts of DDM, before gel filtration chromatography had no effects on the results. Nucleotide-free β_2 AR- G_s complex was prepared by incubating with apyrase (NEB, Ipswich, MA) in the presence of 1 mM $MgCl_2$ for 30 minutes at room temperature.

Reconstitution of β_2 AR and G_s in Vesicles

Lipid stock mixtures of DOPC (3 mg/mL) and CHS (0.3 mg/mL) were prepared in 20 mM HEPES, pH 7.5, 100 mM NaCl including 1% OG, as previously described. The lipid stocks were removed from storage, vortexed, and sonicated for 30 min in an ice

bath. The reconstitution mixture was prepared in 20 mM HEPES, pH 7.5, 100 mM NaCl + 0.1% DDM containing a 10-fold dilution of the lipid stock and β_2 AR \pm G_s. The final DOPC and CHS concentrations in this mixture were 0.3 and 0.03 mg/mL, respectively. The reconstitution mixture was inverted several times and incubated for 2 h on ice. Detergent removal and concomitant formation of vesicles were attained by gel filtration chromatography on a Sephadex G-50 Fine (GE Healthcare, Piscataway) column (135, 136)

Saturation Radioligand-Binding Assays

Binding reactions were prepared in 100 μ l volumes in 96 well plates. Samples were incubated with various concentrations of β_2 AR antagonist [³H] dihydroalprenolol ([³H]DHAP) (0.1-20 nM) in 50 mM Tris-HCl, pH 8.0, 150 mM NaCl (TBS) in the presence or absence of nucleotide (GTP γ S or GDP). Nonspecific binding was determined in the presence of 10 μ M propranolol. Receptor particles were incubated for 90 minutes at RT for saturation isotherms. For separating free [³H]DHAP from bound, the samples were filter on glass fiber plates.

For glass filtering, GF/B 96 well filter plates (Whatman) were used in conjunction with a vacuum manifold. Wells were pre-wet with 0.3 % polyethyleneimine (PEI) for 30 minutes. Samples were applied and washed 3X with 200 μ l of TBS. Scintillation mixture was added (Microscint0, Packard) and plates were counted on a TopCount scintillation counter (Packard). Specific binding was determined by subtracting nonspecific binding from total binding.

[³⁵S]GTP γ S Binding

Purified G_s (100 nM final) was added to β_2 AR reconstituted in rHDL (50 nM final) as above. ISO-stimulated [³⁵S]-GTP γ S-binding assays (10 μ M ISO) were performed on nucleotide-free β_2 AR-G_s in rHDL essentially as described by Asano *et al.* (136). The reactions were initiated by the addition of isotopically diluted [³⁵S]GTP γ S (100 nM). Reactions were terminated at different times (between 15 s and 5 min) by the addition of ice-cold 20 mM HEPES, pH 8.0, 100 mM NaCl, 1 mM EDTA (HNE) with 10 mM MgCl₂. Free [³⁵S]GTP γ S was removed by rapid filtration.

Agonist Competition Assays

Agonist competition assays were performed on G protein-reconstituted sample under similar conditions as used in the saturation binding assays except that a fixed concentration of [³H] DHAP (2 nM) was competed with various concentrations of isoproterenol or ICI-118, 551 (1 X 10⁻¹² – 1 X 10⁻³ M) with or without the addition of 10 μ M GTP γ S (in the presence or absence of GDP). Binding reactions contained 0.02% ascorbic acid to prevent oxidation of the isoproterenol. Samples were incubated for 90 minutes at RT and then filtered on glass fiber plates as above. Normalized data were fit to a two-site competition-binding model using Prism (GraphPad).

CHAPTER 4

CONCLUSIONS

Summary and Discussion

Historically, G protein-coupled receptors have been studied and considered great drug targets in pharmacology due to their abundance in humans. The human genome project identified more than 800 different GPCRs genes and currently over 30% of the drugs in clinical practice exert their effects on them. The molecular mechanisms involved in receptor functions, particularly the molecular modes of receptor activation, G protein recognition and activation, have therefore become the research focus of an increasing number of labs. Still after decades of research the mechanism by which G_s couples to agonist-free receptor is still unknown.

The emergence of various crystal structures of ligand-bound GPCRs (*41, 42, 85, 86*) and the recent structure of a complex between opsin and the carboxyl-terminal peptide of transducin (*40*) have given insights into the mechanisms by which G proteins couple to their partner receptor. In addition, fluorescence lifetime (*108*) data has shown that receptors exist in an ensemble of various conformations and that each conformation is stabilized or favored by specific ligands and/or G proteins. A part of our work focused on the isolation and characterization of these conformations.

We were able to characterize the properties of a fluorescently labeled receptor (mB- β_2 AR) in complex with G_s in rHDL particles. This system allowed us to incorporate monomeric and functional mB- β_2 AR into a homogenous lipid bilayer that allows access to both receptor termini allowing the interaction with both ligands and protein partners.

The bimane spectra illustrate how G protein binding stabilizes an active conformation that resembles the agonist-bound form of β_2 AR. Interestingly, the addition of the agonist isoproterenol to a pre-formed mB- β_2 AR- G_s complex induces a more dramatic change in the spectrum (further decrease in fluorescence intensity and increase in the λ_{\max}). This could indicate that the presence of agonist favors the formation of the β_2 AR- G_s complex or that the receptor conformation in the presence of both differs from that of each of them alone. The effect of G protein and isoproterenol can be abolished by adding saturating amounts of GDP or GTP γ S as demonstrated by the reversal of the bimane spectra to that of the β_2 AR alone.

The behavior of inverse agonists is fascinating, as it seems that these ligands stabilize specific inactive conformations. Our results indicate that the inverse agonists ICI 118, 551 and carazolol bind to the receptor alone and upon binding stabilize a conformation that cannot couple to G proteins. Similarly, addition of saturating amounts of ICI had no effect on the pre-formed β_2 AR- G_s complex suggesting that it cannot bind to G_s -coupled β_2 AR. These results are in agreement with what is observed in functional assays, as it is observed that inverse agonists block the binding of other ligands and decrease the basal activity of receptor. Also, previous studies demonstrated that the

number of binding sites for [³H] ICI-118, 551 was reduced in cells overexpressing G_s (111).

