Biased Signaling at the β2-adrenergic Receptor is Established by Receptor-Transducer Interactions

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Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Biochemistry in the Graduate School of Duke University

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ABSTRACT

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Abstract

β-Adrenergic receptors (βAR) are one of the key modulators of cardio-pulmonary functions and belong to a large family of membrane proteins, termed G-protein coupled receptors (GPCRs). β-blockers (βAR antagonists) and βAR agonists are the mainstay treatments for heart failure and asthma respectively, which reflects the significance of βARs as therapeutic targets. The binding of catecholamines (e.g. adrenaline) to βARs activates intracellular transducer proteins such as hetero-trimeric guanosine triphosphate (GTP) binding proteins (G-proteins) or β-arrestins (βarr) that results in regulation of cardiac output and bronchodilation.

The bifurcated signaling pathways initiated by G-protein or β-arrestin downstream of βAR can be selectively activated, a phenomenon termed ‘biased agonism’. Biased ligands, which can pharmacologically separate these pathways, are of major therapeutic interest due to their potential for improving the specificity of drug actions. For βAR, biased agonism towards β-arrestin is expected to render cardioprotective benefits, while selective activation of G proteins is hypothesized to subdue major side effects from current asthma therapy. Therefore, elucidation of how βARs can preferentially interact with their transducers is at the core of developing next generation therapeutics, beyond conventional β-blockers and agonists.

Thus far, the exact mechanism behind GPCR biased agonism remains obscure. The leading hypothesis in the field is that GPCRs adopt distinct conformations that preferentially couple to G proteins or β-arrestins. In order to test this hypothesis, we developed and established a G protein biased mutant β2AR (Chapter 2), since efficacious biased ligands for this receptor are yet to be found. Subsequent assessment of GPCR kinase (GRK)-mediated phosphorylation states of this mutant receptor and
phosphorylation rescue experiments revealed unexpected findings that contradict the initial hypothesis (Chapter 3). Next, we initiated a biophysical characterization of this mutant β2AR (Chapter 4) to comprehend the conformational and structural basis for its apparent biased phenotype. The cumulative insight gained from experiments described in Chapters 2-4 highlights the under-appreciated role of GRKs in determining GPCR biased agonism – the mutant β2AR is biased towards G protein due to conformational selection against GRKs, rather than β-arrestins. Furthermore, to obtain a comprehensive understanding of biased agonism, we devised a strategy to map the interface between β2AR-β-arrestin, which can also be used to form stable complexes for further biophysical characterization (Chapter 5). In summary, this dissertation improves the current understanding of the molecular mechanism behind biased agonism at the prototypical GPCR, β2AR.
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List of Abbreviations

3-BTFMA N-(4-bromo-3-(trifluoromethyl)phenyl)acetamide
7TMR Seven transmembrane receptor
AC Adenylyl cyclase
ADP Adenosine diphosphate
ANOVA Analysis of variance
AT1AR Angiotensin II type 1A receptor
ATP Adenosine triphosphate
β2AR β2-adrenergic receptor
β2ARpp V2Rpp-ligated β2AR receptor (at the C-terminal tail)
β2V2R A chimeric receptor which C-terminal tail of β2AR is replaced with that of V2R
βarr β-arrestin
BI-167107 Boehringer Ingelheim, high-affinity β2AR agonist
BRET Bioluminescence resonance energy transfer
BSA bovine serum albumin
cAMP 3’-5’ cyclic adenosine monophosphate
CEST Chemical exchange saturation transfer
CGP-12177 4-[3-[(1,1-Dimethylethyl)amino]2-hydroxypropoxy]-1,3-dihydro-2H-benzimidazol-2-one hydrochloride
CHS Cholesterol hemisuccinate
CYP Cyanopindolol
DDM n-Dodecyl β-D-maltoside
DHA Dihydroalprenolol
DNA Deoxyribonucleic acid
DSA Disuccinimidyladipate
EPR Electron paramagnetic resonance
EDTA Ethylene diamine tetraacetic acid
EM Electron microscopy
ERK Extracellular signal-regulated kinases
F-19 NMR Fluorine-19 nuclear magnetic resonance
Fab Fragment antigen binding
FDA Food and drug administration
FLAG Peptide sequence DYKDDDDK, an affinity tag
GAP GTPase activating protein
Gα α subunit of heterotrimeric G protein
Gβγ βγ dimers of heterotrimeric G protein
GDP Guanosine diphosphate
GEF Guanine nucleotide exchange factor
GFP Green fluorescent protein
GPCR G protein coupled receptor
G protein Guanine nucleotide binding protein
GRK G protein-coupled receptor kinase
GTP Guanosine triphosphate
HDL High-density lipoprotein
HDX Hydrogen-deuterium exchange
HEK293 Human embryonic kidney 293 cell
HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), a buffering agent
HRP Horseradish peroxidase
IB Immunoblot
ICI ICI-118,551 a β2AR inverse agonist
ICL Intracellular loop
IP Immunoprecipitation
ISO Isoproterenol, a β2AR full agonist
kDa kilodalton
Kd Dissociation constant representing binding affinity
mBBr Monobromobimane
MNG Maltose Neopentyl glycol
Nb Nanobody
NLBD N-terminal lipid binding domain
PAGE Polyacrylamide gel electrophoresis
PAR Protease-activated receptor
PBS Phosphate-buffered saline
PCR Polymerase chain reaction
PDB Protein data bank
PKA Protein Kinase A
RF Radiofrequency
RNA Ribonucleic acid
RNAi RNA interference
SDS Sodium dodecyl sulfate
SEM Standard error of the mean
SEC Size exclusion chromatography
V2R V2 Vasopressin receptor
$\text{V}_2\text{Rpp}$ a synthetic peptide derived from the $\text{V}_3\text{R}$ C-terminal tail

$\text{WB}$ Westron blot
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Chapter 1. Introduction

1.1 What are adrenergic receptors?

Adrenergic receptors are the metabotropic gateway of endocrinologic effects of the hormones adrenaline and noradrenaline. These catecholamine-derivative messengers play central roles in the regulation of sympathetic nervous system regulated functions, such as neural, cardiac and pulmonary physiology, by stimulating adrenergic receptors distributed widely throughout the body (1). Activation of adrenergic receptors results in variety of effects, from the classical fight-or-flight response to the modulation of heart rate and bronchodilation (2-4).

Originally, adrenergic receptors were divided into two major types, $\alpha$ and $\beta$; then several sub-classifications emerged based on pharmacological differences, G protein subtype specificity and sequence divergence uncovered by molecular cloning (5, 6). In this dissertation, we focused on elucidating the molecular mechanism of the $\beta_2$-adrenergic receptor ($\beta_2$AR), due to its physiological significance and extensive history as a prototypical receptor model for demonstrating key discoveries in the field of receptor biology (7-9).

1.1.1. Adrenergic receptors belong to the G protein coupled receptor superfamily

Adrenergic receptors, including $\beta_2$AR, belong to a membrane protein superfamily referred to as G protein-coupled receptors (GPCRs), also known as seven transmembrane receptors (7TMRs) (10). GPCRs are critical regulators of a multitude of physiological processes as they transduce wide-range of extracellular stimuli—from ions, monoamines, peptides to small proteins—into intracellular signalsSeven-
transmembrane receptors (11). Due to their diverse and critical roles in multitude of physiological processes, GPCRs are one of the richest classes of druggable proteins, attested by the fact that approximately one-third of all FDA-approved drugs target members of the GPCR family (12).

### 1.1.2 Conserved GPCR regulation mechanism

The GPCR family has over 800 members in the human genome (13), yet its members share highly conserved functional and regulatory mechanisms that are predominantly governed by three classes of proteins (heterotrimeric G proteins, GPCR kinases and β-arrestins) (10) (Figure 1-1).

**Figure 1-1: The conserved GPCR regulation by G proteins, GPCR kinase (GRKs) and β-arrestins.**

Stimulation of GPCRs with agonists activates G proteins and subsequent phosphorylation by GRKs (2,3,5,6 isoforms) recruit β-arrestins (1 or 2). Listed ligands and subtypes of proteins represent β2AR interacting partners. Receptor figures modified from original illustrations by Winger. L (Lefkowitz Lab, Duke University).

Binding of the agonist to the extracellular orthosteric binding site of a GPCR induces conformational changes that activate specific G proteins at the plasma
membrane, which triggers the exchange of G protein-bound GDP to GTP and ultimately complete dissociation of the heterotrimeric G protein subunits (α, β, γ) (14). Gα subunits stimulated effectors that generate secondary messengers such as cyclic AMP and IP₃ (15, 16) and Gβγ dimers additional effectors downstream (17). Furthermore, some GPCRs couple to more than one G protein subtypes, such as G protein switching by βAR between Gs (stimulatory) and Gi (inhibitory) proteins (18, 19).

Subsequently, agonist-activated receptor C-terminus or intracellular loops are phosphorylated by GPCR kinases (GRK1-7), which form a serine/threonine kinase subfamily (20). Among 7 GRK isoforms, GRK 1 and 7 are confined to the visual system in eyes (21), and GRK4 expression is limited to testis (22). GRK 2, 3, 5, 6 are ubiquitously expressed, and their kinase activity is an essential trigger of the termination of G protein mediated signaling cascades (23).

The phosphorylation of agonist-activated GPCRs by GRKs promotes β-arrestin recruitment, which in turn induces receptor desensitization (24) by sterically blocking additional G protein coupling and triggers receptor internalization (25). Moreover, β-arrestins also function as a transducer by activating signaling pathways independent of G proteins (26). Out of the 4 arrestins in this subfamily, arrestins 1 and 4 are specifically co-expressed with photoreceptors in eyes (27), and globally expressed arrestins 2 and 3 are referred to as β-arrestins 1 and 2 (28).

1.1.3 A barcode system established by GRKs and β-arrestin

In the complex network of GPCR functions, GRK and β-arrestins feedback system render a pivotal role by steering the kismet of a receptor after activation by agonists (29). One of the crucial factors which governs the path of a GPCR- β-arrestin
complex is hypothesized to be the phosphorylation patterns of an agonist-activated receptor. This phosphorylation-driven differentiation of β-arrestin functions is termed as the “Barcode Hypothesis.”(30)

Analysis of ligand-induced phosphorylation states of the three distinct GPCRs, V₂ vasopressin receptor (V₂R) (31), Angiotensin II type1A (AT1AR) (32), and β₂AR (33) revealed relationships between phosphorylation by different GPCR Kinases (GRKs) and their consequential cellular functions. The authors found that GRK2/3 are responsible for the overall abundance of phosphorylation, as well as recruitment of β-arrestin and internalization of the receptor-β-arrestin complex, whereas phosphorylation by GRK5/6 results in the β-arrestin-mediated activation of extracellular signal-regulated kinases (ERK).

1.2 Dynamic process of receptor activation and regulation

1.2.1 Two-state model vs conformational ensemble

The traditional two-state model explains the receptor activation as a simple on-and-off bimodal switch. According to this model, stimulation of a GPCR with an agonist shifts the equilibration from an inactive form of the receptor (R) to an active state of the receptor (R*) (34). However, increasing biophysical and structural evidence suggests that receptors exist in ensembles of multiple conformations (Rn, R*n) (35-38).

The conformational heterogeneity and plasticity of GPCRs can be perceived as a spectrum of structures, and this additional complexity is changing the classical definition of ligand efficacy. Instead of an agonist being considered a key to “turn on” a receptor, it is now viewed as an external influence which redistributes the population of conformations or redefines the kinetics of interconversion between different states (39-41).
1.2.2 Receptor-transducer complexes

The signal transduction by agonist-activated GPCRs occurs primarily through interactions with intracellular effectors (also known as transducers), such as G proteins, GRKs and β-arrestins. Therefore, the structural and biophysical characterization of receptor-transducer complexes are of great interest for in-depth understanding of GPCR biology (42, 43). GPCRs are notoriously challenging targets for structural analysis due to the difficulty in obtaining functional proteins in abundance and their inherent structural instability (44). Nevertheless, GPCR structural biology experienced an exponential growth in the past decade due to the culmination of multiple technological breakthroughs (45, 46). Thus far, total of 233 receptor crystal structures, 47 unique receptor structures and 3 receptor-G protein complex structures have been reported (47), and the following sections detail current structural information related to β2AR-transducer complexes.

