**INTRODUCTION**

G protein–coupled receptors (GPCRs) are critical regulators of a multitude of physiological processes because they transduce extracellular stimuli into intracellular signals (1). About one-third of all U.S. Food and Drug Administration–approved drugs target members of the large GPCR family, highlighting the importance of GPCRs as viable drug targets (2). The GPCR family has over 800 members in the human genome (3), yet its members share highly conserved functional and regulatory mechanisms that are predominantly governed by three classes of proteins: heterotrimeric G proteins, GPCR kinases (GRKs), and β-arrestins. Agonist binding to the extracellular orthosteric binding site of a GPCR induces conformational changes that activate specific G proteins at the plasma membrane. For most GPCRs, subsequent agonist-dependent phosphorylation of the receptor C-terminal tail, or intracellular loops by GRKs promotes β-arrestin recruitment, which, in turn, induces receptor desensitization (4) by sterically blocking additional G protein coupling and stimulating receptor internalization (5). Moreover, β-arrestins can also act as protein adaptors to initiate signaling pathways independently of G proteins (6).

The preferential activation of G protein– or β-arrestin–mediated signaling pathways downstream of GPCRs, referred to as “biased agonism,” offers substantial therapeutic potential (7). Biased ligands can selectively activate signaling pathways that lead to desired therapeutic outcomes without affecting pathways that contribute to side effects (8). For example, a G protein–biased ligand of the μ-opioid receptor (MOR) in clinical trials displays enhanced analgesic potency compared to morphine but causes reduced side effects, such as constipation and respiratory depression (9–11). 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 proteins or β-arrestins (12). Whereas 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 the GRK-dependent phosphorylation of the receptor C-terminal tail, intracellular loops, or both. The enzymatic activity of GRKs also requires binding to the agonist-activated receptor intracellular core, suggesting that 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 (13), biophysical, and structural characterization (14). β2AR agonists are used as frontline medications for asthma by promoting dilation of bronchial airways, but the sustained activation of β-arrestin–mediated pathways can cause severe side effects. Despite their therapeutic potential (7, 15), efficacious G protein–biased ligands for β2AR have yet to be identified. Therefore, we turned to a mutagenesis strategy to study G protein bias at the β2AR, and we demonstrate that such bias can be achieved at the level of GRK regulation.

**RESULTS**

**Mutation of Tyr219 to alanine (Y219A) converts β2AR into a G protein–biased receptor**

We used a directed mutagenesis strategy around β2AR residues Thr68, Tyr132, and Tyr219, which have been predicted to be involved in transducer binding by evolutionary trace analysis (Fig. 1A) (16). A similar
strategy by our laboratory previously yielded a β-arrestin–biased β2AR triple mutant (T68F, Y132G, Y219A) (16). During the process of saturation mutagenesis of each individual residue, we discovered that an alanine substitution at Tyr219 (Y219A) in transmembrane 5 (TM5) stimulated G protein–biased signaling through the β2AR. More specifically, stimulation of β2AR wild type (WT) or Y219A with the agonist isoproterenol (ISO) induced comparable maximal G protein activation as measured by an increase in cellular cyclic adenosine monophosphate (cAMP; Fig. 1B). The decrease in G protein efficacy (9.4-fold reduction in the half maximal effective concentration (EC50) of ISO; Fig. 1B and table S1) in β2AR Y219A was largely the result of a decrease in ISO affinity (4.1-fold reduction in the half maximal inhibitory concentration (IC50) of ISO; Fig. 1C and table S1).

We measured β-arrestin recruitment to β2AR Y219A with two different enzyme-based assays (Tango and DiscoverX). Whereas β-arrestin was recruited to β2AR WT in an agonist-dependent manner, recruitment to β2AR Y219A was negligible (Fig. 1D). To maximize the sensitivity of the assay, we repeated these experiments with a chimeric β2V2R construct, in which the C-terminal tail of the β2AR was replaced with that of the V2 vasopressin receptor (V2R), thereby increasing the stability of its interaction with β-arrestin without altering its pharmacological properties (17). However, β-arrestin recruitment to the Y219A receptor remained undetectable (fig. S1, A and B). Note that the cell surface abundances of β2AR WT and β2ARY219A were comparable (Fig. 1E). Thus, the biased phenotype of the mutant receptor could not be accounted for by this potential variable, which could differentially affect assays with varying degrees of signal amplification (18). Together, these data indicate that the β2AR Y219A mutant has functional selectivity toward G protein activation (fig. S1C).