Reconstitutions of β₂AR-G_s treated with apyrase made possible the isolation of the nucleotide-free β₂AR-G_s complex, unliganded or agonist-bound. Under these conditions, antagonist (alprenolol) and as previously mentioned, ICI-118, 551 are unable to disrupt the pre-formed complex even after long incubations with saturating amounts, most likely because of the allosteric effects of the G protein on the ligand-binding pocket. Comparison of the inactive (83, 84) and active (48) structures of the β₂AR suggests that G protein coupling may cause the extracellular domain of the receptor to collapse thus restricting access to the ligand binding pocket. This would explain the inability of alprenolol to disrupt the pre-formed complex but bind with high affinity to receptor alone. We set out to characterize the nucleotide-free β₂AR-G_s complex using radioligand binding studies.

The G protein in the ternary complex that constitutes the high-affinity agonist state is believed to be nucleotide-free and in its trimeric form. Association of the α-subunit with the βγ-dimer is modified by receptor-interaction and GDP presence. Although it was believed that GDP binding was dependent on agonist-occupied receptor, our data suggests otherwise. The nucleotide-free β₂AR-G protein complex is active and can re-bind nucleotide in the absence of agonist (ISO) most likely due to its basal activity. Furthermore, antagonist binding to β₂AR-G protein complex depends on the presence of guanine nucleotide.

The nucleotide-free $\beta_2\text{AR-G}_s$ complex was also observed in native membranes (lung) and *Sf9* membranes over-expressing $\beta_2\text{AR}$ and G_s , demonstrating the generalizability of our results. As observed with reconstituted rHDL, the addition of nucleotides to these preparations revealed more [^3H]DHAP binding sites (increases B_{max}) confirming that the presence of GDP (and/or $\text{GTP}\gamma\text{S}$) restores antagonist binding. Our findings imply that alprenolol is not a “neutral” antagonist as it can discriminate and prefer certain receptor conformations. A similar behavior is observed with inverse agonists: radioligand binding assays reveal that ICI-118, 551 appears to inhibit [^3H]DHAP binding to the uncoupled receptor in a competitive manner, binding with high affinity to receptor alone. In contrast, agonists bind to all active conformations regardless of the amount of nucleotide present. [^3H]Epinephrine was able to bind to the nucleotide-free $\beta_2\text{AR-G}_s$ complex but could not be displaced by alprenolol but only by other agonists such as isoproterenol or “cold” epinephrine. It should be noted that we are not discarding the possibility that a receptor population was not bound by [^3H]Epinephrine as its K_d has not been accurately determined, for the purpose of this study we assumed saturation.

Finally, analysis of agonist inhibition of antagonist binding to the nucleotide-free $\beta_2\text{AR-G}_s$ complex displayed a monophasic curve with a low affinity site that resembled the uncoupled receptor. In addition, it also showed ~30-50% less maximal binding sites than the uncoupled complex (treated with 10 μM $\text{GTP}\gamma\text{S}$) suggesting that antagonists do not bind a distinct receptor population. Addition of nucleotide restores the biphasic nature of the curve and increased the number of binding sites detected confirming that antagonists are not able to detect the nucleotide-free complex. In summary, we have been

able to isolate the β_2 AR-G_s (nucleotide-free) high-affinity complex that cannot bind antagonists.

Implications for GPCR Research (and Drug Discovery)

The characterization of ligand affinity and efficacy has been classically done using guanine nucleotide binding assays (129) and allosteric competition assays (130). The former assay is attractive because guanine nucleotide exchange is a proximal step to receptor activation and is not subject to regulation by other pathways (129, 137). However, competition assays offer a more accurate estimation of drug affinity, which is extremely valuable when considering potential therapeutic drugs.

These findings have significant implications on *in vitro* GPCR research, more specifically in sample preparation. Membrane protein research is highly dependent on our ability to purify and functionally reconstitute these proteins in membrane-like systems. Such preparations will be used in screens such as those previously mentioned. In order to arrive at an accurate affinity value one must be certain that all the active receptors are being labeled. If the probe, such as a ‘neutral’ antagonist, is used in an attempt to label all receptor sites it is not likely to detect all receptor conformations. Assessing the affinities of a competing ligand, particularly if the competing ligand is an agonist, may produce a misleading IC₅₀, and K_i value. As demonstrated in this study, agonist isoproterenol inhibited the ‘neutral’ antagonist [³H]DHAP with low affinity at the nucleotide-free G protein•receptor complex. Primary screens are often designed to find candidate drugs using functional assays in cells. These assays should not be affected, as

the guanine nucleotide concentrations in the cell are in the micromolar range, making the formation of a stable nucleotide-free receptor-G protein complex difficult (93, 94). However, a critical step in these screens is the subsequent hit validation using binding assays that estimate the EC_{50} (K_i). The EC_{50} (K_i) not only confirms the functional assay but also ensures that the dose required for a full response falls in a reasonable range for therapeutics. Incorrect K_i values could lead to the disposal of a potential therapeutic drug. Once again demonstrating the importance of guanine nucleotide in ligand binding.

Implications for GPCR Theory

The isolation of the high-affinity nucleotide-free β_2R - G_s complex, which cannot bind antagonists, has opened some gaps in the current theory of allosteric binding. The concepts of GPCR basal activity and the G protein high-affinity state must be re-evaluated in order to fit with our current results.

The fact that GPCRs exist in an ensemble of various conformational states has been demonstrated. Current theory postulates that one conformational state, the inactive ' R ' state, binds agonist at a relatively low affinity and, the active ' R^* ' state, binds agonist at a high affinity. Supposedly, antagonists are able to bind both states with approximately equal affinity, thus occupying the orthosteric binding site without altering the distribution of R and R^* states. This prevents the receptors from binding any other drugs while still allowing a certain percentage of the receptor population to enter the R^* state needed to catalyze guanine nucleotide exchange on the G protein, leading to a basal level of activation. In contrast, inverse agonists stabilize either the R or other states that are not

able to catalyze nucleotide exchange on the G protein. Binding of a G protein will also stabilize the R^* state, allowing the allosteric modulation of agonist binding to GPCRs by the G protein.