1.2.2.1 β2AR-Gs complex

The activation of downstream effectors by heterotrimeric G proteins in response to agonist-activated GPCRs is at the heart of the GPCR-mediated signal transduction. The crystallographic structure of β2AR in complex with nucleotide-free heterotrimeric Gs protein revealed major conformational changes from that of the inactive β2AR (48). Large outward movements of TM6, an outward shift of TM5, and subtle inward shifts of TM7 and Helix8 are the key features of the G protein coupled β2AR. In addition, authors found that the Gα subunit is exclusively responsible for forming the interface with the β2AR, and no contacts with the receptor were found from Gβγ subunits.
1.2.2.2 β₂AR- β-arrestin complex

Despite added complexity by GRK-mediated phosphorylation of the receptor and multi-step binding mode, structural information on receptor-β-arrestin complexes is emerging from multiple fronts (49-52). In particular, a combination of electron microscopic (EM) imaging and hydrogen-deuterium exchange (HDX) analysis identified two distinct β₂AR- β-arrestin1 conformations (49).

One is a “loose” conformation where β-arrestin1 is hanging from the phosphorylated C-terminal tail of the β₂AR and does not interact with the receptor’s 7 transmembrane helix bundle. The other is “tight” conformation in which β-arrestin1 makes contact with both phosphorylated C-terminal tail and agonist-activated intracellular core of the β₂AR.

Moreover, decades of biochemical and mutagenesis data have implicated a conformational change in a region called the “finger loop” of arrestin as being the primary mediator of the latter interaction with the receptor transmembrane core (53). This has been revealed in higher resolution in a recent crystallographic structure of rhodopsin-arrestin (50).

1.2.2.3 β₂AR- GRK5 complex

While the agonist-activated receptor phosphorylation by GRKs is the pivotal first step towards the initiation of multi-faceted β-arrestins functions, structural characterization of receptor-GRK complexes has been the least explored area of research due to the fact that the complex between these two proteins has low affinity, high flexibility and is sensitive to lipid environments (54).
In order to circumvent these challenges, complementary approaches of chemical crosslinking and HDX, followed by mass spectrometry analysis have been employed to investigate the β2AR-GRK5 complex (55). One of the prominent features of the interaction between β2AR and GRK5 is nestling of the GRK5 N-terminal lipid binding domain (NLBD) inside the intracellular core of the β2AR. This feature is similar to that of alpha helix domain of the Gs protein in complex with β2AR (48) as well as the finger-loop of arrestin1 coupled with rhodopsin (50).

1.3 Biased agonism and its therapeutic potential

1.3.1 Definition of biased agonism

Prior to the discoveries of β-arrestin mediated signaling pathways, G proteins were believed to have a monopoly on GPCR signal transduction (56). This paradigm has been replaced by the revelation that a trifecta of proteins (G protein, GRK, β-arrestin) regulate GPCRs, leading to the appreciation of the multi-dimensional nature of the GPCR physiology. Intriguingly, the multifarious signaling pathways triggered by G protein or β-arrestin are pharmacologically separable (57) (Figure1-2A). Although finer distinctions can be made, the term “biased agonism” is used in the context of the selective activation of G protein- or β-arrestin-mediated signaling pathways downstream of GPCRs in this dissertation.

1.3.2 Therapeutic potential of biased agonism at the β2AR

Biased agonism has extensive therapeutic ramifications as one can preferentially activate signaling pathways that lead to desired therapeutic outcomes without affecting pathways that contribute to side effects (58, 59). For example, carvedilol, an FDA-approved mainstay therapeutic for heart failure, is a weak β-arrestin biased β2AR
agonist and an antagonist for G protein functions with cardio-protective properties (60, 61). On the other hand, β2AR agonists with bias in the opposite direction, towards G protein activation, are expected to be the next generation of asthma therapeutics (62) due to the deleterious effects exerted by β-arrestin mediated functions in the pathophysiology of asthma, such as tachyphylaxis and implications in allergic lung inflammation (63-65).

1.4 Focus of this dissertation

To date, the molecular mechanisms underlying biased agonism remain poorly understood. A widely accepted hypothesis is that biased ligands stabilize distinct receptor conformations that preferentially engage G protein or β-arrestin (66). While β-arrestin interacts with the intracellular “core” of the GPCR seven transmembrane helix bundle, overlapping the G protein binding pocket, initial recruitment of β-arrestin requires GRK-dependent phosphorylation of the receptor C-terminal tail and/or intracellular loops. Interestingly, the enzymatic activity of GRKs also requires binding to the agonist-activated receptor intracellular core, suggesting specific receptor conformations might also differentially modulate GRK coupling to influence signaling through β-arrestin indirectly.

To understand the molecular mechanisms of biased agonism, we used the β2-Adrenergic Receptor (β2AR) as a model system given its extensive pharmacological (10), biophysical, and structural characterization (9). Despite their therapeutic potential (58), efficacious G protein-biased ligands for β2AR have yet to be identified. Therefore, we turned to a mutagenesis strategy (Figure1-2B) to study G protein bias at the β2AR, and we demonstrate that such bias can indeed be achieved at the level of GRK regulation. In
addition, we also devised a disulfide trapping strategy to form a stable complex between β₂AR-β-arrestin1 which can be further subjected to structural characterization.

Figure 1-2: Multiple ways to achieve biased agonism at GPCRs. The ligand-induced (A) and mutation-induced (B) selective activation of bifurcated signaling pathways by G protein or β-arrestin.
Chapter 2. G Protein Biased β2AR Mutant


### 2.1 Mutation induced G protein biased agonism

G protein-biased β2AR agonists hold invaluable potential as the next generation of asthma therapeutics (62), since sustained activation of β-arrestin-mediated pathways are associated with eliciting side effects (63-65). Despite their therapeutic potential, efficacious G protein-biased ligands for β2AR have yet to be identified. Therefore, we turned to a mutagenesis strategy (Figure1-2B) to study G protein bias at the β2AR, which can spur the subsequent efforts for finding biased ligands.

We employed a directed mutagenesis strategy around β2AR residues T68, Y132, and Y219 (Figure 2-1A), which have been predicted to be involved in transducer binding by evolutionary trace analysis (67). A similar strategy by our laboratory previously yielded a β-arrestin-biased β2AR triple mutant (T68F, Y132G, Y219A) (67). During the process of saturation mutagenesis of each individual residue, we discovered that an alanine substitution at Y219 (Y219A) in transmembrane 5 (TM5) (Figure 2-1B) triggers G protein-biased signaling through the β2AR.
Figure 2-1: The location of Y219A substitution on the β2AR.
(A) The snake diagram of β2AR representing the position of Y219A (Red outlined-G protein biased) and previously reported mutations that cause a β-arrestin-biased phenotype of β2AR (Blue-T68F, Y132G, Y219A) (67). (B) The structural model of β2AR with Y219A highlighted in red (PDB ID: 2RH1). Mutagenesis screening experiments were performed by Staus.D and Wingler. L (Lefkowitz Lab, Duke University).

2.1.1 G protein activation by β2AR Y219A

More specifically, stimulation with agonists induced increase in cellular cAMP levels and G protein mediated GTP hydrolysis in vitro by both β2AR wild-type (WT) and Y219A, as a direct consequence of G protein activation (Figure 2-2 A.1). While the ability to activate G protein is preserved for β2AR Y219A, the efficacy of agonists appears to be diminished, as shown by overall decrease in maximal responses and kinetics of G protein activation (Figure 2-2 B.2). However, a full agonist isoproterenol (ISO) induced comparable levels of maximal G protein activation, albeit decrease in apparent EC$_{50}$ (Figure 2-2 A.2). The decrease in G protein efficacy in β2AR Y219A is largely the result of a decrease in isoproterenol affinity for Y219A (4.1-fold reduction in affinity, Figure 2-4A).
Figure 2-2: The agonist-dependent activation of G protein is retained but diminished at β2AR Y219A.

(A.1) A diagram depicting an increase in cAMP level as a direct consequence of G protein activation via agonist stimulated β2AR upstream. (A.2-A.4) G protein activation by transiently expressed β2AR WT or β2AR Y219A was measured in HEK293 cells that stably express a cellular cAMP biosensor (Glosensor, Promega). SEM of n=5 in triplicate. Cells were stimulated with a full agonist ISO (A.2) or a partial agonist Salbutamol (A.3)
or a weak partial agonist DCI (A.4). (B.1) A diagram illustrating GTP hydrolysis as a readout of measuring kinetics of G protein activity. (B.2, B.3) G protein activation by purified β2AR WT or β2AR Y219A reconstituted in HDL particles was measured in vitro through monitoring GTP hydrolysis (GTPase-Glo, Promega). Purified receptors were stimulated with ISO (B.2) or no ligands (B.3) SEM of n=3 in duplicate.

2.1.2 β-arrestin recruitment to β2AR Y219A

While the ability to activate G protein was preserved at β2AR Y219A, the interaction between β-arrestin and β2AR Y219A (Figure 2-3A) was under the detection limit of two different enzyme-based assays (Tango and DiscoverX) and confocal microscopy (Figure 2-3B-E). In a protease-triggered transcription factor assay, β-arrestin was recruited to β2AR wild-type in an agonist-dependent manner, but recruitment to β2AR Y219A was negligible (Figure 2-3B). To maximize the assay’s sensitivity, we repeated these experiments with a chimeric β2V2R construct, in which the β2AR's C-terminal tail is replaced with that of the V2 vasopressin receptor (V2R), thereby increasing the stability of its interaction with β-arrestin without altering its pharmacological properties (68). However, β-arrestin recruitment to Y219A receptor remained undetectable (Figure 2-3C, D). Moreover, GFP-βarrestin2 did not show relocalization towards the plasma membrane upon stimulation with ISO in β2AR Y219A-expressing cells (Figure 2-3E). Collectively, these data indicate that β2AR Y219A is a novel mutant with unequivocal biased agonism towards G protein activation.
Figure 2-3: The agonist-dependent recruitment of β-arrestin is abolished at β2AR Y219A.

(A) A pictorial representation of β-arrestin recruitment to agonist-activated receptors. (B-D) β-arrestin recruitment to transiently expressed β2AR WT or β2AR Y219A (also in β2V2R format) was measured using cell-based assays. (B) The protease-triggered
transcription factor assay (Tango, Thermo Fisher). SEM of n=3 in triplicate. (C) The enzyme complementation assay (Pathhunter, DiscoverX). SEM of n=3 in triplicate. (E) β-arrestin recruitment to plasma membrane visualized by confocal microscopy. U2OS cells were transiently transfected with β2AR WT or β2AR Y219A and GFP-β-arrestin2, followed by stimulation with ISO for indicated durations. (B)-(D) Experiments were performed by Ahn. S, Staus. D, Chatterji. A (Lefkowitz lab, Duke University).

2.1.3 Pharmacological characterization of β2AR Y219A

In order to determine if there are any changes in pharmacological properties of β2AR Y219A that may affect its biased behavior, the affinities of various agonist and antagonists to β2AR Y219A were measured by competitive radio-labeled ligand binding (69). During this analytical procedure, fixed concentrations of receptors and radio-labeled ligands, [125I]-cyanopindolol (CYP) in this case, were incubated with increasing amounts of unlabeled ligands. The amount of radio-labeled ligand bound to receptors, which decreases as the amount of unlabeled ligand increases, is fitted to a decreasing sigmoidal curve. Any change in affinity of an unlabeled ligand to the receptor is manifested as a horizontal shift of this sigmoidal curve.

Overall, the affinities of both agonists and antagonists to β2AR Y219A are reduced compared to those of β2AR wild-type to varying degrees (Figure 2-4A). Notably, it appears that the extent of reduction in affinity follows the trend of ligand efficacy in G protein activation (Figure 2-4B). The observed alterations in affinity of unlabeled ligands were not due to changes in affinity of the radio-labeled probe [125I]-CYP, as Kds of [125I]-CYP, determined by saturation binding, to both β2AR wild-type and Y219A are comparable (Figure 2-4C). Moreover, when over expressed in HEK cells, β2AR wild-type and Y219A have comparable surface expression (Figure 2-4D); therefore, the mutant receptor’s biased phenotype cannot be accounted for by this potential variable, which
could differentially impact cell-based assays with varying degrees of signal amplification (70).