Given the inability of β2AR Y219A to recruit β-arrestin, we hypothesized that other β-arrestin–mediated events, such as GPCR internalization (5) and extra-cellular signal–regulated kinase 1 and 2 (ERK1/2) activation (16), would also be impaired. Whereas stimulation of β2AR WT with ISO induced the plasma membrane localization of β-arrestin2 and receptor internalization, as assessed by

Fig. 1. Functional characterization of the G protein–biased mutant β2AR Y219A. (A) Snake diagram of β2AR representing the location of Y219A and the residues phosphorylated by GRKs and PKA. Serrated line indicates palmitoylation. (B) G protein activation by transiently expressed β2AR WT and β2AR Y219A in response to the indicated concentrations of ISO was measured in HEK 293 cells that stably expressed a cellular cAMP biosensor. Data are means ± SEM of five experiments performed in duplicate. (C) The affinity of ISO for β2AR WT and β2AR Y219A expressed in HEK 293 cell membranes was measured by competitive radioligand binding with [125I]-CYP. Data are means ± SEM of five experiments performed in duplicate. (D) β-Arrestin recruitment to transiently expressed β2AR WT or β2AR Y219A was measured using the protease-triggered transcription factor assay as described in Materials and Methods. Data are means ± SEM of three experiments performed in duplicate. (E) The cell surface abundances of β2AR WT and β2AR Y219A on transiently transfected HEK 293 cells were measured by whole-cell binding with [3H]-CGP 12177. Data are means ± SEM of three experiments performed in duplicate.
confocal microscopy (Fig. 2A) and analysis of surface receptor density (Fig. S2), respectively, neither event occurred with $\beta_2$AR Y219A. Consistent with these findings, an enzyme complementation GPCR internalization assay confirmed that the chimeric $\beta_2$V2R Y219A failed to translocate $\beta$-arrestin to endosomes (Fig. 2B). Because both G protein– and $\beta$-arrestin–mediated signaling can affect ERK1/2 activation, we assessed ERK1/2 phosphorylation after stimulation of $\beta_2$AR WT and $\beta_2$AR Y219A with carvedilol, which induces $\beta$-arrestin–mediated but not G protein–mediated phosphorylation of ERK1/2 (19). To assess ERK activation only in transfected cells, we cotransfected cells with plasmids encoding enhanced green fluorescent protein (EGFP)–ERK2 (distinguishable from endogenous ERK2 by its greater molecular weight) and $\beta_2$AR WT or $\beta_2$AR Y219A. Stimulation of $\beta_2$AR Y219A with the balanced agonist ISO activated ERK2, which was maximal at 5 min after stimulation (fig. S3, A and B), albeit to a lesser extent than occurred after stimulation of $\beta_2$AR WT. On the other hand, the $\beta$-arrestin–biased ligand carvedilol induced substantial ERK2 activation only through $\beta_2$AR WT and not through $\beta_2$AR Y219A (Fig. 2C and fig. S3C).

**The G protein–biased properties of $\beta_2$AR Y219A stem from impaired phosphorylation by GRK**

Phosphorylation of the C terminus or third intracellular loop of GPCRs by GRKs is a critical intermediary step for $\beta$-arrestin recruitment. To determine whether changes in GRK-mediated phosphorylation could contribute to the biased properties of $\beta_2$AR Y219A, we compared the agonist-dependent phosphorylation pattern of $\beta_2$AR Y219A to that of $\beta_2$AR WT in cells. We used phospho-specific antibodies against residues known to be phosphorylated by GRK2 (Ser$^{355}$ and Ser$^{356}$), GRK5 or GRK6 (Ser$^{355}$ and Ser$^{356}$), and a non-GRK kinase, protein kinase A (PKA; Ser$^{361}$ and Ser$^{362}$) (20, 21). Although we observed a large increase in agonist-dependent phosphorylation of $\beta_2$AR WT Ser$^{355}$ and Ser$^{356}$, no substantial phosphorylation of $\beta_2$AR Y219A was observed (Fig. 3, A and B) even after 30 min of agonist stimulation. Similarly, phosphorylation of Ser$^{355}$ and Ser$^{356}$ (targets of GRK2) was reduced in $\beta_2$AR Y219A compared to that in $\beta_2$AR WT (Fig. 3, A and C). The reduction in phosphorylation of $\beta_2$AR Y219A was specific to GRK-phosphorylated residues because we observed no substantial difference between the WT and mutant receptors in the phosphorylation of Ser$^{361}$ and Ser$^{362}$ by PKA, which is activated downstream of G protein (Fig. 3, A and D).