Under this theory, the interpretation of an agonist competition assay with a radiolabeled antagonist is seen as two affinity sites, one that corresponds to G protein-coupled and another for uncoupled-receptors. The observed K_i of the sample treated with GTP γ S is just as expected if there was no G protein at all in the sample, since it has all been functionally dissociated by addition of the non-hydrolyzable GTP analogue. The two observed K_i 's in the samples treated with moderate amounts of GDP correspond to the proportion of receptor that is not associated with G protein (K_{low}) and the proportion of the receptor that is pre-coupled with G protein (K_{high}). However, this theory does not take into account the nucleotide state of the G protein regarding allosteric communication with the orthosteric binding site, but rather views nucleotide exchange as a separate reaction that is only catalyzed by the GR^* state but does not affect ligand binding.

The finding that antagonists cannot bind to the state of the receptor that can also catalyze nucleotide exchange requires modification of this theory. The interpretation for the K_i with GTP γ S and the K_{low} with GDP are similar to current theory, with the only difference being that there is no antagonist bound R^* state. The K_{high} involves an initial population of antagonist-bound R state associated with GDP-bound G protein. The G protein loses the guanine nucleotide, allowing it to interact with the unliganded R^* state that stabilizes the nucleotide-free state of the G protein. The stabilized, unliganded R^* state then binds free agonist at a high affinity. It should be noted that for this particular assay, the association between receptor and G $_s$ -GDP-liganded receptor could be either a

distinct conformational state or simply reflect the fact that G protein and receptor are 'near' each other (i.e., able to collide with one another in the time frame of the assay).

However, other evidence suggests the existence of a GDP bound pre-association of receptor and G protein. Also, the assay cannot suggest the nature of the agonist-bound K_{high} complex, be it receptor bound to nucleotide-free G protein, liganded receptor-GDP-G protein, or simply dissociated agonist-bound R^* and G protein. Finally, the gap between the maximal amount of bound antagonist in the nucleotide containing samples is interpreted as that any proportion of the sample that starts as a R^* state stabilized by nucleotide-free G protein will never be detected in the assay, as it has no chance to bind radiolabeled antagonist.

In light of these new data, the question that comes up is how would an antagonist allow for the basal level of GPCR activation if it does not bind to (and therefore select against) the R^* state of the receptor? The question is discussed below, primarily for ligands that bind to the GPCR orthosteric site. Ligands that bind to allosteric sites and extended N-terminal domains may influence the system in much different ways, although it is highly likely that most or all of such ligands must pass conformational information through the orthosteric site in order to communicate with cytoplasmic GPCR effector proteins.

An emerging idea in GPCR pharmacology is that from a mechanistic standpoint, true neutral antagonists are extremely rare (perhaps non-existent). Certainly, there are drugs that cause negligible changes in GPCR activation, but still prevent other drugs affecting the receptors. However, when examined in detail with ever improving

techniques, we find that many antagonists must be re-classified as weak partial agonists or inverse agonists. Our *in vitro* binding data suggests that alprenolol behaves as an inverse agonist.

Even more dramatic reclassification of antagonists happens when considering more than a single signaling output. Recent studies have shown that GPCRs can signal through multiple pathways in both G protein dependant and independent manners. Thus, in order to really understand what a drug does upon receptor binding, we must look simultaneously at a number of distinct signaling outputs. By doing this we can observe that the classification of drugs for one output of GPCR signaling often has little predictive power for activity measured with another output. Viewed from this perspective, the finding that antagonist binding is incompatible with the traditional assumption that GPCR basal activity is due to an inherent tendency of the receptor to enter the R^* state in the absence of agonist is not surprising. Instead, antagonist binding probably causes real changes in GPCR signaling, but the sum of all the various signaling inputs and outputs ends up being similar enough to the unliganded basal activity of the receptor that overall activation level is considered negligible.

One possibility for rationalizing both the phenomenon of GPCR basal activity and the inability of antagonist to bind the R^* state is a departure from the traditional assumptions but potentially modest explanation. It could be that the receptor-GDP·G protein complex is both a distinct conformational state and resembles the source of GPCR basal activity. Most importantly, recent studies on rhodopsin have observed a transient state of the receptor, metarhodopsin-Ib, that is bound to GDP-heterotrimeric

transducin (138) . Other studies have long implicated the existence of some type of pre-association between receptor and G protein.

APPENDIX

FORMATION AND STABILITY OF A GPCR-ARRESTIN 2 COMPLEX IN RECONSTITUTED HIGH-DENSITY LIPOPROTEINS

Introduction

Three families of regulatory proteins modulate signal transduction by the G protein-coupled receptors: G proteins, GPCR kinases (GRKs) and arrestins. Activation of a GPCR by an agonist promotes a conformational change that leads to coupling and subsequent activation of the heterotrimeric G protein (dissociation into the α -GTP and $\beta\gamma$ subunits, each of which signals to downstream effectors) (139). The transduction cascade is terminated by receptor phosphorylation (by GRKs) followed by arrestin recruitment and receptor internalization that leads to its degradation or recycling to the membrane (*Fig. A-1*) The mammalian arrestin family is composed of four members: arrestin 1 and 4 are confined to retinal rods and cones respectively, and arrestin 2 (β -arrestin 1) and arrestin 3 (β -arrestin 2) are ubiquitously expressed (140).