Figure 2-4: β2AR Y219A displays overall decreases in affinity to various agonists and antagonists. (A) Competitive radio-ligand binding assays performed with [125I]-cyanopinolol (CYP) for the measurement of affinities between ligands and β2AR WT or β2AR Y219A. (B) A bar graph representation of differences in IC50 of ligands on β2AR Y219A determined in (A) compared to that of β2AR WT. (C) [125I]-CYP affinity to HDL-β2AR WT or HDL-β2AR
Y219A determined by saturation binding. (D) Cell surface expression levels of β2AR WT and β2AR Y219A from transiently transfected HEK293 cells were measured by whole cell binding with [3H]-CGP. (A-D) SEM of n≥3 in duplicate.

2.2 Absent downstream β-arrestin mediated functions at β2AR Y219A

Given the inability of β2AR Y219A to recruit β-arrestin, we hypothesized that other β-arrestin-mediated events, such as GPCR internalization (25) and extracellular-signal regulated kinase 1 and 2 (ERK1/2) activation (67), would be impaired as well.

2.2.1 β-arrestin-mediated internalization of β2AR Y219A

While stimulation of β2AR wild-type with isoproterenol induced plasma membrane localization of β-arrestin2 and receptor internalization, neither event occurred with β2AR Y219A as assessed by confocal microscopy (Figure 2-5A). Consistent with these findings, an enzyme complementation GPCR internalization assay (Figure 2-5B) confirmed that the chimeric β2V2R Y219A failed to translocate β-arrestin to endosomes (Figure 2-5C).
Figure 2-5: β-arrestin mediated internalization is not observed at β2AR Y219A.
(A) U2OS cells were transiently transfected with β2AR WT or β2AR Y219A and GFP-β-arrestin2. Subsequently, cells were fixed, permeabilized, blocked and stained for imaging with a laser-scanning microscope. (B) An illustration elucidating the enzyme complementation-based detection of β-arrestin dependent receptor internalization. (C) The translocation of β-arrestin2 to endosomes in response to the agonist ISO was measured in U2OS cells stably expressing β-arrestin and endosomes each tagged with fragments of β-galactosidase (PathHunter, DiscoverX) and transiently expressing β2V2R WT or β2V2R Y219A. SEM of n=3 in triplicate. (A) Microscopic images were obtained by Staus. D (LeKowitz lab, Duke University).

2.2.2 β-arrestin-mediated activation of EKR1/2 downstream of β2AR Y219A

While β-arrestin recruitment and internalization assays are direct readouts of β-arrestin involvements, activation of ERK1/2 is governed by G protein activities (Figure 2-6A) as well (67). Since both G protein- and β-arrestin-mediated signaling can affect ERK1/2 activation, we assessed ERK1/2 phosphorylation after stimulation of β2AR wild-type and Y219A with carvedilol, which induces β-arrestin but not G protein-mediated phosphorylation of ERK1/2 (71). In order to probe for ERK activation only in transfected cells, we probed the phosphorylation of an ERK2 fusion protein, which has a different electrophoretic mobility than endogenous ERK, co-transfected with β2AR wild-type or Y219A. Indeed, stimulation of β2AR Y219A with the balanced agonist ISO activated ERK2, peaking at 5 minutes post-stimulation (Figure 2-6B, C), albeit to a lesser extent than β2AR wild-type. On the other hand, the β-arrestin biased ligand carvedilol induced significant ERK2 activation only through β2AR wild-type β2AR Y219A (Figure 2-6D, E).
Figure 2-6: Impaired β-arrestin-mediated activation of ERK2 downstream of β2AR Y219A.

(A) A diagram illustrating multi-faceted ERK1/2 activation through both G protein and β-arrestin. (B-E) β-arrestin-mediated activation of ERK2 was measured by transiently expressing β2AR WT or β2AR Y219A and ERK2-EGFP in HEK293 cells and stimulating with indicated ligands at 37°C for the indicated duration. (B) Representative immunoblots of ISO (balanced agonist) induced pERK2 and (C) its quantified immunoblots. $$$ P<0.001 between WT 5min versus Y219A 5min, Tukey’s multiple comparisons test after two-way ANOVA, SEM of n=4. (D) Representative immunoblots of carvedilol (β-arrestin-biased agonist) induced pERK2 and (E) its quantified immunoblots. **** P<0.0001 compared to WT (0min) control, Dunnett’s multiple comparisons test after one-way ANOVA. SEM of n=5.
Multiple lines of evidence thus far, such as absent recruitment of β-arrestins to β2AR Y219A (plotted in bias calculation graphs Figure2-7) and its downstream functions, demonstrate that the introduction of a single mutation Y219A on β2AR unequivocally converts the wild-type receptor become to become profoundly biased towards activation of G proteins.

Figure 2-7: Equimolar bias calculation (70) of β2AR WT (Black, close circle) and β2AR Y219A (Red, closed square).

The downstream cellular responses upon ISO stimulation of β2AR WT or β2AR Y219A were measured by two different assays (G protein activation vs β-arrestin recruitment) at the same concentrations of ligand (ISO) and then plotted against each other. (A)-(C) Three graphs each represents the equimolar bias comparison between G protein activation measured by increase in cellular cAMP level (Figure 2-2A.2) and against three different β-arrestin recruitment assays (A)-Figure 2-3B, (B)-Figure 2-3D, (C)-Figure 2-3C.
Chapter 3. GRKs Orchestrate Biased Agonism


3.1 GRK-mediated phosphorylation states of β2AR Y219A

Phosphorylation of the C-terminus or intracellular loops (ICL) of GPCRs by GRKs is a critical intermediary step for β-arrestin recruitment (20). To determine if changes in GRK phosphorylation could contribute to the biased properties of β2AR Y219A, we compared the agonist-dependent phosphorylation pattern of β2AR Y219A to that of β2AR wild-type in cells and in vitro.

![Figure 3-1: The snake diagram of β2AR phosphorylation sites.](image)
3.1.1 In cellulo phosphorylation

Thirteen serine/threonine phosphorylation sites have been identified on the β2AR by an orthogonal approach, integrating RNA interference and mass spectrometry analysis (Figure 3-1). Among these, certain residues have been shown to be phosphorylated by GRK2 (β2AR Ser407 and Ser411), GRK6 (β2AR Ser355 and Ser356) or a non-GRK kinase, protein kinase A (PKA, β2AR Ser261 and Ser262) specifically (33) (72).

In order to investigate the agonist-induced changes in phosphorylation state of β2AR Y219A and to compare to that of β2AR wild-type, we overexpressed respective receptor constructs in cells and stimulated with an agonist isoproterenol. The phosphorylation pattern of both β2AR wild-type and Y219A were probed with previously developed phospho-specific antibodies (33) against residues mentioned to be phosphorylated by various intracellular kinases. Although we observed a large increase in agonist-dependent phosphorylation of β2AR wild-type Ser355 and Ser356, no significant phosphorylation of β2AR Y219A was observed even after 30 minutes of agonist stimulation (Figure 3-2A, quantified in 3-2B). Similarly, phosphorylation of Ser407 and Ser411 (GRK2) was reduced in β2AR Y219A compared to β2AR wild-type (Figure 3-2A, quantified in 3-2C). The reduction in phosphorylation of β2AR Y219A was specific to GRK-phosphorylated residues, as we observed no significant difference in the phosphorylation of Ser261 and Ser262 by protein kinase A (PKA), which is activated downstream of G protein (Figure 3-2A, quantified in 3-2D).
Figure 3-2: The agonist-dependent phosphorylation pattern of β2AR Y219A in cell.
Expi293f cells were transiently transfected with FLAG-tagged β2AR WT or β2AR Y219A and stimulated with agonist ISO at 37C for the indicated duration. The receptor was immunoprecipitated with anti-FLAG M1 agarose beads. Eluted samples were subjected to immunoblotting with phospho-specific antibodies. (A) Representative immunoblots and (B) quantified immunoblots of pS355 and S356 (****P<0.0001 compared to WT (0min) control, $$$ P<0.0001 compared to equivalent conditions of Y219A), (C) pS407 and S411 ($$ $ P<0.001, overall comparison) and (D) pS261 and S262 (*P<0.05 compared to WT (0min) control, ### P<0.001 compared to Y219A (0min) control). (B)-(D) The densitometry values of phospho immunoblots were normalized by total receptor immunoblots and represented as % values of the maximum signal obtained from WT. SEM of 4 independent experiments. Tuckey’s multiple comparisons test after two-way ANOVA.
3.1.2 In vitro phosphorylation

To verify that the deficiency in GRK-mediated β2AR Y219A phosphorylation in cells is a direct consequence of an altered interaction between GRKs and the receptor, we measured the in vitro GRK5-mediated phosphorylation of purified β2AR wild-type or Y219A reconstituted in liposomes. In this system, isoproterenol is required to phosphorylate Ser355 and Ser356 of β2AR wild-type (Figure 3-3A, quantified in 3-3B). Consistent with the cellular data, the Y219A mutation abrogated isoproterenol-induced phosphorylation of Ser355 and Ser356 by GRK5 in vitro (Figure 3-3A, quantified in 3-3B). Moreover, steric blockade of the G protein-binding pocket by Gs heterotrimer or the single domain antibody Nb80 (73) is sufficient to prevent this phosphorylation (Figure 3-3A, quantified in 3-3B), in agreement with a previous report (55). This further supports that GRKs respond to agonist activation by sensing the large conformational changes that occur in the intracellular core of the receptor (illustrated in Figure 3-3C).

**Figure 3-3: The agonist-dependent phosphorylation pattern of β2AR Y219A in vitro.** Receptors were reconstituted in lipid vesicles and stimulated with ISO in the presence of purified GRK5 and ATP. (A) Representative and (B) quantified immunoblots of phosphorylation at residue Ser355 and Ser356. Comparisons between WT ISO (second black bar) to all other conditions within WT (****P<0.0001) and all other conditions of Y219A (#### P<0.0001). The densitometry values of phospho immunoblots were
normalized by total receptor immunoblots and represented as % values of the maximum signal obtained from WT. SEM of 3 independent experiments. Tuckey’s multiple comparisons test after two-way ANOVA. (C) A cartoon representation of interaction between receptor intracellular core and a GRK and its allosteric effects.

3.2 Artificial restoration of phosphorylation rescues β-arrestin mediated functions

Engagement of β-arrestin with agonist-activated GPCRs occurs in two steps (Figure 3-4): first, β-arrestin recruitment to the phosphorylated receptor C-terminus induces conformational changes within β-arrestin, which then promote β-arrestin coupling to the receptor intracellular core (74). β2AR Y219A fails to interact with β-arrestin due to a deficiency in GRK-dependent phosphorylation, which interferes with the first step of this interaction. This raises the question of whether the agonist induced conformations of β2AR Y219A would be capable of promoting the binding of β-arrestin at the intracellular core if C-terminal phosphorylation is somehow rescued.

Figure 3-4: The 2-step recruitment of a β-arrestin to a GPCR.
3.2.1 *in vitro* ligation of V₂Rpp phospho-peptide

Since β₂AR Y219A cannot be phosphorylated by GRKs in cellulo or in vitro (Figure 3-2, Figure 3-3), we restored the phosphorylation on the C-terminal tail by we enzymatically ligating (Figure 3-5A) (75, 76) a synthetic phosphopeptide (pp). The enzymatic conjugation of the phosphopeptide was carried out by sortase, a transpeptidase of Gram-positive bacteria, which catalyzes the joining of the recognition sequence LPXTG and multi-glycine substrate (77). The sequence of homogenously phosphorylated synthetic peptide was derived from the C-terminal tail of V₂ vasopressin receptor (V₂R), which provides more stable interactions with β-arrestins (68).