To verify that the deficiency in GRK-mediated phosphorylation of $\beta_2$AR Y219A in cells was a direct consequence of an altered interaction between GRKs and the receptor, we measured in vitro GRK5-mediated phosphorylation of purified $\beta_2$AR WT or $\beta_2$AR Y219A reconstituted in liposomes. In this system, ISO was required to phosphorylate Ser$^{355}$ and Ser$^{356}$ of $\beta_2$AR WT (Fig. 3, E and F). Steric blockade of the G protein–binding pocket of the receptor by Gs or the single-domain antibody Nb80 (22) was sufficient to prevent this phosphorylation (Fig. 3, E and F), consistent with a previous report (23). This further supports the idea that GRKs respond to agonist activation by sensing the large conformational changes that occur in the intracellular core of the receptor. Consistent with the cellular data, the Y219A mutation abrogated ISO-induced phosphorylation of Ser$^{355}$ and Ser$^{356}$ of the mutant receptor by GRK5 in vitro (Fig. 3, E and F).

**Restoring phosphorylation at the $\beta_2$AR Y219A C terminus rescues $\beta$-arrestin–mediated functions**

Engagement of $\beta$-arrestin with agonist-activated GPCRs occurs in two steps. First, $\beta$-arrestin recruitment to the phosphorylated C terminus of the receptor induces conformational changes within $\beta$-arrestin, which then promote the coupling of $\beta$-arrestin to the receptor intracellular
β2AR Y219A.

to Y219A (0 min) control. (Tukey’s multiple comparisons test after two-way ANOVA.)

were normalized by those for the total receptors and are presented as percentage values of the maximum signal

obtained from WT receptor. Data are means ± SEM of three or four independent experiments and were analyzed by

compared to all Y219A conditions. For (B) to (D) and (F), the densitometry values of the phosphorylated receptors

were analyzed by Tukey’s multiple comparisons test after two-way ANOVA.

were subjected to Western blotting analysis with the indicated phospho-specific antibodies and with anti-FLAG anti-

body to determine total receptor. (A) Western blots are representative of four independent experiments. (B) Densitometric analysis of pS261 and pS262 bands. *P < 0.05 compared to WT (0 min) control; ###P < 0.0001 compared to all other WT conditions; $$$$. (C) Densitometric analysis of pS407 and pS411 bands. $$$$. (D) Densitometric analysis of pS355 and pS356 bands. ****P < 0.0001 compared to unligated receptors (fig. S4, B to D). These was specific to phosphopeptide-ligated β-arrestin1 tent. The allosteric effect of C-terminal phosphorylation was some-

how rescued. Because β2AR Y219A could not be phosphorylated by GRKs in cells or in vitro (Fig. 3), we enzymatically

ligated (25, 26) a synthetic phosphopeptide (pp) derived from the V3R (27) to the C-terminal tail of purified β2AR WT and β2AR Y219A (Fig. 4A and fig. S4A) to provide a more stable interaction with β-arrestin (17). Restoration of β-arrestin binding to the phosphorylated C terminus was verified by coimmunoprecipitation experiments. Both agonist-activated β2ARpp WT and β2ARpp Y219A specifically pulled down similar amounts of β-arrestin1 and Fab30 (27), an antibody fragment that sta-

bilizes phosphopeptide-activated β-arrestin1 (Fig. 4B, lanes 3 and 5). In contrast, β-arrestin1 was not communoprecipitated with nonphosphorylated β2AR WT or β2AR Y219A, underscoring the necessity of receptor phosphorylation for β-arrestin binding (Fig. 4B, lanes 2 and 4).