In addition to its role in desensitization, it is now appreciated that arrestin binding initiates different signaling cascades. These signals are often both spatially and temporally distinct, and result in unique cellular and physiological consequences from those regulated by G proteins. The role of arrestins acting as signal transducers through

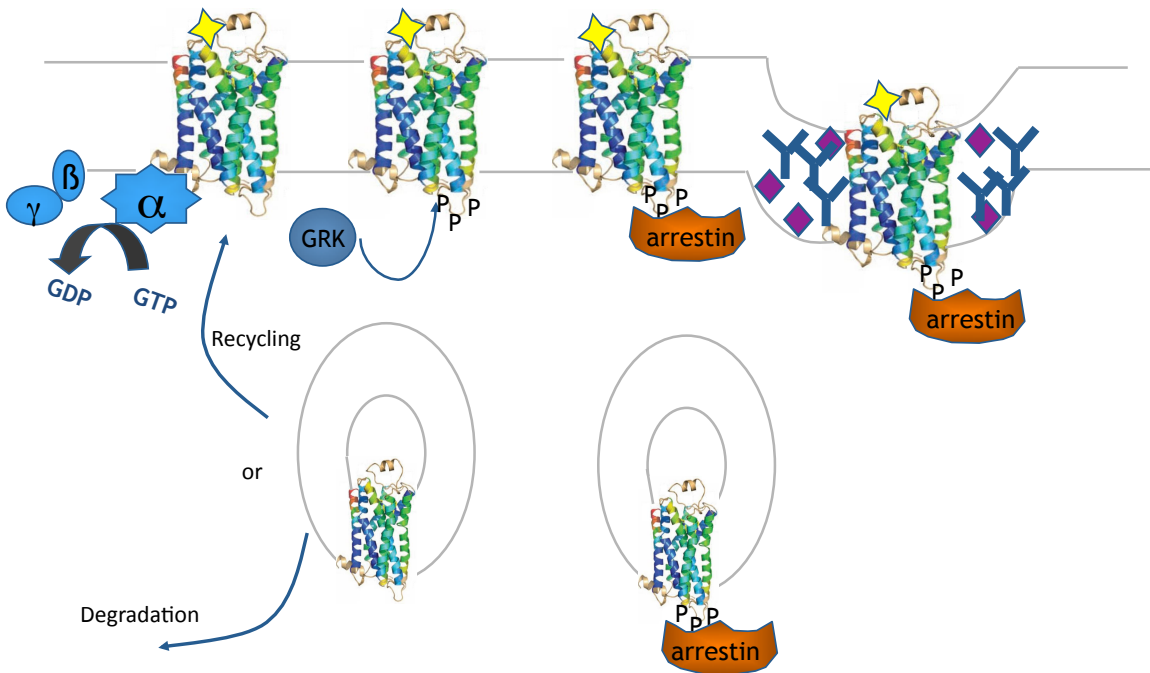


Figure A-1. Regulation of GPCR trafficking by GRKs and arrestins. Agonist (star) binding to GPCR leads to receptor activation, G protein coupling, and signal transduction. GRKs then phosphorylate the agonist-activated GPCR on intracellular domains, promoting arrestin recruitment. Arrestin binding to the receptor inhibits G protein coupling and terminates signaling (desensitization). Receptor/arrestin complexes are targeted to clathrin-coated pits where they interact with different components of the internalization machinery. Internalized GPCRs are sorted to either degradation or recycling compartments. Image adapted from Diane Calinski, University of Michigan.

the formation of scaffolding complexes with accessory effector molecules such as Src, ERK1/2, JNK3 and MAPK is becoming increasingly recognized (9, 141-143).

The potential signaling diversity of GPCRs suggests the existence of multiple “discrete” active receptor conformations. Each conformation can be stabilized by specific ligands that direct distinct signaling responses. These ligands that directly target GPCRs have been classically described as agonists, antagonists or inverse agonists for G protein signaling. Agonists are defined as drugs that can stabilize the active receptor

conformation, which promote G protein activation. In contrast, inverse agonists stabilize the inactive conformation thereby reducing G protein signaling (144). Antagonists theoretically bind all receptor conformations indiscriminately having no effect on basal activity but blocking further activation by agonists. The fact that arrestins are capable of initiating distinct signaling pathways through GPCRs independently of G proteins has established the need to re-evaluate all drugs and has introduced the term of biased agonism.

Biased agonism refers to the preferential activation of one of a number of possible downstream pathways of a receptor by a particular ligand (140). Over the last decade a diversity of biased ligands for GPCRs have been identified that selectively activate G proteins or arrestins, and several of them seem to have distinct functional consequences when compared to traditional ligands (15). For example, the compound ICI-118, 551, is a well established inverse agonist for the β_2 AR that leads to down-regulation of adenylyl cyclase levels (145), yet it induces ERK phosphorylation that completely depends on arrestin-3 expression (8). Moreover, arrestins have been implicated in numerous aspects of physiology and pathophysiology of disease (146). All these findings make the arrestin-receptor complex a tempting target for more detailed characterization that could lead to new therapeutics.

To date, it is still not exactly clear how arrestins interacts with the cytoplasmic face of a GPCR. Even more, the stoichiometry of the arrestin-GPCR complex in cells has not been determined accurately and some conflicting models have been proposed (147, 148). Interestingly, arrestins have been shown to allosterically regulate hormone binding to GPCRs, in a similar fashion to G proteins (149). An interaction with nM affinity has

been demonstrated between arrestin and purified β_2 AR reconstituted into phospholipid vesicles. Unfortunately the heterogeneous nature of phospholipid vesicles makes it impossible to delineate even basic questions about the interaction, such as the β_2 AR-arrestin stoichiometry. Any structural information would therefore be difficult to assess.

In order to understand this ternary complex we utilized the rHDL system to characterize the interaction between the β_2 AR and arrestin 2 from a functional and structural perspective. We used mB- β_2 AR reconstituted in rHDL to investigate arrestin interactions. Our goal was to determine how arrestin may induce changes in bimane fluorescence as a result of conformational changes in mB- β_2 AR structure upon binding as well as ligand binding. Ongoing studies in the lab are trying to determine the affinity between the β_2 AR receptor, in various phosphorylated states, with arrestin isoforms and mutants using various approaches. Immunoprecipitations and size exclusion chromatography will be used to verify complex formation in a qualitative manner and we will determine its affinity with the Flow Cytometry Protein Interaction Assay (FCIP) (150). In addition, the high degree of efficiency of receptor reconstitution and homogeneity of β_2 AR-HDL preparations suggest that it may be useful for structural studies.