Following ligation of the phosphopeptide, the purified receptors were reconstituted in high-density lipoprotein (HDL) particles and evaluated for their physical interaction with β-arrestin. Restoration of β-arrestin binding to the phosphorylated C-terminus was verified by co-immunoprecipitation (IP) experiments. Both agonist-activated β₂ARpp wild-type and β₂ARpp Y219A were able to specifically pull-down similar amounts of β-arrestin1 and Fab30 (78), an antibody fragment that stabilizes phosphopeptide-activated β-arrestin1 (Figure 3-5C, lane 3, 5). In contrast, β-arrestin1 does not survive co-IP with non-phosphorylated β₂AR wild-type or Y219A, underscoring the necessity of receptor phosphorylation for β-arrestin binding (Figure 3-5C, lane 2, 4).
Figure 3-5: In vitro ligation of phosphopeptide (V2Rpp) to β2AR Y219A reinstates interaction with β-arrestin1.

(A) A schematic diagram of V2Rpp ligation to β2AR WT and β2AR Y219A. The enzyme sortase was used to conjugate the synthetic phosphopeptide V2Rpp (a 29-amino acid peptide with 8 phosphorylated residues) to β2AR WT and β2AR Y219A at residue 365, where the sortase recognition sequence LPETGGH was inserted. (B) The ligation efficiency of the phosphopeptide was determined by gel electrophoresis and visualized by coomassie staining. (C) The physical interaction between V2Rpp ligated receptors and purified β-arrestin1 was verified by co-immunoprecipitation (IP) experiment. Purified β-arrestin1 and Fab30 were added in all lanes. The receptors in eluates were deglycosylated with PNGaseF (n=3).
3.2.2 β-arrestin mediated desensitization is restored

β-arrestins are scaffolding proteins that mediate a multitude of functions such as desensitization of G protein activity, receptor internalization, and initiation of downstream signaling pathways. While several of these functions require interaction only with the phosphorylated receptor C-terminus (79, 80), the interaction between the intracellular core of the β2AR and β-arrestin appears to be indispensable for the desensitization of G protein activity (80).

Initially, based on β2AR Y219A's impairment in β-arrestin-mediated functions in cellular assays, we hypothesized that β-arrestin would be unable to interact with the intracellular core of β2ARpp Y219A. Accordingly, we used an in vitro G protein-dependent GTP hydrolysis assay to measure the ability of β-arrestin to inhibit G protein activation (Figure 3-6A). Stimulation of the β2AR by isoproterenol significantly enhanced G protein-dependent GTP hydrolysis, which could be blocked by Nb80 binding to both phosphorylated and non-phosphorylated receptors (Figure 3-6B, C, D, E). By contrast, β-arrestin1 attenuated GTP hydrolysis only for phosphorylated wild-type receptor (Figure 3-6B, C). β-arrestin1 similarly decreased GTP hydrolysis for β2ARpp Y219A (Figure 3-6D, E), confirming that β-arrestin1 properly engages the intracellular core of the mutant receptor and successfully blocks coupling to Gs protein.

Taken together, experimental results from both Chapter2 (Characterization of the biased phenotype of β2AR Y219A) and the current chapter on the role of GRK-mediated phosphorylation in the biased agonism at β2AR Y219A indicate that the intracellular core of the Y219A receptor is modestly impaired in coupling to both G protein (Figure 2-2) and β-arrestin (Figure 2-3) compared to the wild-type receptor, but completely deficient in coupling to GRKs (Figure 3-3) thus giving rise to the G protein biased phenotype.
Figure 3-6: Restoration of phosphorylation on β2AR Y219A rescues β-arrestin mediated desensitization.

(A) Desensitization of Gs protein activity in the presence of β-arrestin1 was measured by a decrease in G protein-dependent GTP hydrolysis in vitro (GTPase-Glo, Promega). HDL-β2ARpp WT(B) or HDL-β2AR WT(C) or HDL-β2ARpp Y219A(D) or HDL-β2AR Y219A(E) was incubated with Gs protein and GTP in presence of indicated ligands and Nb80 or β-arrestin+Fab30 (Comparisons between indicated pairs in WT (**) P<0.01) or Y219A (## P<0.01) from Tuckey’s multiple comparisons test after one-way ANOVA, SEM of n=3 in duplicate). HDL reconstitution of purified receptors was performed by Pani.B (Lefkowitz Lab, Duke University).
4. Chapter 4. β2AR Y219A Conformations


Collective experimental results from chapter 2 and 3 suggest that the selective activation of G proteins by β2AR Y219A is due to conformational selection against GRK coupling, rather than its inherent inability to couple to β-arrestins. In order to dissect the structural basis of this unexpected origin of biased agonism towards G proteins, we initiated conformational characterization of β2AR Y219A.

4.1 β2AR Y219A – transducer interactions

According to the ternary complex model, the coupling of transducer proteins, such as G protein (81) and β-arrestins (82), to the intracellular core of β2AR exerts an allosteric effect on the receptor that increases agonists’ affinity for the orthosteric ligand binding pocket. The allosteric interaction between a receptor and its transducers manifests as a change in agonist affinity that can be directly measured by competitive radiolabeled ligand binding. Therefore, we measured β2AR Y219A's ability to interact with Gs protein, GRK5 and β-arrestins relative to that of β2AR wild type (Figure 4-1). The increase in agonist isoproterenol affinity results in a left-shift of competitive binding curves.
Figure 4-1: Allosteric effect of transducer binding to the intracellular core of $\beta_2$AR Y219A.

(A), (D), (F), (I) Schematic illustration of allosteric interactions between the extracellular ligand binding pocket and transducer binding at the intracellular core of $\beta_2$AR. Purified $\beta_2$AR WT (Black) or $\beta_2$AR Y219A (Red) were reconstituted in HDL particles. Radio-
labeled ligand competition binding experimental results with Gs protein (B-C), GRK5 (E) and β-arrestins (G-K).

For heterotrimeric Gs proteins (Figure 4-1C) and β-arrestins (Figure 4-1K), both transducers were able to allosterically couple to β2AR Y219A and phosphopeptide-ligated β2ARpp Y219A, albeit to a lesser extent compared to β2ARpp wild-type (Figure 4-1B, J). Interestingly, the addition of nanobody 35 (Nb35), which stabilizes the nucleotide-free state of Gs protein (48), seemed to rescue the strength of allosteric interaction between β2AR Y219A and Gs protein (Figure 4-1C, open triangles). Moreover, β-arrestins’ allosteric effect is specific to phosphopeptide ligated receptors and was not observed for the unligated receptors (β2AR wild-type and β2AR Y219A) (Figure 4-1G, H). For GRKs, no allosteric effect was detected at β2AR wild-type, even at high concentrations of GRK5 (1µM) (Figure 4-1E). Therefore, the receptor-GRK interaction was measured by probing the degree of phosphorylation (Figure3-3) instead.

These data are consistent with the restoration of desensitization at β2ARpp Y219A (Figure3-6) and bolster the claim that a G protein-biased receptor can retain the conformational capability of coupling to β-arrestin through its intracellular core.

4.2 β2AR Y219A conformations

4.2.1 Outward movement of the TM6 is a hallmark of receptor activation

Activation of GPCRs through binding of an agonist at the extracellular orthosteric ligand binding pocket results in substantial conformational changes of the intracellular transmembrane (TM) domains. For instance, the large outward movement (14Å) of TM6 observed in the crystal structure of the β2AR-Gs protein complex (48) is one of the hallmarks of receptor activation. This mobility of TM6 was measured with multiple biophysical techniques: bimane fluorescence emission analysis and Fluorine-19
(19F) NMR use cysteine reactive chemical probes (Figure 4-2B) on the β2AR construct (Figure 4-2C, β2AR Δ4C) of which all non-essential cysteines are replaced. β2AR Δ4C used for these studies retained pharmacological properties of β2AR wild-type (83, 84).

**Figure 4-2: Chemical probes and the receptor construct used for biophysical analysis of β2AR Y219A conformations.**

(A) A cartoon representation of the outward movement of TM6, which is characteristic of receptor activation (85). (B) The chemical probes used for bimane fluorescence and 19F NMR experiments. (C) A snake diagram of the receptor construct used for bimane
fluorescence and F-19 NMR experiments. Filled red circles indicate replaced cysteine residues and open red circles highlight the remaining cysteine residues.

4.2.2 G protein induced conformational changes in β₂AR Y219A

Using bimane-labeled β₂AR Δ4C Y219A, we measured the changes in the bimane fluorescence emission spectra upon binding with an agonist isoproterenol and Gs protein. Such perturbations of β₂AR Δ4C wild-type is associated with a decrease in overall fluorescence intensity and an increase in the peak emission wavelength (84). In agreement with the cellular (Figure 2-2) and pharmacological data (Figure 4-1), TM6 of β₂AR Y219A responds to the stimulation of an agonist and couples to Gs protein (Figure 4-3C), but to lesser extent compared to β₂AR wild-type (Figure 4-3B). Further assessments of the interactions between β₂AR Δ4C Y219A and GRKs or β-arrestins are underway.

![Figure 4-3: Gs protein-induced β₂AR Y219A conformational changes measured by bimane fluorescence emission.](image)

(A) A cartoon representation of the bimane-labeled β₂AR. Bimane fluorescence emission spectra of β₂AR WT(B) and β₂AR Y219A (C). (B),(C) Average spectra from 4 independent experiments.

4.2.3 Distinct β₂AR Y219A conformational states

While the analysis of bimane-labeled β₂AR Δ4C Y219A revealed that the TM6 of this mutant receptor experiences chemical environments distinct from that of β₂AR wild-
type during activation, the nature of these differences was not clear. In order to dissect the impact of Y219A modification on receptor conformations, we employed $^{19}$F NMR measurements on the identical residue (C265) that was used in bimane experiments (Figure 4-6A). Changes in chemical environments around the $^{19}$F NMR probe (3-BFTMA, Figure 4-2B) is reflected as distinct chemical shifts (position of the peak on x-axis) and/or shape of the peak.

The use of Fluorine-19 in protein NMR experiments offers enhanced sensitivity and specificity relative to other NMR active nuclei, owing to its high gyromagnetic ratio and rarity in biological samples. Due to these advantages, it has been extensively used to characterize $\beta_2$AR conformations (86-89). In particular, the dynamic nature of $\beta_2$AR activation was investigated through a combination of $^{19}$F NMR and electron paramagnetic resonance (EPR) experiments (88). In this study, 4 distinct states (S1-4) of $\beta_2$AR were detected (Figure 4-5): S1 refers to an inactive state with the ionic-lock (charged-residue interactions that anchor $\beta_2$AR in an inactive state (90)) intact and S2 represents another inactive state with the ionic-lock broken; S3 was observed as an intermediary state towards activation and S4 represents the fully activated state of $\beta_2$AR.

![Figure 4-5: Dynamic process of $\beta_2$AR activation represented by 4 states (88).](image)
Grey: inactive states, Red: active states.

The one-dimensional $^{19}$F NMR spectra of $\beta_2$ARΔ4C wild-type displayed typical signatures reported in the previous study; one broad peak from the unliganded receptor (fast exchanging mixture of S1 and S2 states, Figure 4-7A) and the emergence of an S3 peak in response to the addition of BI-167107, a high-affinity agonist (Figure 4-7B). Notably, changes in detergent affect the overall position of the $^{19}$F NMR peaks (Figure 4-6B), presumably due to differences in off-rates of detergents (91). All NMR experiments in this chapter were performed in detergent n-Dodecyl $\beta$-D-maltoside (DDM) and every spectrum contains an additional peak termed ‘alternative labeling’, indicated as (A) in Figures 4-6B and 4-7, which had been reported to be insensitive to stimulations with both agonists and antagonists (88).

![Figure 4-6: The effect of detergents on $^{19}$F NMR spectra of $\beta_2$ARΔ4C WT.](image)

(A) A cartoon representation of the 3-BTFMA labeled $\beta_2$AR. (B) 1D $^{19}$F NMR spectra of $\beta_2$AR Δ4C wild-type reconstituted in detergent n-Dodecyl $\beta$-D-maltoside (DDM-Black line) or Maltose Neopentyl glycol (MNG-Blue line) were measured at 25°C on a Bruker 700 MHz spectrometer equipped with a room temperature $^1$H/$^{19}$F/$^{13}$C/$^{15}$N CPQCI probe. The data were processed with NMRPipe with 5 Hz exponential line broadening and cosine window function. In collaboration with Wu.Q (Zhou Lab, Duke University).