We next assessed the interaction between the intracellular core of β2ARpp Y219A and β-arrestin1 by radioligand competition binding experiments. According to the ternary complex model, the coupling of transducer proteins, such as G proteins (28) and β-arrestins (29), to the intracellular core of β2AR exerts an allosteric effect on the receptor that increases agonists’ affinity for the ortho-

steric ligand-binding pocket. We found that β-arrestin1 substantially enhanced the affinity of ISO for β2ARpp WT (eight-

fold; Fig. 4C) and for β2ARpp Y219A (threelfold; Fig. 4D), albeit to a lesser ex-

tent. The allosteric effect of β-arrestin1 was specific to phosphopeptide-ligated receptors and was not observed for the unligated receptors (fig. S4, B to D). These data suggest that a G protein–biased receptor can retain the conformational ca-

pability of coupling to β-arrestin through its intracellular core.

β-Arrestins are scaffolding proteins that mediate a multitude of functions, such as desensitization of G protein activity, receptor internalization, and initiation

of a deficiency in GRK-dependent phosphorylation, which interfered with the first step of this interaction. This raised the question of whether the agonist-induced conformations of β2AR Y219A would be capable of promoting the binding of β-arrestin to the intracellular core if C-terminal phosphorylation was some-

Fig. 3. The agonist-dependent phosphorylation pattern of β2AR Y219A. (A to D) Expi293f cells were transiently transfect with plasmids encoding FLAG-tagged β2AR WT or β2AR Y219A and stimulated with 10 μM ISO at 37°C for the indicated times. Receptors were immunoprecipitated with anti-FLAG M1 agarose beads. Eluted samples were then subjected to Western blotting analysis with the indicated phospho-specific antibodies and with anti-FLAG antibody to determine total receptor. (A) Western blots are representative of four independent experiments. (B) Densitometric analysis of pS355 and pS356 bands. ****P < 0.0001 compared to WT (0 min) control; $$$$. (C) Densitometric analysis of pS407 and pS411 bands. $$$$P < 0.0001, overall comparison. (D) Densitometric analysis of pS261 and pS262 bands. *P < 0.05 compared to WT (0 min) control; $$$$P < 0.0001 compared to Y219A (0 min) control. (E and F) To measure the GRK5-mediated phosphorylation of the receptor residues Ser355 and Ser356 in vitro, we reconstituted receptors in lipid vesicles and stimulated with ISO in the presence of purified GRK5 and adenosine 5′-triphosphate (ATP). (E) Western blots are representative of three independent experiments. (F) Densitometric analysis of pSer355 and Ser356. $$$$P < 0.0001 compared to all other WT conditions; $$$$P < 0.0001 compared to all Y219A conditions. For (B) to (D) and (F), the densitometry values of the phosphorylated receptors were normalized by those for the total receptors and are presented as percentage values of the maximum signal obtained from WT receptor. Data are means ± SEM of three or four independent experiments and were analyzed by Tukey’s multiple comparisons test after two-way ANOVA.
of downstream signaling pathways. Whereas several of these functions require interaction only with the phosphorylated receptor C terminus (30, 31), the interaction between the intracellular core of the β2AR and β-arrestin appears to be indispensable for the desensitization of G protein activity (30). We hypothesized that the allosteric interaction of β-arrestin with β2ARpp Y219A would preserve this desensitization function. Accordingly, we used an in vitro G protein–dependent guanosine 5′-triphosphate (GTP) hydrolysis assay to measure the ability of β-arrestin to inhibit G protein activation. Stimulation of the β2AR by ISO substantially enhanced G protein–dependent GTP hydrolysis, which was blocked by the binding of Nb80 to both phosphorylated and nonphosphorylated receptors (Fig. 4E and fig. S5A). β-Arrestin1 similarly decreased GTP hydrolysis for β2ARpp Y219A (Fig. 4F and fig. S5B), confirming that β-arrestin1 properly engaged the intracellular core of the mutant receptor and successfully blocked its coupling to Gs protein. In summary, the data indicate that the intracellular core of β2AR Y219A is modestly impaired in coupling to both G protein (Fig. 1B) and β-arrestin (Fig. 1D) compared to that of β2AR WT, but that it is completely deficient in coupling to GRKs (Fig. 3), thus giving rise to the G protein–biased phenotype.