Results

rHDL Particles as a Mean to Study the β_2 AR-Arrestin 2 Complex

In order to isolate and characterize the β_2 AR and arrestin 2 complex we took advantage of the rHDL system. We first tested if the rHDL particles would interact non-specifically with arrestin. To address this issue we incubated empty particles (only

containing the lipids: POPC and POPG) with a high concentration of arrestin (50 μ M). Analysis of the sample with size exclusion chromatography (SEC) showed two distinct peaks that eluted at the predicted molecular weights (chromatogram not shown). The fractions corresponding to the two peaks with high absorbance were analyzed by SDS-Page to confirm the identity of the proteins. The peaks corresponded to two distinct proteins. Each ran as a single band at the expected molecular weight when compared to the marker (**Fig. A-2**). This data suggests that arrestin 2 and the main components of rHDL particles do not interact under the conditions used for this assay.

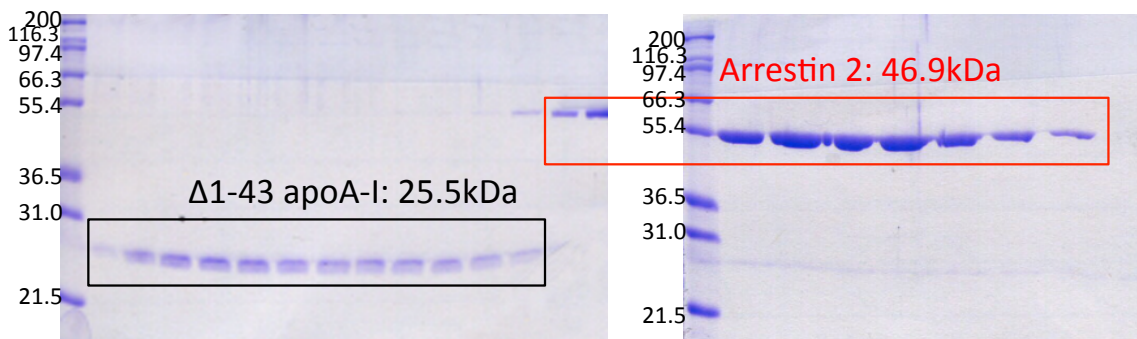


Figure A-2. Arrestin 2 and rHDL do not interact. Empty rHDL particles were prepared with POPC and POPG (3:2 ratio) at the exact conditions used to make β_2 AR containing ones. The empty rHDL particles were incubated with arrestin 2 (50 μ M) for 30 mins at 4°C and then resolved by SEC (Superdex 200). SDS-Page (12.5 % gel) confirmed the presence of both proteins at the expected molecular sizes for Δ 1-43 apo A-I and arrestin 2, 25.5 kDa and 46.9 kDa respectively.

Arrestin Recruitment: Conformational Changes on the Receptor

Most signal transduction mechanisms are guided by ligand-induced conformational changes in the receptor, which are then transmitted, to downstream effectors, resulting in a specific signal output. Plasma membrane translocation of arrestin to activated receptor and its signaling capabilities even in the absence of receptor-G

protein interaction suggests the existence of receptor conformations that are specific for arrestin binding. In order to elucidate distinct arrestin-binding conformations. We decided to use site-specific labeling of the β_2 AR with bromobimane together with rHDL particles to investigate β_2 AR–arrestin 2 active-state. This system was previously used to characterize the conformational changes that the β_2 AR undergoes upon treatment with ligands and/or G proteins (63).

Although it has been established as a critical step in arrestin recruitment, it is still unknown the extent to which receptor phosphorylation is necessary for, or contributes to, arrestin signaling pathways. To address this issue we decided to start our studies with arrestin mutants that were previously shown to bind receptor indiscriminately of their phosphorylation state. Arrestin 2–382, was shown to exhibit phosphorylation-independent receptor binding due to the removal of the key regulatory residues that keep arrestin in its basal conformation (151).

The bimane-labeled β_2 AR (mB- β_2 AR) was incorporated in particles and the response to arrestin 2-382 was determined in the absence or presence of the agonist isoproterenol. Addition of the phosphorylation-independent mutant (Arrestin 2-382) did not cause a significant change in fluorescence intensity on the mB- β_2 AR spectra at low ratio, 2:1 arrestin 2-382. Increasing the ratio of arrestin to receptor to 20:1 caused a 14% decrease in fluorescence intensity and a slight leftward shift in the λ_{\max} . Interestingly, addition of saturating amounts of the agonist isoproterenol together with an excess of arrestin 2-382 causes a moderate decrease in the fluorescence intensity. The fact that arrestin induces a change in fluorescence intensity on top of that induced by ISO suggests

that arrestin binding might contribute to stabilizing an active receptor conformation (**Fig. A-3**). Although these data are encouraging and imply that addition of isoproterenol could promote and stabilize the formation of an arrestin- β_2 AR complex more experiments need to be done before stating a concrete hypothesis. To this end we decided to repeat these experiments using various ligands under different conditions that would resemble those found in the natural environment inside the cell.