Intriguingly, $^{19}$F NMR spectra of the unliganded $\beta_2$AR Δ4C Y219A showed two new peaks that were not observed in that of $\beta_2$AR Δ4C wild-type (labeled S’1 and S’3,
Figure 4-7C). Based on the chemical shift (position on the x-axis) of the new peaks, it is conceivable that they represent the S1 and S3 states (Figure 4-5) of $\beta_{2}AR$ Δ4C wild-type. Yet, it is also possible that they originate from a new species of conformations adopted by $\beta_{2}AR$ Δ4C Y219A. Furthermore, the decrease in peak intensity of $\beta_{2}AR$ Δ4C Y219A S3 peak Figure 4-7D) suggests that it is a unique conformation which is more prominently populated by unliganded $\beta_{2}AR$ Δ4C Y219A.

Figure 4-7: One dimensional $^{19}$F NMR spectra of $\beta_{2}AR$ Δ4C Y219A.
1D $^{19}$F NMR spectra of $\beta_2$AR Δ4C wild-type (Black lines-A, B) and $\beta_2$AR Δ4C Y219A (Red lines-C, D) reconstituted in detergent DDM were collected at 25°C on a Bruker 700 MHz spectrometer equipped with a room temperature $^1$H/$^{19}$F/$^{13}$C/$^{15}$N QXI probe. Data analysis was processed with NMRPipe with 5Hz exponential line broadening and cosine window function. (B), (D) Dotted lines represent the addition of agonist BI167107. In collaboration with Wu.Q (Zhou Lab, Duke University).

In this chapter, we initiated the interrogation of $\beta_2$AR Y219A conformations by pharmacological (Figure 4-1) and biophysical techniques (Figure 4-2~7); experimental results thus far suggest that this G protein biased mutant receptor is conformationally distinct from $\beta_2$AR wild-type.
Chapter 5. β2AR-β-arrestin Interface and Complex Formation

Portions of the experimental results and methods developed in the following chapter are published in


β-arrestins are multi-adaptor proteins that regulate a plethora of GPCR functions, primarily by being recruited to agonist-activated and GRK-phosphorylated GPCRs (29). β-arrestins distinguish active GPCRs by two factors; first through GRK mediated phosphorylation of intracellular loops (ICL) or C-terminal tail of GPCRs and second by conformational changes in the intracellular core of the receptors due to agonists binding (Figure 5-1A). The engagement between GPCRs and β-arrestins is expected to cause substantial conformational changes both proteins.

Several features of structural changes within β-arrestin due to binding to a phosphorylated receptor C-terminal tail was captured by the crystal structure of activated β-arrestin1 bound to a V2 Vasopressin receptor derived phosphopeptide (V2Rpp) (78). The disruption of two major sets of intramolecular constraints in β-arrestin1 by V2Rpp resulted in relative 20 twisting of the N and C domain, as well as rearrangement of several loops which further interacts with the activated GPCR intracellular core. Furthermore, the dynamics of the β-arrestin conformational changes
due to phosphorylated GPCR tails have been investigated by orthogonal biophysical methods such as F-19 NMR and BRET (92-94).

Mounting biochemical, structural and biophysical studies also indicate that the receptors adopt distinct conformations upon coupling with β-arrestins (49-52). Although the rhodopsin-arrestin crystal structure (50) started to shed light on this aspect, detailed information of the conformational changes in hormone activated GPCRs in complex with β-arrestins still remains obscure.

The foremost prerequisite for any structural or biophysical analysis of the interaction of a GPCR with a β-arrestin is to obtain stable protein complexes that will last the duration of data acquisition in mg quantity. Yet formation of the GPCR-β-arrestin complex is a challenging endeavor due to the relatively low affinity, transient and complex nature of their interaction. Numerous attempts without additional stabilizers, such as antibody fragments (49, 78), nanobodies (80) or generation of fusion proteins, did not yield much success. Therefore, we devised a strategy to stabilize the receptor-β-arrestin interaction by covalently linking two proteins through a disulfide bond between mutagenized cysteines on the interface, termed ‘disulfide trapping’ (Figure 5-1B, C).
Figure 5-1: Conformational heterogeneity of the β2AR-β-arrestin1 complexes and disulfide trapping strategies utilized for stabilization of each conformation.

(A) The interaction between the agonist-activated, GRK-phosphorylated β2AR and β-arrestin occurs at the phosphorylated C-terminal tail and the intracellular core of a receptor. This two-step binding mode gives rise to the mixture of “Loose” and “Tight” conformations (49). (B)-(C) Disulfide trapping strategies were devised to stabilize these transient conformations. To find the optimal pair of residues to be replaced with cysteine to form a covalent disulfide bond between two proteins, two regions of the β2AR, C-terminal tail (A) and intracellular core (B), and corresponding parts of the β-arrestin1

5.1 Disulfide trapping of β2AR and β-arrestin

We used disulfide trapping as a method to obtain stable complexes between β2AR and β-arrestin, as well as to verify residues that interact in proximity. The association of β-arrestin with agonist activated β2AR occurs at two parts of the receptor, the phosphorylated C-terminal tail and the intracellular core (Figure 5-1A). Therefore, residues on both the C-terminal tail (Tail Selection, Figure 5-1B) and the core of the β2AR (Core Selection, Figure 5-1C) were screened for the feasibility of forming a covalent disulfide bond with corresponding residues on β-arrestin1 that are predicted to be in close proximity. To maximize the interaction with β-arrestin1 (68) during screening and also due to existing crystallographic structure of activated β-arrestin1 is bound to V2Rpp (78), a chimeric form of β2AR referred to as β2V2R (the C-terminal tail of β2AR is replaced with that of V2 vasopressin) was used.

5.1.1 Tail selection

The residues to be replaced with cysteine on the tail of β2V2R and β-arrestin1 were selected based on the crystallographic structure of active β-arrestin1 bound to a homogenously phosphorylated Vasopressin2 receptor tail peptide (V2Rpp) (PDB ID: 4JQI, (78)) (Figure 5-2A). The phosphorylated serine and threonine residues on the V2Rpp and lysine residues on β-arrestin1 that are within 10Å of each other (measured from Cβ) were mutated to cysteine individually (Figure 5-2B).

The feasibility of the selected residues to form a specific disulfide bond between the β2V2R and β-arrestin1 complex was tested by transiently co-expressing both
proteins in HEK293 cells. The recruitment of β-arrestin1 to β₂V₂R was triggered by the stimulation of cells with an agonist isoproterenol (ISO). Subsequently, H₂O₂ was added to facilitate disulfide bond formation by oxidizing thiol groups on cysteine residues. Successful complex formation between β₂V₂R and β-arrestin1 was assessed by the shift in the apparent molecular weight (50kDa >100kDa indicated by the red arrow) of the β-arrestin1 co-immunoprecipitated with receptors (Figure 5-2C). The dissociation of β-arrestin1 from the complex in the presence of the reducing agent dithiothreitol (DTT) validated that complexes were held together by disulfide bonds (Figure 5-2C, Lane 3s in each condition). Among 12 pairs that were tested, 4 pairs of residues successfully formed disulfide trapped complexes. Notably, these pairs are all between residues approximately 5Å apart in the V₂Rpp activated β-arrestin1 crystal structure successfully formed disulfide trapped complexes (Figure 5-2B).
Figure 5-2: Summary of the tail selection result.

(A) Crystallographic structure of V2Rpp activated β-arrestin1 (PDB ID: 4JQI) with residues that were tested for disulfide trapping are highlighted in red or blue. (B) The list of residues on the V2Rpp and β-arrestin1 that were individually mutated to cysteine and their corresponding Cβ distances measured from the crystal structure. (C) Cysteine mutants of β2V2R and β-arrestin1 were transiently co-expressed in HEK293 cells and disulfide complex was formed upon stimulation with the agonist ISO and oxidant H2O2. Subsequently, FLAG tagged β2V2R was immunoprecipitated (IP) with anti-FLAG M2 and co-IPed β-arrestin1s were detected by immunoblotting. The red arrow represents the location of the disulfide trapped β-arrestin1 and the positive hits are highlighted in black.
5.1.2 Core selection (Interface mapping between β₂V₂R and β-arrestin1)

The affinity between the tail of β₂V₂R and β-arrestin1 is relatively strong due to favorable electrostatic interactions between negatively charged phosphorylated residues on the receptor tail and the positively charged lysine residues on β-arrestin1. However, the interaction between intracellular core of the β₂V₂R and β-arrestin1 is rather weak and transient, which gives rise to a mixture of “loose” and “tight” conformations when visualized by electron microscopy (49) (Figure 5-1A).

In order to obtain conformationally homogenous complexes, we expanded the disulfide trapping strategy used in the tail selection (Figure 5-2) to stabilize the interaction between the intracellular core of the receptor and β-arrestin1. We hypothesized that the stabilization of the receptor core and β-arrestin1 through disulfide trapping would convert the previously observed mixed population of “loose” (single contact with the phosphorylated C-terminal tail) “tight” conformation of β₂V₂R – β-arrestin1 complexes to uniformly “tight” one.

Unlike the tail selection, no structural information was available to guide the selection of residues to be mutated to cysteine in the intracellular core region of β₂V₂R. Thus, a combination of chemical cross-linking and mass spectrometry analysis was used to identify residues on β₂V₂R and β-arrestin1 that are in close proximity. β₂V₂R – β-arrestin1 complexes were reacted with a homo-bifunctional amine cross linker, disuccinimidyl glutarate adipate (DSA, Figure 5-3B, C) which crosslinked two lysine residues on β₂V₂R K235 and β-arrestin1 K77 (Figure 5-3D), identified by mass spectrometry analysis.
Based this result, residues surrounding $\beta_2V_2R$ K235 and $\beta$-arrestin1 K77 were mutated to cysteine and tested in pairwise combinations for the formation of a covalent disulfide bond between the two proteins. Six single cysteine mutants of $\beta_2V_2R$ (231-236) and five single cysteine mutants of $\beta$-arrestin1 (75-79) were tested against each other in total of thirty combinations using the same in cellulo crosslinking assay as for the tail selection. Among five $\beta$-arrestin1 (75-79) cysteine mutants, robust complex formations were observed with $\beta$-arrestin1 D78C. Therefore, we further tested its disulfide trapping efficiency with all six receptor cysteine mutants in detail (Figure 5-3E). Among the tested combinations, $\beta_2V_2R$ K235C and $\beta$-arrestin1 D78C yielded the highest cross-linking efficiency (Figure 5-3E, quantified in 5-3F). The disulfide bond formation between $\beta_2V_2R$ K235C and $\beta$-arrestin1 D78C is dependent upon the presence of both engineered cysteines (WT control, Figure 5-3E first lane), as well as agonist and oxidant (Figure 5-3E, last 3 lanes) specific.
Figure 5-3: $\beta_2 \text{V}_2 \text{R}\text{-}\beta$-arrestin1 interface mapping by chemical crosslinking and disulfide trapping revealed pairs of residues in proximity.

(A) A structural model of the $\beta_2 \text{V}_2 \text{R}\text{-}\beta$-arrestin1 complex with DSA crosslinked residues highlighted in red. (B) The chemical structure of the homo-bifunctional amine reactive cross-linker (DSA) used to crosslink $\beta_2 \text{V}_2 \text{R}$ and $\beta$-arrestin1. (C) A representative coomassie stained SDS-PAGE gel showing crosslinking efficiency of $\beta_2 \text{V}_2 \text{R}\text{-}\beta$-arrestin1 complexes with DSA. (D) $\beta_2 \text{V}_2 \text{R}$ K235 and $\beta$-arrestin1 K77 were identified as crosslinked by mass spectrometry analysis. (E)-(F) Cysteine mutants of $\beta_2 \text{V}_2 \text{R}$ and $\beta$-arrestin1 were
transiently co-expressed in HEK293 cells and disulfide complex was formed upon stimulation with the agonist ISO and oxidant H$_2$O$_2$. Subsequently, FLAG tagged $\beta_2$V$_2$R was immunoprecipitated (IP) with anti-FLAG M2 and co-IPed $\beta$-arrestin1s were detected by immunoblotting. Representative immunoblots (E) and quantified immunoblots (F) of the IP experiment. The densitometry values of the disulfide trapped $\beta$-arrestin1s were normalized by that of IPed total receptor and represented as % values of the $\beta_2$V$_2$R K235C-$\beta$-arrestin1 D78C complex. SEM of n=4. (B)-(D) Experiments were performed by Jiang, Q and Xiao, K (Duke University, Lefkowitz Lab.