**DISCUSSION**

Although it has long been known that GRK-dependent phosphorylation of agonist-activated GPCRs is critical for the initial recruitment of β-arrestins (32), the role of GRKs in modulating biased signaling is often overlooked. Here, we used directed mutagenesis to identify a mutation in the β2AR, a single alanine substitution at tyrosine 219 (Y219A) in TM5, that resulted in a strongly G protein–biased phenotype. We found that the proximal cause of the failure of the mutant receptor to recruit β-arrestin (Fig. 1D and fig. S1, A and B) and undergo internalization (Fig. 2, A and B, and fig. S1C) was its impaired phosphorylation by GRKs (Fig. 3). Furthermore, the rescue of β-arrestin functions by in vitro ligation of a synthetic phosphopeptide to β2AR Y219A contradicts the generally held view that the “G protein–biased” phenotype is due to a deficiency in β-arrestin binding to the agonist-induced conformation of the receptor intracellular core (Fig. 4). Rather, our results suggest that the distinct conformational ensemble adopted by β2AR Y219A was deficient in coupling to GRKs, highlighting the importance of these kinases in establishing biased signaling.
Crystallographic structures of β2AR-Gs (33) and rhodopsin-arrestin (34) reveal that Y219 (Y5.58 by 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 (35) between the extracellular orthosteric ligand-binding pocket and the intracellular transmembrane core, where all three transducers bind. High-resolution (2.1 Å) active-state structures of MOR (in contrast to the 3.5 Å of β2AR structures) uncovered a highly conserved extensive polar network that likely plays important roles in this allosteric linkage (36). In particular, several residues in this network that rearrange upon activation are conserved in the β2AR, including Tyr219. Thus, the replacement of Tyr219 by alanine may disrupt the fine balance of interactions, leading to conformational states distinct from that of β2AR WT. In addition, a decrease in the solvent exchange rate at β2AR TM5 upon binding with GRK5 in hydrogen-deuterium exchange experiments (23) suggests that TM5, where Tyr219 resides, plays a major role in the allosteric interaction between the orthosteric ligand-binding pocket and the intracellular GRK5-binding interface. Furthermore, the topologically equivalent residue of β2AR Tyr219 (Y5.58) is present in 205 of 286 receptors throughout the class A family of GPCRs but is absent in members of the class B (secretin), class C (glutamate), and class F (Frizzled) families (37). Considering the highly conserved nature of Y5.58 and its involvement in receptor activation, the G protein bias of the β2AR Y219A mutant may translate to other family A GPCRs.

Receptor mutagenesis has long been a valuable approach to expose unexpected aspects of the mechanisms used by GPCRs (38), including biased signaling pathways (16, 39). Our results underscore the idea that elucidating the molecular basis of the signaling mechanisms of such mutants can expand our concepts of how these pathways can be rewired to bias receptor signaling and perhaps inspire new strategies to search for biased ligands. In addition, because 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 (40), facilitating early drug discovery efforts.

The bias of GPCR ligands is typically assessed by cellular end-points downstream of G protein and β-arrestin activation; this has created an implicit assumption that biased ligands’ selectivity lies in their ability to induce receptor coupling to G protein or β-arrestin. However, elucidating the mechanism underlying G protein bias at the β2AR Y219A highlights the fact 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, would 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.

**MATERIALS AND METHODS**

**Materials**

All mutagenesis PCRs were performed with the QuikChange Mutagenesis Kit (Agilent Technologies) in accordance with the manufacturer’s protocol. Gs protein (33), β-arrestin1 (minimal cysteine construct, truncated at residue 393) (27), Fab30 (27), and surtose (25) were purified as previously described. The V2Rpp-GGG phosphopeptide was synthesized and purified by the Tufts University Peptide Synthesis facility.

**Cell surface β2AR expression measurement**

The cell surface abundances of β2AR WT and β2AR Y219A were measured by whole-cell binding with the hydrophilic radioligand [1H]-CGP 12177 (PerkinElmer). Human embryonic kidney–293 (HEK 293) cells were transiently transfected with pBK-β2AR WT or pBK-β2AR Y219A with Fugene 6 (Roche), and the cells were harvested 48 hours later by gentle agitation with 0.02% EDTA at 4°C. Cells were resuspended in assay buffer (10 mM Hepes in minimum essential medium) and incubated with 30 nM [1H]-CGP 12177 for 3 hours on ice. Propranolol (10 μM) was used to account for nonspecific binding. Unbound [1H]-CGP 12177 was separated by filtration onto grade B glass fiber filters soaked in water with a 96-well-format Brandel harvester. The GF/B filters were then rapidly washed three times with ice-cold buffer [50 mM tris-HCl (pH 7.5), 12.5 mM MgCl2, and 2 mM EDTA (pH 8.0)] and soaked in scintillation fluid (Research Products International) overnight. Bound [1H]-CGP 12177 was quantified by the Tri-Carb 2800TR Liquid Scintillation Analyzer (PerkinElmer). All data represent at least three independent experiments; SE and analysis were performed in GraphPad Prism.