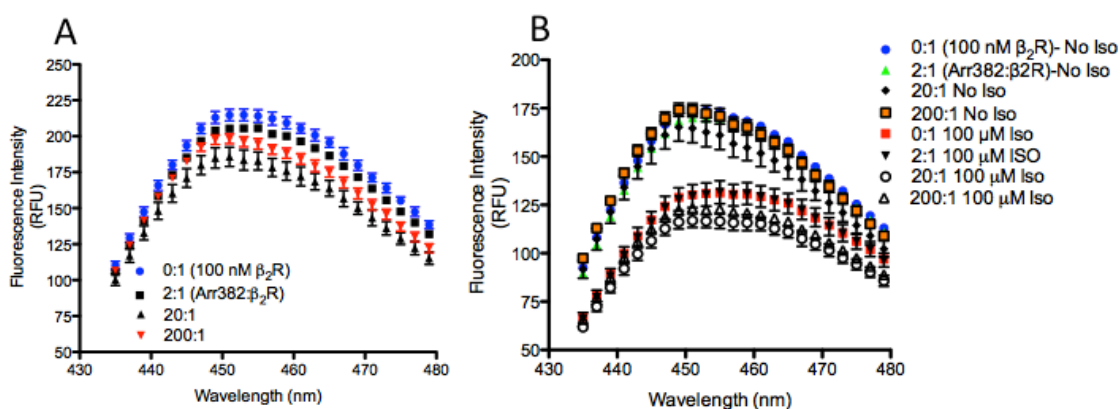


Figure A-3. The effect of arrestin 2-382 on the mB- β_2 AR conformation. Initial emissions scans (blue spectra) of mB- β_2 AR were obtained. **A)** Addition of increasing amounts of arrestin 2-382 to pre-formed rHDL-mB- β_2 AR particles. Addition of a 20-fold excess of the arrestin mutant caused a slight decrease in fluorescence intensity. **B)** rHDL-mB- β_2 AR \pm arrestin 2-382 at various ratios were incubated with isoproterenol. After 20 mins the fluorescence intensity of the β_2 AR spectra decreased as previously observed (63). The presence of arrestin 2-382 caused a further decrease in the fluorescence intensity.

The role of phosphorylation in arrestin recruitment and endocytosis was demonstrated by the finding that GRK2 overexpression promoted agonist-induced internalization of the M2 muscarinic acetylcholine (M2) receptor, whereas expression of catalytically inactive GRK2 inhibited internalization (152). Since, numerous studies have demonstrated that GRK-mediated phosphorylation promotes endocytosis. Yet its role in

signaling is controversial as it has been showed that some GPCRs like the AT1_AR can recruit/signal through arrestin regardless of the phosphorylation state of the receptor (153). However, for the β_2 AR it was proven necessary, a mutant that lacks both GRK and PKA phosphorylation sites was incapable of recruiting arrestin, and could not mediate arrestin-dependent ERK activation. In light of these observations we measured the conformational changes of the phosphorylated- β_2 AR reconstituted in rHDL in the absence or presence of WT arrestin would be more physiologically relevant.

rHDL- β_2 AR was phosphorylated in the presence of GRK2 with or without the $\beta_1\gamma_2$ subunits of the G_s heterotrimer. It has been shown that GRK2 interacts with $\beta_1\gamma_2$ (154) and this interaction leads to an increase in phosphorylation levels (155). Western blot analysis with a phospho-serine (355, 356) antibody specific for the phosphorylated form of the β_2 AR (156) shows a robust increase in the level of phosphorylation of the receptor when compared to a non-treated sample that showed basal phosphorylation. Addition of isoproterenol caused a further increase in the phosphorylation levels, more than GRK2 alone. The most dramatic increase in phosphorylation was observed in the presence of agonist, kinase and the $\beta_1\gamma_2$ subunits (**Fig. A-4**). These results show that the β_2 AR incorporated into rHDL particles can be phosphorylated *in vitro* by GRK2 and that kinase activity is enhanced by the presence of the $\beta_1\gamma_2$ subunits. Moreover, these results are in agreement with published data that states that agonist binding induces a receptor conformation suitable for GRK2 recruitment and binding.

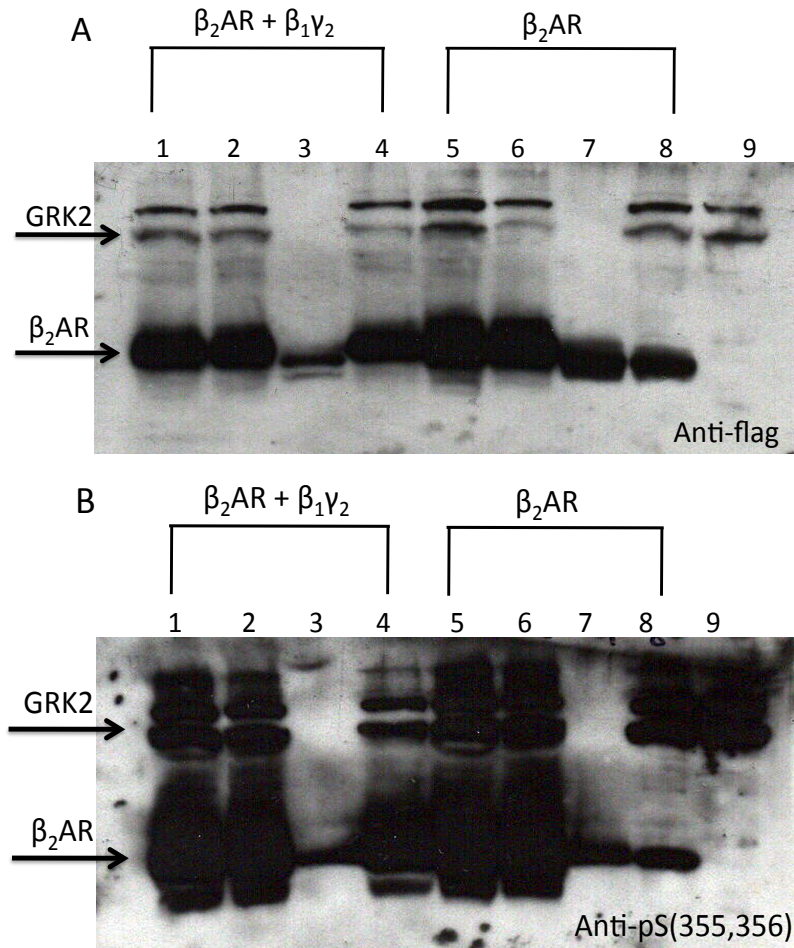


Figure A-4. Phosphorylation of the β_2 AR in rHDL by GRK2. The β_2 AR was reconstituted in rHDL particles and phosphorylated with GRK2 in the presence of agonist $\pm \beta_1\gamma_2$. Western blot analysis **A)** Anti-flag **B)** Anti-pS(355-356). Lanes 1-4: rHDL- β_2 AR with $\beta_1\gamma_2$ 1) β_2 AR:GRK2 (1:10), 2) β_2 AR:GRK2 (1:5), 3) No GRK₂, 4) No Iso. Lanes 5-8: rHDL- β_2 AR alone 5) β_2 AR:GRK2 (1:10), 6) β_2 AR:GRK2 (1:5), 7) No GRK₂, 8) No Iso. Lane 9: GRK2 alone. Primary Ab: Anti-flag (1:5,000), Anti-pSer(355-356) (1:2,000). Secondary: Goat anti mouse (1:10,000) for both.