5.2 Pharmacological evaluation of disulfide trapped $\beta_2$AR-$\beta$-arrestin complexes

To ensure the physiological relevance of disulfide trapped $\beta_2$AR-$\beta$-arrestin1 complexes, pharmacological assays with radio-labeled ligands were used. There are two properties of ligands that make this assay suitable for the purpose of quality control. First, ligands (either agonists or antagonists) only bind to functional receptors that are properly folded, which can be used to interrogate any changes in expression due to cysteine mutation. Second, a receptor binds to its agonist with higher affinity when a transducer (such as G protein (81) or $\beta$-arrestin (82)) is also bound, due to allosteric effects. Thus, a shift in agonist affinity for $\beta_2$AR is expected if the disulfide-linked complex exhibits a physiological conformation.

5.2.1 Expression level of single cysteine mutants of $\beta_2$V$_2$R

The protein expression level of $\beta_2$V$_2$R mutants that were selected from both tail and core selections was measured with whole-cell radio-ligand binding experiments. The total receptor expression was determined with the cell membrane permeable $\beta_2$AR antagonist $[^3H]$-dihydroalprenolol (DHA), and the receptor cell surface density was quantified with the cell membrane impermeable $\beta_2$AR antagonist $[^3H]$-CGP 12177 (95).

Three $\beta_2$V$_2$R mutants from the tail selection (T360C, S363C and S364) expressed in 5 pmol/mg range (Figure 5-4A, B), which is approximately 50% of the $\beta_2$AR wild-type
expression level (Figure 5-4A, B, first bars). However, the introduction of these single cysteine mutations markedly increased the expression level compared to that of $\beta_2V_3R$ (Figure 5-4A, B, second bars). Also, the overall expression of the six $\beta_2V_2R$ cysteine mutants from the core selection was comparable to that of the $\beta_2AR$ wild-type (Figure 5-5A).
Figure 5-4: Pharmacological characterization of the disulfide trapped β2V2R S364C-β-arrestin1 K107C (tail complex).

(A) Cell surface density and (B) total receptor expression levels of the β2V2R cysteine mutants (positive hits) from the tail selection were measured by whole cell binding with...
radio-labeled ligands. (C) A cartoon representation of the allosteric effects between intracellular core of β₂V₂R S364C and disulfide crosslinked β-arrestin1 K107C, measured by the radiolabeled ligand competition binding experiments. (D)-(H) Radiolabeled ligand competition binding experiments were performed with HEK293 membranes expressing the disulfide trapped β₂V₂R S364C-β-arrestin1 K107C complexes or indicated proteins. β₂V₂R-β-arrestin2 fusion protein was used as a positive control. (A),(B) Experiments were performed by Wingler. L. (Lefkowitz Lab, Duke University).

Figure 5-5: Pharmacological characterization of the disulfide trapped β₂V₂R K235C-β-arrestin1 D78C (core complex).

(A) Total receptor expression levels of the β₂V₂R cysteine mutants from the core selection were measured by whole cell binding with radio-labeled ligands. (B) A cartoon
representation of the allosteric effects between intracellular core of β2V2R K235C and disulfide crosslinked β-arrestin1 D78C, measured by the radio-labeled ligand competition binding experiments. (C)-(F) Radiolabeled ligand competition binding experiments were performed with HEK293 membranes expressing the disulfide trapped β2V2R K235C - β-arrestin1 D78C complexes or indicated proteins. β2V2R-β-arrestin2 fusion protein was used as a positive control. SEM of n=2 in duplicate.

5.2.2 Allosteric effects of disulfide trapping β-arrestin1 to the β2V2R

Pairs of β2V2R and β-arrestin1 cysteine mutants with the highest disulfide trapping efficiencies from both the tail and core selection were subjected to further pharmacological characterization. The radio-labeled ligand competition assay is a well-established method for assessing the allosteric effect of transducer binding. As explained in chapter 4, the presence of transducer at the intracellular core of a receptor increases the affinity of an agonist in the extracellular binding pocket. This change in agonist affinity is reflected as a curve shift towards the left in the radio-labeled ligand competition assay.

Upon agonist (ISO) and oxidant (H2O2) treatment, disulfide crosslinked β-arrestin1 mutants in both the tail (β2V2R S364C-β-arrestin1 K107C, Figure 5-4F) and core (β2V2R K235C- β-arrestin1 D78C, Figure 5-5F) complexes enhanced the agonist affinity. The allosteric effect exerted by these β-arrestin1 mutants were specific to disulfide linked complexes, as it was not observed in the absence of agonist and oxidant treatment (Figure 5-4E, Figure 5-5E).

5.3 Electron microscopic (EM) evaluation of disulfide trapped β2AR-β-arrestin1 complexes

For screening experiments during the tail and core selection, a chimeric β2V2R was used due to its enhanced affinity to β-arrestins, since it was not feasible to form a stable receptor- βarrestin1 complex with the wild type, non-chimeric β2AR. However, we
successfully disulfide trapped the non-chimeric β2AR K235C (a closer form of the receptor to the physiological β2AR wild-type) with β-arrestin 1 D78C by applying the pair of cysteine mutations identified from the core selection.

### 5.3.1 Expression and purification of disulfide trapped β2AR-β-arrestin1 complexes

For most biophysical analysis, it is essential to secure a substantial amount of proteins in mg quantity. Therefore, we used baculovirus-insect cell (Sf9) expression system to obtain purified β2AR-β-arrestin complexes. Sf9 cells are eukaryotic insect cells that have the membrane environment and post-translational modification machinery that are necessary for receptor expression (96). The majority of GPCR structural and biophysical studies to date have been carried out with proteins purified from the Sf9 insect cell system (97).

Two components, β2AR K235C (N-terminal FLAG tagged) and β-arrestin1 D78C (C-terminal 6X Histidine tagged) were co-expressed in Sf9 cells. Upon harvesting, the cells were stimulated with high affinity agonist BI-167107 to recruit β-arrestin1 D78C. During cell lysis, diamide was included in the lysis buffer to create an oxidizing environment that favors formation of a disulfide bond between β2AR K235C and β-arrestin1 D78C.

Subsequently, a 3-step purification scheme was employed to isolate disulfide trapped β2AR K235C and β-arrestin1 D78C. First, all detergent solubilized N-terminal FLAG-tagged β2AR K235Cs were separated by passing through M1 resin conjugated to anti-FLAG antibodies. Then the elutes were exposed to Ni-NTA resin to select β2AR K235Cs in complex with C-terminal histidine tagged β-arrestin1 D78Cs. Lastly, the purified β2AR K235C-β-arrestin1 D78C complexes were further cleaned up through size
exclusion chromatography (SEC). Among the purified complexes, approximately 70% of \( \beta_2 \text{AR K235C} \) were successfully disulfide trapped with \( \beta \)-arrestin1 D78C (Figure 5-6A).
Figure 5-6: Visualization and pharmacological characterization of the disulfide trapped β₂AR K235C-β-arrestin1 D78C complexes.

(A) β₂AR K235C-β-arrestin1 D78C complexes were expressed, disulfide-trapped in Sf9 insect cells. Subsequent 3-step purification with FLAG affinity (M1-antiFLAG resin), Histidine affinity (Ni-NTA resin) and Size Exclusion Chromatography (SEC) steps yielded purified complexes in mg quantity. Representative SEC A280 trace and a corresponding coomassie stained SDS-PAGE gel and an immunoblot of the eluted fractions. (B) Purified β₂AR K235C-β-arrestin1 D78C complexes were visualized by negative stain-EM followed by particle analysis. Previously reported rhodopsin-arrestin1 negative stain-EM images (50) were added (top row) for a visual reference. (C) Radiolabeled ligand competition binding experiments were performed to measure the allostERIC effect exerted by disulfide trapped β-arrestin1 D78C on β₂V₂R K235C reconstituted in detergent Maltose Neopentyl Glycol (MNG). A G-protein mimetic nanobody (Nb80) (73) was used as a positive control. (B) EM images were obtained by Cahill. T (Lefkowitz Lab, Duke University).

5.3.2 Visualization of disulfide trapped β₂AR-β-arrestin1 complexes by electron microscope (EM)

Biological samples such as proteins are challenging objects to visualize by Electron Microscopy (EM), as they provide poor contrast due to low electron density. However, this limitation is surmounted by staining samples with heavy atom salts which enhances contrast (negative stain) (98). We used this negative stain EM imaging followed by single particle analysis to ensure conformational homogeneity and protein quality of the disulfide-trapped β₂AR K235C-β-arrestin1 D78C complex. We expected this complex to be uniformly in a “tight” conformation, since cysteine residues are located to stabilize β-arrestin1 interaction with the receptor intracellular core, of which was responsible for the origin of “loose” conformation in the previous assessment (99).

Among the observed particles, most appeared to be in the expected “tight” conformation (Figure 5-6B), similar to that of the negative stain EM images and crystal structure of the rhodopsin-arrestin1 complex (50). Yet, there were particles suspected to
be in “loose” conformation as well, presumably due to disulfide trapping efficiency not reaching to completion or the disulfide bonds dissociating in EM imaging conditions.

Furthermore, when these purified β₂AR K235C-β-arrestin1 D78C complexes were subjected to competitive radio-ligand binding experiment, no allosteric effect on the agonist affinity by the disulfide trapped β-arrestin1 was observed (Figure 5-6C). This result was rather unexpected, given the increase in the isoproterenol affinity observed from the disulfide trapped β₂V₂R K235C- β-arrestin1 D78C complexes embedded in HEK293 cell membranes (Figure 5-5F). The perplexing absence of allostERIC connection between the disulfide linked β₂V₂R K235C and β-arrestin1 D78C is most likely due to detrimental effects of detergents on β-arrestin functionality (100). Perhaps forming disulfide complexes with receptors reconstituted in HDL particles, which is devoid of any detergents and closer to the cellular membrane environment, may mitigate the deleterious effects of detergents on proper folding of β-arrestins.
Chapter 6. Materials and Methods


6.1 Materials

All mutagenesis PCRs were performed using the QuikChange mutagenesis kit (Agilent Technologies) in accordance with the manufacturer’s protocol. Gs protein (48), β-arrestin1 (minimal cysteine construct, truncated at residue 393) (78), Fab30 (78) and sortase (75) were purified as previously described.

6.2 Pharmacological assays

6.2.1 Cell surface β2AR expression measurement

The cell surface expression level of β2AR wild-type and β2AR Y219A was measured by whole cell binding with the hydrophilic radioligand [3H]-CGP 39653 (PerkinElmer). pBK-β2AR wild-type or pBK-β2AR Y219A was transiently transfected into HEK293 cells with Fugene 6 (Roche) and harvested 48 hours post-transfection with gentle agitation using 0.02% EDTA at 4ºC. Cells were resuspended in assay buffer (10mM HEPES in Minimum Essential Medium) and incubated with 30nM [3H]-CGP for 3 hours on ice. 10μM Propranolol was used to account for non-specific binding. Unbound [3H]-CGP was separated by filtration onto GF/B glass microfiber filters soaked in water using a 96 well-format Brandel harvester. The GF/B filters were then rapidly washed three times with ice-cold buffer (50mM Tris-HCl pH7.5, 12.5mM MgCl2, 2mM EDTA pH8.0) and soaked in scintillation fluid (Research Products International) overnight. Bound [3H]-CGP was quantified by a Tri-Carb 2800TR Liquid Scintillation
Analyzer (PerkinElmer). All data represent at least three independent experiments; standard error and analysis were performed in GraphPad Prism.