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

Competition binding assays that measure the affinity of ISO for β2AR WT and β2AR Y219A were performed in a 250-μL reaction volume consisting of 60 pM [125]I]-CYP, a serial dilution of ISO, and 60 fmol of functionally active receptors [from HEK 293 cell membranes or purified receptors reconstituted in high-density lipoprotein (HDL) particles] in assay buffer [20 mM Hepes (pH 7.4), 100 mM NaCl, and 0.2% bovine serum albumin (BSA)]. For experiments with transducers, 1 μM β-arrestin1 was added within the total volume of 250 μL. Nonspecific binding was measured with 10 μM propranolol, and total binding was determined in the absence of ISO. To ensure that the binding reaction reached equilibrium, the mixed components were incubated at room temperature for 90 min. Unbound [125]I]-CYP was separated by filtration onto GF/B glass microfiber filters treated with 0.3% polyethyleneimine using a 96-well-format Brandel harvester. The GF/B filters were then rapidly washed three times with ice-cold buffer (20 mM Hepes and 100 mM NaCl). Bound [125]I]-CYP was quantified with a 2470 automatic gamma counter (PerkinElmer). The affinity of [125]I]-CYP for β2AR WT and β2AR Y219A was determined by saturation binding (fig. S4B). All data represent at least three independent experiments; SE and analysis were performed in GraphPad Prism. For Fig. 4 (C and D), the difference in log (IC50) of ISO between a control curve (receptor only, closed circles) and a β-arrestin1 curve (open squares) was verified by Welch’s unpaired t test.
Measurement of cAMP concentration in cells

G protein activation by agonist-stimulated β2AR WT and β2AR Y219A was examined by measuring cAMP concentrations in HEK 293 cells stably expressing the GloSensor cAMP biosensor (Promega) (41). HEK 293 cells at ~70% confluency in 10-cm dishes were transiently transfected with 2 μg of plasmid encoding either β2AR WT or β2AR Y219A using the Fugene 6 transfection reagent (Roche). After 24 hours, the transfected cells were plated at 80,000 cells per well in 96-well, clear-bottom white plates. Forty-eight hours after transfection, the cells were treated with the GloSensor reagent, which contains luciferase substrates, for 90 min at 27°C. The cells were then incubated with ISO (10⁻¹² to 10⁻⁵ M) for 5 min at room temperature. The luminescence signals were detected by a NOVOstar microplate reader (BMG Labtech).

β-Arrestin recruitment assays

Tango (Thermo Fisher Scientific), a transcription factor–based assay, was used to measure β-arrestin recruitment to the agonist-stimulated receptor (42). HEK 293T cells stably expressing a tetracycline transactivator (tTA) protein-driven luciferase reporter and β-arrestin2 fused to the tobacco etch virus protease were transiently transfected using the Fugene 6 transfection reagent with plasmids encoding β2AR-tTA WT, β2AR-tTA Y219A, β2V2R-tTA WT, or β2V2R-tTA Y219A. Twenty-four hours after transfection, the cells were seeded onto 96-well dishes at 60,000 cells per well, maintained for an additional 24 hours, and then stimulated with ISO (10⁻¹³ to 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 WT or β2V2R Y219A was also measured with PathHunter (DiscoverX), a chemiluminescence-based enzyme fragment complementation assay (43). U2OS cells stably expressing enzyme acceptor (EA) β-arrestin2 were transiently transfected with plasmids expressing ProLink (PK)–tagged β2V2R WT or β2V2R Y219A with the Fugene 6 transfection reagent (Roche). Twenty-four hours after transfection, the cells were plated at 25,000 cells per well in 96-well, clear-bottom white plates. Forty-eight hours after transfection, the cells were stimulated with ISO (10⁻¹² to 10⁻⁵ M) for 1 hour at 37°C, which was followed by the addition of PathHunter detection reagent and incubation for 1 hour at 27°C. The luminescence signals were detected by a NOVOstar microplate reader (BMG Labtech).