Discussion

In light of our current knowledge of the different functions of the GPCRs-arrestin complex a logical step is the evaluation of the conformational changes they imposed on each other upon binding. We have previously taken advantage of fluorescence spectroscopy to answer a similar question but looking closely at the GPCR-G protein complex specifically monitoring the interactions between the β_2 AR and G_s . The study of a β_2 AR-arrestin 2/3 complex in rHDL will provide insights into distinct receptor conformations that will be invaluable in the design of new drugs specially biased agonists.

Our preliminary data suggests that arrestin 2-382 binding induces a modest decrease in the fluorescence intensity and a decrease in λ_{\max} indicative of an outer movement of TM6; this change suggests the formation of a β_2 AR-arrestin 2-382 complex. Interestingly, using a 200-fold excess of arrestin caused a slight increase in fluorescence intensity, opposite of what was observed at the lower concentrations. This could be due to steric hindrance induced by arrestin on the receptor. A possible explanation for this response is that the amount of protein used was too high and it created a constraint that prevented the changes in the bimane environment required to observe a difference in the spectra.

The addition of isoproterenol together with arrestin 2-382 (20-fold excess) promotes a larger change in the bimane spectra (decrease in fluorescence intensity and increase in λ_{\max}). The formation of an active receptor conformation would be in agreement with data that states that isoproterenol can be an agonist for arrestin

recruitment leading to ERK1/2 activation (10). However this change is not as dramatic as the one induced by G_s , suggesting that the conformation adopted by the β_2AR in the presence of arrestin differs from the one observed in the presence of G protein. More experiments looking at the interaction between phosphorylated β_2AR , WT arrestin and different ligands will be instrumental in determining if this change is significant. Also, we cannot discard the possibility that the formation of this complex does not lead to an outer movement of TM6, explaining the lack of change in bimane fluorescence. In this case, we would need to look at different transmembrane domains to find out which ones undergo conformational changes that can be monitored with our system.

We are still in the process of optimizing the assay. Different receptor concentrations are being tested to ensure optimal signal that will allow detection of any changes in the fluorophore environment. The preliminary western blot data on receptor phosphorylation is encouraging. However, with this assay we cannot accurately quantitate phosphate levels. This experiment has to be done in the presence of a tracer such as radiolabeled [^{32}P] ATP (phosphate donor) that will allow a quantification of phosphate moles. Additionally more studies have to be conducted to assesses how the presence of GRK2 and/or $\beta_1\gamma_2$ will affect subsequent experiments. $\beta_1\gamma_2$ has post-translational modifications that allow it to be embedded in the membrane making its removal difficult. Our rHDL particles have a Stokes radius of 10 nm; such a constricted environment will promote an interaction with the receptor thus likely having an effect on bimane spectra. Finally, we will be performing agonist affinity shift assays in which we will evaluate the ability of arrestin 2/3 to form a high-affinity complex with the monomeric β_2AR .

The goal of these experiments is to define the allosteric interaction between ligand binding and arrestin recruitment by using a conformational reporter on the β_2 AR in hopes of getting a better understanding of the concept of biased agonism. The ability of biased ligands to differentiate between arrestin and G protein functions at the receptor level is an emerging area of research. Understanding the molecular and structural framework will allow us to delineate the physiological consequences of these two signaling mechanisms. We expect that a better definition of arrestins and G proteins role in GPCR signaling will facilitate the development of improved therapeutic agents that target this receptor family with improved specificity, efficacy and fewer side effects.

Materials and Methods

I purified apo A-I, CFP- β_2 AR, and arrestin 2 (WT and Δ 382). I also did the rHDL reconstitutions and the fluorescence spectroscopy studies. Helen (X.J. Yao) and Soren Rasmussen from Stanford University purified and bimeane-labeled the β_2 AR.

Expression, Purification and Labeling of β_2 AR

A modified version of the β_2 AR where 4 reactive cysteines were mutated (C77V, C327S, C378A, and C406A) was made. The modified β_2 AR was expressed in Sf9 insect cells by using recombinant baculovirus and solubilized using methods previously described (99). The DDM solubilized receptor was purified by sequential M1-Flag antibody affinity and alprenolol affinity chromatography as described (99). Briefly, CaCl_2 was added to the DDM solubilized extract to a final concentration of 1mM and

loaded to the M1-Flag column. The receptor was eluted from the flag resin with 20 mM Hepes, pH 7.5, 100 mM NaCl, 0.1% dodecylmaltoside (Buffer A) with 1 mM EDTA. The concentration of functional, purified receptor was determined using a saturating concentration (10 nM) of [³H] Dihydroalprenolol as previously described (112). Flag purified receptor was then purified by alprenolol-Sepharose chromatography as described (112). The receptor was eluted from alprenolol-Sepharose with Buffer A with 300 μM alprenolol and 1 mM CaCl₂ and loaded directly onto M1-Flag resin. The M1-Flag resin was washed with Buffer A to remove free alprenolol and eluted with Buffer A plus 1 mM EDTA. Two liters of sf9 cells typically yield 500 μl of a 5 μM solution of β₂AR.

The purified β₂AR and monobromobimane were mixed at the same molarity and incubated overnight on ice in the dark. The fluorophore-labeled receptor was purified right before use by gel filtration.