### 6.2.2 Radiolabeled ([¹²⁵I]-CYP) ligand competition assay

Competition binding assays to measure isoproterenol affinity for β₂AR wild-type and β₂AR Y219A were performed in a 250µl reaction volume comprised of [¹²⁵I]-CYP (60pM), a serial dilution of isoproterenol and 60 fmol of functionally active receptors (from HEK membranes or purified receptors reconstituted in HDL particles) in assay buffer (20mM HEPES pH7.4, 100mM NaCl, 0.2% BSA). For experiments with transducers, purified Gs (100nM) or β-arrestin1 (1µM) were added within the total volume of 250ul. Non-specific binding was measured with 10µM propranolol, and total binding was determined in the absence of isoproterenol. In order to ensure that the binding reaction reached equilibrium, the mixed components were incubated at room temperature for 90 minutes. Unbound [¹²⁵I]-CYP was separated by filtration onto GF/B glass microfiber filters treated with 0.3% PEI using a 96 well-format Brandel harvester. Then GF/B filters were then rapidly washed three times with ice-cold buffer (20mM HEPES, 100mM NaCl). Bound [¹²⁵I]-CYP was quantified with a 2470 automatic gamma counter (Perkin Elmer). [¹²⁵I]-CYP affinity for β₂AR wild-type and β₂AR Y219A was determined by saturation binding (Figure 2-4C). All data represent at least three independent experiments; standard error and analysis were performed in GraphPad Prism.

### 6.3 Cell-based assays

#### 6.3.1 In cell cAMP (G protein activation) measurement
G protein activation by agonist-stimulated β2AR wild-type and β2AR Y219A were examined by measuring cAMP levels in HEK293 cells which stably express the GloSensor cAMP biosensor (Promega) (101). HEK293 cells at ~70% confluency in 10cm dishes were transiently transfected with 2µg of either β2AR wild-type or β2AR Y219A using the Fugene 6 transfection reagent (Roche). After 24 hours, the transfected cells were plated at 80,000 cells/well in 96-well, clear-bottomed white plates. Forty-eight hours post-transfection, the cells were treated with the GloSensor reagent that contains luciferase substrates for 90 minutes at 27°C. The cells were then incubated with isoproterenol (10^{-12} ~10^{-5}M) for 5 minutes at room temperature. The luminescence signals were detected by a NOVOstar microplate reader (BMG Labtech).

6.3.2 β-arrestin recruitment assays

Tango (Thermo Fisher), a transcription factor-based assay, was used to measure β-arrestin recruitment to the agonist-stimulated receptor (102). HEK293-T cells stably expressing a tetracycline transactivator (tTA) protein-driven luciferase reporter and β-arrestin2 fused to the tobacco etch virus (TEV) protease were transiently transfected using the Fugene 6 transfection reagent with β2V2R-tTA or β2AR-tTA in the presence and absence of the Y219A mutation. Twenty-four hours post transfection, the cells were seeded onto 96-well dishes at 60,000 cells/well, maintained for an additional 24 hours, and then stimulated with isoproterenol (10^{-12} ~10^{-5}M) for a period of 18 hours. Luciferase activity was measured after incubation with the Bright-Glo luciferase substrate (Promega).

β-arrestin recruitment to agonist-stimulated β2V2R wild-type or β2V2R Y219A was also measured with PathHunter (DiscoverX), a chemiluminescence-based enzyme
fragment complementation assay (103). U2OS cells stably expressing enzyme acceptor (EA) β-arrestin2 were transiently transfected with ProLink™ (PK) tagged β2V2R wild-type or β2V2R Y219A using the Fugene 6 transfection reagent (Roche). Twenty-four hours post-transfection, the cells were plated at 25,000 cells/well in 96-well, clear-bottomed white plates. Forty-eight hours post-transfection, the cells were stimulated with isoproterenol (10^{-12} \text{ M} \text{ to } 10^{-5} \text{ M}) for 90 minutes at 37°C; stimulation was terminated by incubating the cells in PathHunter detection reagents for one hour at 27°C. The luminescence signals were detected by a NOVOstar microplate reader (BMG Labtech).

### 6.3.3 Confocal microscopy

U2OS cells were seeded in CC² coated 4-well chamber slides (NUNC) and transfected with 75ng of pBK-flag-β2AR wild-type or Y219A and 25ng of GFP-β-arrestin2. Forty-eight hours post-transfection, the cells were treated with vehicle (DMSO) or 10µM isoproterenol for 15 min and fixed using 3.7% paraformaldehyde. The cells were permeabilized using 0.4% Triton X-100 in phosphate-buffered saline (PBS) for 3 min, blocked for 1 hour in PBS containing 3% BSA and 20% goat serum, and incubated with rabbit-anti-FLAG (Sigma, 1:500) in blocking buffer overnight at 4°C. Cells were then incubated with secondary anti-rabbit antibody conjugated to Texas-red (Molecular Probes, 1:1000) and DAPI (1:5000) for 1 hour at room temperature. Confocal images were obtained on a Zeiss LSM510 laser-scanning microscope.

### 6.3.4 Internalization assay

The internalization of β-arrestin2 into endosomes by agonist-stimulated β2V2R wild-type or β2V2R Y219A was monitored by PathHunter (DiscoverX), a
chemiluminescence-based enzyme fragment complementation assay. Enzyme Acceptor (EA) tagged β-arrestin2 and a ProLink™ (PK) tag localized to the endosomes form a functional enzyme that generates a chemiluminescence signal upon internalization of β2V2R wild-type or β2V2R Y219A and β-arrestin complexes to endosomes. U2OS cells stably expressing both EA-tagged β-arrestin and PK-tagged endosomes were transiently transfected with 3µg of β2V2R wild-type or β2V2R Y219A in 10cm dishes using the Fugene 6 transfection reagent (Roche). After 24 hours, the transfected cells were seeded at 25,000 cells/well in 96-well, clear-bottomed white plates. At 48 hours post transfection, cells were treated with isoproterenol (10⁻¹²–10⁻⁵M) for one hour at 37°C, followed by the addition of PathHunter detection reagent and incubation for one hour at 27°C. The luminescence signals were detected by a NOVOstar microplate reader (BMG Labtech).

6.3.5 ERK activation assay

The carvedilol (β-arrestin biased agonist) and isoproterenol (balanced agonist) dependent activation of ERK2 was measured in HEK 293 cells transiently expressing ERK2 and β2AR wild-type or β2AR Y219A. HEK293 cells were seeded at 12x10⁶ cells/15cm dish and transfected the following day with 2µg of ERK2-EGFP (104), 2µg of empty vector plasmid and 6µg of pBK-β2AR wild-type or pBK-β2AR Y219A using Fugene6 transfection reagent (Roche). Twenty-four hours post-transfection, the cells were plated at 2x10⁶ cells/well in 6-well plates. Forty-eight hours post-transfection, the cells were starved in serum-free media for 4.5 hours prior to stimulation. Subsequently, cells were treated with ligands (10µM) at 37°C for indicated durations. The stimulation was quenched by the addition of 2X Laemmli sample buffer (250µl/well), followed by sonication. Phosphorylated ERK2, total ERK2 were detected by western blotting with
anti-p44/42 MAPK (Cell Signaling, 1:4000), anti-MAPK 1/2 (EMD Millipore, 1:10,000), respectively.

Western blots were quantified by densitometry with Image J (NIH) and GraphPad Prism was used for data analyses. pERK densitometry values were divided by their respective total ERK densitometry values and represented as % of the average maximum β2AR wild-type signals (5-minute post-stimulation). The significance of ERK activation in response to carvedilol was verified by setting either WT (0min) or Y219A (0min) as a point of comparison in Dunnett’s multiple comparisons test after one-way ANOVA analysis. The difference in ERK activation between maximum signals of β2AR wild-type (5min) and Y219A (5min) in response to isoproterenol (Figure 2-6C) was verified by Tuckey’s multiple comparisons test after two-way ANOVA analysis.

6.3.6 **In cellulo phosphorylation assay**

The agonist-dependent phosphorylation of β2AR wild-type and β2AR Y219A in Expi293f cells was measured with overexpressed receptors and endogenously expressed kinases. Expi293f cells (2.9X10⁶ cells/ml, 8.5ml) were transiently transfected with 10µg of pBK-β2AR wild-type or pBK-β2AR Y219A and the Expifectamine293 transfection reagent (Gibco™). Forty-eight hours post-transfection, 2.5ml of cells/condition were treated with the respective ligands (10µM) at 37 °C, and 2ml of pelleted cells/condition were resuspended in 1ml of Hypotonic Lysis Buffer (10mM Tris-HCl pH7.4, 2mM EDTA, Halt™ Protease Inhibitor Cocktail and Halt™ Phosphatase Inhibitor). Receptors were extracted in 300µl of solubilization buffer (20mM HEPES pH7.4, 100mM NaCl, 1% DDM, 0.1%CHS, Halt™ Protease Inhibitor Cocktail and Halt™ Phosphatase Inhibitor) for one hour at 4⁰C. Equal micrograms of protein from clarified lysates were loaded onto M1
anti-FLAG resin (resin volume 20µl) to immunoprecipitate N-terminal FLAG-tagged β2AR wild-type or β2AR Y219A. The eluted samples (20mM HEPES pH7.4, 100mM NaCl, 0.1% MNG, Halt™ Protease Inhibitor Cocktail and Halt™ Phosphatase Inhibitor) were subjected to western blot analysis using phospho-specific antibodies. The phosphorylation at β2AR Ser^{355} and Ser^{356} was detected by commercially available anti-β2AR pS^{355} and S^{356} (1:1000, Santa Cruz) and phosphorylation at β2AR Ser^{261} and Ser^{262} (1:500) and Ser^{407} and Ser^{411} (1:500) were measured using phospho-specific antibodies that were developed in a previous study (20). The total amount of β2AR was measured with an anti-FLAG-HRP antibody (1:1000, Sigma).

Western blots were quantified by densitometry with Image J (NIH) and GraphPad Prism was used for data analyses. Densitometry values from phospho western blots were divided by their respective total receptor densitometry values and represented as % of the average maximum β2AR wild-type signals. The significance of differences in phosphorylation of β2AR wild-type and Y219A for indicated pairs was verified by Tuckey’s multiple comparisons test after two-way ANOVA analysis.

### 6.3.7 Disulfide trapping of β₂V₂R and β-arrestin1 complex

Cysteine mutants of β₂V₂R and β-arrestin1 were transiently co-expressed with Fugene6 (Roche) in HEK293 cells. Forty-eight hours post-transfection, cells were stimulated with the β₂AR agonist isoproterenol (10 µM) and treated with H₂O₂ (1 mM) at different time points to induce the formation of disulfide bond. Subsequently, cells were washed and lysed in lysis buffer (50 mM HEPES, 250 mM NaCl, pH 7.4, 2 mM EDTA, 10% glycerol, 0.5% NP40, 1 mM NaV, 57 mM NaF, EDTA-free complete protease inhibitor). In order to separate N-terminal Flag-tagged β₂V₂Rs, cell lysates were
subjected to immunoprecipitation with anti-Flag antibody conjugated to agarose beads. Eluted proteins were separated by SDS–PAGE and co-immunoprecipitated β-arrestin1 as part of a disulfide trapped complex with β2V2R was detected by western blotting. Expression levels of the receptor and β-arrestin1 mutants were measured by radioligand binding and western blotting, respectively. Densitometry analysis of the β-arrestin-1 bands was done using ImageJ software.

6.4 Expression and purification of the β2AR

Full-length N-terminal FLAG-tagged β2AR wild-type and β2AR Y219A were expressed in Sf9 insect cells and purified via M1 anti-FLAG affinity and alprenolol-ligand affinity chromatography as previously described (105). The β2AR-LPETGGH wild-type or β2AR-LPETGGH Y219A (LPETGGH is a sortase recognition sequence inserted after residue 365) used for enzymatic (sortase) ligation of V2Rpp (sequence derived from (78)) was expressed and purified from tetracycline-inducible Expi293f cell system. pcDNA3.1/Zeo plasmids containing the CMV promotor with two tet operator sequences in tandem, followed by β2AR-LPETGGH wild-type or β2AR-LPETGGH Y219A were transiently transfected into tetracycline-inducible Expi293f cells (76) using the expifectamine293 transfection reagent (Gibco®). Forty-eight hours post transfection, the cells were induced with doxycycline (4µg/ml) and sodium butyrate (5µM), and harvested 24 hours post induction. The cells were then lysed in a buffer containing 10mM Tris-HCl pH7.4, 2mM EDTA, and 10mM MgCl2 and benzonase. Subsequently, the receptors were extracted from cell membranes into a solubilization buffer containing 1% dodecylmaltoside (DDM), 0.1% cholesterol hemisuccinate (CHS), 20mM HEPES pH7.4, 100mM NaCl, 10mM MgCl2 and benzonase at 4C for one hour. The solubilized lysate was loaded on to the M1 anti-FLAG resin and washed with a buffer comprised of
20mM HEPES pH7.4, 100mM NaCl, 2mM CaCl$_2$, 0.1% DDM, 0.01%CHS. Then the receptors were eluted with 0.2mg/ml FLAG peptide, 5mM EDTA, 20mM HEPES pH7.4, 100mM NaCl, 0.1% DDM, 0.01%CHS. All buffers listed above contained 1µM alprenolol and the following protease inhibitors – benzamidine and leupeptin. M1-pure receptors were subjected to size exclusion chromatography using a Superdex200 Increase 10/300 GL column (GE healthcare) in 20mM HEPES pH7.4, 100mM NaCl, 0.1% DDM, 0.01%CHS.