Purification of Recombinant ApoA-I

Wild type human apoA-I was purified from expired serum as previously described (70). A recombinant apoA-I with an N-terminal 43 amino acid deletion and a hexa-histidine tag (Δ(1-43)-his₆-apoA-I) was expressed using a pET15b vector to transform competent *Escherichia coli* cells (BL21). Cells were resuspended and lysed by gently vortexing in 10 mM Tris-HCl, pH 8.0, 100 mM NaH₂PO₄, 6 M guanidine hydrochloride (GuHCl), 1 % Triton X-100. Lysate was fractionated by centrifugation at 10, 000 G and the supernatant was loaded onto a Ni-NTA column by gravity flow. The column was washed with 10 mM Tris-HCl, pH 7.0, 100 mM NaH₂PO₄, 6 M guanidine hydrochloride, 1 % Triton X-100 and then with 50 mM NaH₂PO₄, pH 8.0, 300 mM NaCl

and 1 % Triton X-100. Bound $\Delta(1-43)$ -his₆-apoA-I was eluted with 50 mM NaH₂PO₄, pH 8.0, 300 mM NaCl, 250 mM imidazole, 1 % Triton X-100. Peak fractions were further purified on a Superdex 75 gel filtration chromatography column in 20 mM Hepes, pH 8.0, 100 mM NaCl, 1 mM EDTA, 20 mM sodium cholate. Pooled apoA-I was then dialyzed against 20 mM Hepes, pH 8.0, 100 mM NaCl, 1 mM EDTA, 5 mM sodium cholate. Purified apoA-I was concentrated to ~10 mg/mL and stored at -80°C until use.

In Vitro Reconstitution of β_2 AR into rHDL

High-density lipoproteins were reconstituted *in vitro* according to a protocol adapted from Jonas (113). Briefly, a mixture of POPC and POP (3:2 molar ratio) in combination was used to mimic the zwitterionic environment of a cell membrane (114). A typical rHDL reconstitution consisted of the following components: 24 mM detergent (cholate or DDM), 8 mM lipids, and 100 μ M apoA-I. Lipids were solubilized with a solution of 20 mM Hepes pH, 8.0, 100 mM NaCl, 1 mM EDTA and 50 mM detergent. To reconstitute the bimane labeled β_2 AR (mB- β_2 AR), purified β_2 AR was added to at least 10-fold excess ([apoA-I]:[β_2 AR]) to receptor preparations diluted in solubilized lipids. Following an incubation of 1-2 hrs at the TM of the lipid combination, samples were added to an equal volume of hydrated BioBeads (BioRad) for an additional 3 hrs to remove detergents, resulting in the formation of rHDL particles. Samples were stored on ice until used. If necessary, β_2 AR-rHDL particles were separated from receptor-free rHDL by M1-anti-Flag immunoaffinity chromatography. Purified β_2 AR-rHDL particles were eluted with EDTA (10 mM) and stored on ice until further use.

Purification of Arrestin 2

Recombinant arrestin 2 and arrestin 2-382 were expressed using a modified pGEX4T-1 vector (GST tag and thrombin site removed) to transform competent *Escherichia coli* cells (BL21). Both isoforms were purified as described (157) but with modified procedures. Cells were grown in LB (Luria Broth) containing 0.1 mg/mL ampicillin at 30°C for 6-8 hours (O.D.₆₀₀ ~ 0.1-0.4) then induced with 30 µM IPTG for an additional 12-16 hours. The cells were harvested by centrifugation; the pellet was resuspended in 50 mM Tris-HCl, pH 8.0, 5 mM EGTA, 1 mM DTT, 2 mM benzamidine, 1 mM PMSF, 10 µM leupeptin, 0.7 µg/mL pepstatin, 5µg/mL chymostatin and lysed using a French Press. The lysate was centrifuged (12, 000 rpm for 60 mins), and the supernatant protein was then precipitated with (NH₄)₂SO₄ (35 g/100 mL of supernatant). The sample was centrifuged (13, 000 rpm for 30 mins), and the pellet was dissolved in column buffer A (10 mM Tris -HCl, pH 7.5, 2 mM EDTA, 2 mM EGTA, 1 mM DTT, 2 mM benzamidine, 1 mM PMSF, 10 µM leupeptin, 0.7 µg/mL pepstatin, 5µg/mL chymostatin) and clarified by centrifugation (19, 000 rpm for 20 mins). The supernatant was dialyzed against column buffer A containing 100 mM NaCl. The sample was then loaded on a heparin-Sepharose column and eluted with a 150-700 mM linear NaCl gradient in column buffer A. Peak fractions were pooled, concentrated using an Amicon Ultra 10, 000 MW, and prepared for phenyl-Sepharose column by adding (NH₄)₂SO₄ to a final concentration of 1 M. The sample was loaded into the phenyl-Sepharose column, and eluted with a 1-0 M (NH₄)₂SO₄ gradient in column buffer B (50 mM NaPO₄, pH 7.5, 200 mM NaCl, 2 mM EDTA, 2 mM EGTA, and protease inhibitors. Peak fractions were pooled and dialyzed overnight against 20 mM Hepes, pH 7.2, 200 mM NaCl, 1 mM

EDTA. The sample was concentrated, aliquoted, frozen in liquid nitrogen and stored at -80°C until needed. Protein purity was ~ 95%, based on SDS-Page and Coomassie blue staining, and typical yields were 5-10 mg of purified arrestin-2/L of culture. *Note:* For different isoforms and mutants with low expression levels and/or *in vivo* proteolysis problems, grow the cultures for 10-12 hours without IPTG, and then add 20-30 µM IPTG and grow for an additional 3-5 hours.

Immunoblotting

Antibody for the Flag epitope was obtained from Invitrogen (Carlsbad, CA). β_2 AR phosphorylation was detected with anti-phospho-Ser(355, 356) β_2 AR antibody that was kindly provided by Dr. Richard Clark (The University of Houston, Texas). Aliquots (10 µL) of samples were resolved on SDS-polyacrylamide 12.5 % gels and transferred to PVDF membranes for immunoblotting. The membranes were probed with the monoclonal β_2 AR anti-phosphoserine-specific antibody pSer(355,356) at a dilution of 1:2,000. The membranes were stripped and re-probed with Anti-Flag (1:5,000). Blots were washed three times and incubated with the appropriate secondary antibodies [goat anti-mouse IgG horseradish peroxidase at a 1:10,000 and detected by ECL Plus chemiluminescence reagents.

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