6.5 In vitro assays

6.5.1 In vitro Phosphorylation Assay

Phosphorylation of agonist stimulated $\beta_2$AR wild-type and $\beta_2$AR Y219A reconstituted in liposomes by purified GRK5 was measured in vitro. Tube A, comprised of 250ng of $\beta_2$AR wild-type or $\beta_2$AR Y219A reconstituted in liposomes (2.5mM crude soybean PC), ligand (final 10µM) and Gs or Nb80 (final 1µM), and Tube B, consisting of GRK5 (final 100nM), ATP (final 100µM) and MgCl$_2$ (final 10mM), were assembled separately on ice. The phosphorylation reaction was initiated by mixing tubes A and B (total volume of 20µl), followed by a 5-minute incubation at room temperature. The reaction was quenched by adding 20µl of 2X Laemmli sample buffer. The total volume was then expanded by adding 120µl of 1X Laemmli sample buffer for even loading. The reaction mixture was loaded (50µl/ well) on to nitrocellulose membranes (BioRad) via dot blotting. The degree of $\beta_2$AR Ser$^{355}$ and Ser$^{356}$ phosphorylation (Santa Cruz), total receptor (N-terminal FLAG tagged) and total GRK5 were measured by western blot using anti-$\beta_2$AR pS$^{355}$ and S$^{356}$ (1:1000, Santa Cruz), anti-FLAG-HRP (1:1000, Sigma) and anti-GRK5 (1:1000, Santa Cruz) antibodies, respectively.
Western blots were quantified by densitometry with Image J (NIH) and GraphPad Prism was used for data analyses. Densitometry values from pS\textsuperscript{355} and S\textsuperscript{356} western blots were divided by their respective total receptor densitometry values and represented as % of the average maximum β\textsubscript{2}AR wild-type signals. The significance of differences between phosphorylation of β\textsubscript{2}AR wild-type in response to isoproterenol (Figure 3-3B, second black bar) and all other conditions was verified by Tuckey’s multiple comparisons test after two-way ANOVA analysis.

### 6.5.2 Enzymatic ligation of synthetic phosphopeptide V\textsubscript{2}Rpp

Purified β\textsubscript{2}AR-LPETGGH wild-type or β\textsubscript{2}AR-LPETGGH Y219A (10µM or higher final concentration) was incubated with sortase (1/5 of receptor), CaCl\textsubscript{2} (5mM), NiSO\textsubscript{4} (200µM) and the synthetic phosphopeptide V\textsubscript{2}Rpp-GGG (5-fold excess of receptor, Tufts) on ice overnight. The ligation efficiency of V\textsubscript{2}Rpp to receptors via sortase under this condition was close to hundred percent, assessed by coomassie staining (Figure 3-5B). The ligated receptors were reconstituted into HDL particles as previously described (106). The excess V\textsubscript{2}Rpp-GGG was removed by dialysis overnight at 4C.

### 6.5.3. Co-immunoprecipitation assay

The physical interaction of purified β-arrrestin1 with FLAG-β\textsubscript{2}V\textsubscript{2}Rpp wild-type or FLAG-β\textsubscript{2}V\textsubscript{2}Rpp Y219A was validated by an in vitro co-immunoprecipitation experiment. Isoproterenol (10µM), 6µg of FLAG-receptor, molar equivalent amounts of β-arrrestin1 and Fab30 were incubated in an assay buffer containing 20mM HEPES pH7.4, 100mM NaCl, 2mM CaCl\textsubscript{2}, 0.1%DDM and 0.01%CHS for 30 minutes at room temperature. Next, 20µl of M1 anti-FLAG resin were added and incubated for 30 minutes at room
temperature with rotation. Subsequently, the resin was rapidly washed in the assay buffer mentioned above for 3 times and eluted with 1mg/ml FLAG peptide, 5mM EDTA, 20mM HEPES pH7.4 and 100mM NaCl. Co-immunoprecipitated β-arrestin1 was visualized by coomassie staining.

6.5.4 Desensitization measured by in vitro GTPase assay

β-arrestin1-mediated desensitization of Gs protein activity was measured by quantifying the decrease in GTP hydrolysis using the GTPase-Glo assay (Promega). The β2V2Rpp wild-type (4nM) or β2V2Rpp Y219A (4nM) reconstituted in HDL particles was incubated with isoproterenol (10µM), rGTP (2.5µM), β-arrestin1 (5µM), Fab30 (10µM) or Nb80 (5µM) for 10 min at room temperature in a 96-well, clear bottomed white plate. The GTP hydrolysis reaction was initiated by addition of the Gs protein (443nM) and DTT(1mM) to the pre-incubated receptor mixture at a total volume of 25µl per well. The listed concentrations represent the concentrations after mixing. The GTP hydrolysis activity of Gs protein was allowed to proceed at room temperature for two hours. The reaction was quenched by addition of the reconstituted GTPase-Glo reagent (25µl per well), followed by incubation at room temperature for 30 minutes with shaking. The GTPase-Glo detection reagent (50µl/well) was then applied and incubated at room temperature for 10 minutes in the dark. The luminescence signal was detected by a SpectraMax M5 plate reader (Molecular Devices). Control experiments with unligated receptors (β2AR-LPETGGH wild-type and β2AR-LPETGGH Y219A reconstituted in HDL particles) were performed under identical conditions, in parallel within the same 96-well plates.
The luminescence values from individual conditions were normalized to values of the respective total GTP input and represented as % of the total GTP hydrolyzed. The desensitization due to β-arrestin1 was verified by calculating statistical significance between indicated pairs in Figure 3-6 with Tuckey’s multiple comparisons test after one-way ANOVA analysis.
Chapter 7. Conclusion


### 7.1 GRKs orchestrate biased agonism at the β2AR Y219A

Although it has long been known that GRK-dependent phosphorylation of agonist-activated GPCRs is critical for the initial recruitment of β-arrestin (107), the role of GRKs in modulating biased signaling is often overlooked. Here we utilized directed mutagenesis to identify a mutation in the β2AR, a single alanine substitution at tyrosine 219 (Y219A) in transmembrane five, that exhibits a strongly G protein-biased phenotype. We found that the proximal cause of this mutant’s failure to recruit β-arrestin (Figure 2-3) and internalize (Figure 2-5) was its impaired phosphorylation by GRKs (Figure 3-2,3). Importantly, the rescue of β-arrestin functions by in vitro ligation of a synthetic phosphopeptide to β2AR Y219A expressly refutes the generally held view that the “G biased” phenotype is due to a deficiency in β-arrestin binding to the agonist induced conformation of the receptor intracellular core (Figure 3-3, 4-1). Rather, our results suggest that the distinct conformational ensemble adopted by β2AR Y219A is principally deficient in coupling to GRKs, highlighting the importance of these kinases in establishing biased signaling.

### 7.2 Y219A mutation allosterically alters β2AR conformations

Crystallographic structures of β2AR-Gs (48) and rhodopsin-arrestin (50) reveal that Y219 (Y5.58- Ballesteros-Weinstein numbering) does not directly contact these transducers. Likewise, it is improbable that the small alanine substitution would sterically interfere with or alter the affinity of GRK binding profoundly enough to
entirely abrogate its activity at β2AR Y219A. A more likely explanation is that the Y219A mutation alters the allosteric connection (108) between the extracellular orthosteric ligand binding pocket and the intracellular transmembrane core, where all three transducers bind.

High-resolution (2.1Å) active-state structures of the µ-opioid receptor (in contrast to 3.5Å β2AR structures) uncovered a highly conserved extensive polar network that likely plays important roles in this allosteric linkage (109). In particular, several residues in this pathway that rearrange upon activation are conserved in the β2AR, including Y219. Thus, the replacement of Y219 by alanine may disrupt the fine balance of interactions, leading to conformational states distinct from that of β2AR wild-type. In addition, a decrease in solvent exchange rate at β2AR TM5 upon binding with GRK5 in hydrogen deuterium exchange experiments (55) suggests that TM5, where Y219 resides, is one of the major allosteric pathways between the orthosteric ligand binding pocket and the intracellular GRK5 binding interface.

7.3 Y219 residue on β2AR is also highly conserved among other class A GPCRs

Intriguingly, the topologically equivalent residue of β2AR Y219 (Y5.58) is present in 205 out of 286 receptors throughout the class A family of GPCRs, but is absent in class B (secretin), class C (glutamate) and F (Frizzled) families (110). Considering the highly conserved nature of Y5.58 and its involvement in receptor activation, the G protein bias of the β2AR Y219A mutation may translate to other family A GPCRs.

7.4 Implications of mutagenesis approach in GPCR biology

Receptor mutagenesis has long been a valuable approach to expose unexpected aspects of GPCRs’ mechanisms (111) including biased signaling pathways (67, 112). Our
results underscore that elucidating the molecular basis of such mutants’ signaling mechanisms can expand our concepts on how these pathways can be rewired to bias receptor signaling and perhaps inspire new strategies to search for biased ligands. In addition, since robust biased ligands are as yet unavailable for many GPCRs such as the β2AR, biased receptor mutants can be used in cellular and animal disease models as an alternative route to validate the potential of biased ligands (113), facilitating early drug discovery efforts.

7.5 Impact on biased agonist drug discovery

The bias of GPCR ligands is typically assessed by cellular endpoints downstream of G protein and β-arrestin activation; this has created an implicit assumption that biased ligands’ selectivity lies in their ability to induce coupling to G protein or β-arrestin. However, elucidating the mechanism underlying G protein bias at the β2AR Y219A highlights that a more complex network of receptor-transducer interactions regulates biased agonism. The implication is that more nuanced signaling profiles than the binary categories of “G protein-biased” and “β-arrestin-biased” ligands will be necessary.

For example, one could imagine that specific receptor conformations could selectively promote G protein coupling and GRK-dependent receptor phosphorylation (and thereby β-arrestin recruitment) but not β-arrestin coupling to the receptor intracellular core. In this permutation, only β-arrestin functions dependent on interaction with the receptor intracellular core, such as desensitization, will be absent. Therefore, evaluating ligand-induced GRK phosphorylation in parallel with β-arrestin binding to the receptor intracellular core may facilitate the discovery of ligands that are capable of activating subsets of β-arrestin functions. In summary, these findings
illuminate the importance of the GRK-receptor interaction in orchestrating biased agonism at GPCRs.
Bibliography


Biography

I was born June 5, 1989 in Seoul, South Korea. In 2007, I attended Hankuk University of Foreign Studies (HUFS), South Korea in pursuit of Arabic language as a major for one year. Then in 2011, I graduated with a Bachelor’s of Science (B.S.) in Biochemistry with Honors and Distinction from the University of Iowa, IA, USA. In 2012, I matriculated at Duke university through Structural Biology and Biophysics program and become affiliated with the department of biochemistry PhD program after joining Dr. Robert Lefkowitz’s Lab in 2013.

Peer-Reviewed Publications


Fellowships and Scholarships

American Heart Association Predoctoral Fellowship (2016-2018)
Competitive pre-doctoral research grant for cardiovascular disease

James B. Duke Fellowship (2012-2016)
Duke University’s most prestigious and competitive fellowship for incoming graduate students

Chancellor’s Scholarship (2012-2014)
Merit based scholarship for incoming graduate students in biosciences at Duke University