Confocal microscopy

U2OS cells were seeded in CC²-coated, four-well chamber slides (Nunc) and transfected with 75 ng of pBK-FLAG-β2AR WT or pBK-FLAG-β2AR Y219A and 25 ng of plasmid encoding GFP-β-arrestin2. Forty-eight hours after transfection, the cells were treated with vehicle (DMSO) or 10 μM ISO for 15 min and fixed with 3.7% paraformaldehyde. The cells were permeabilized with 0.4% Triton X-100 in phosphate-buffered saline (PBS) for 5 min, blocked for 1 hour in PBS containing 3% BSA and 20% goat serum, and incubated with rabbit anti-FLAG (1:500; Sigma) in blocking buffer overnight at 4°C. The cells were then incubated with secondary anti-rabbit antibody conjugated to Texas Red (1:1000; Molecular Probes) and DAPI (1:5000) for 1 hour at room temperature. Confocal images were obtained on a Zeiss LSM510 laser scanning microscope.

β-Arrestin–mediated internalization assay

The internalization of β-arrestin2 into endosomes by agonist-stimulated β2V2R WT or β2V2R Y219A was monitored by PathHunter (DiscoverX), a chemiluminescence-based enzyme fragment complementation assay. EA-tagged β-arrestin2 and a PK tag localized to the endosomes form a functional enzyme that generated a chemiluminescence signal upon internalization of β2V2R WT 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 3 μg of plasmid encoding β2V2R WT or β2V2R Y219A in 10-cm dishes using the Fugene 6 transfection reagent (Roche). After 24 hours, the transfected cells were seeded at 25,000 cells per well in 96-well, clear-bottom white plates. At 48 hours after transfection, the cells were treated with ISO (10⁻¹² to 10⁻⁵ M) for 1 hour at 37°C, which was followed by the addition of PathHunter detection reagent and incubation for 1 hour at 27°C. The luminescence signals were detected by a NOVOstar microplate reader (BMG Labtech).

Total receptor internalization assay

The extent of receptor internalization was quantified by measuring the cell surface abundance of β2AR WT and β2AR Y219A by whole-cell binding with the hydrophilic radioligand [³H]-CGP 12177 (PerkinElmer). Exp293f cells (3 ml) were transiently transfected with a plasmid encoding pBK-β2AR WT (3 μg) or pBK-β2AR Y219A (3 μg) with the Expifectamine 293 transfection reagent ( Gibco). After 48 hours, the cells were treated with 10 μM ISO at 37°C for the times indicated in the figure. The cells were then immediately placed on ice and washed twice with ice-cold medium (Exp293 expression medium, Gibco). The cells were then resuspended in medium (10 mM Hepes in minimum essential medium), and 30 μl of cells was incubated with 30 nM [³H]-CGP 12177 for 3 hours on ice. Propranolol (10 μM) was used to account for nonspecific binding. Unbound [³H]-CGP 12177 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 [20 mM Hepes (pH 7.4) and 100 mM NaCl] and soaked in scintillation fluid (Research Products International) overnight. Bound [³H]-CGP 12177 was quantified by the Tri-Carb 2800TR Liquid Scintillation Analyzer (PerkinElmer). The amounts of surface receptor quantified as specific counts of [³H]-CGP 12177 (counts per minute) from individual conditions were divided by their respective total protein inputs (in micrograms) and presented as a percentage of total receptor (fig. S1C, ISO 0-min vehicle control) internalized. The extent of receptor internalization in response to ISO was verified by setting either WT (0-min) or Y219A (0-min) as a point of comparison in Dunnett’s multiple comparisons test after one-way ANOVA analysis.

Analysis of pERK

The activation of ERK2 by carvedilol (β-arrestin–biased agonist) and ISO (balanced agonist) was measured in HEK 293 cells transiently expressing ERK2 and β2AR WT or β2AR Y219A. HEK 293 cells were seeded at 12 × 10⁶ cells per 15-cm dish and transfected the following day with 2 μg of plasmid encoding ERK2-EGFP (44), 2 μg of empty plasmid, and 6 μg of pBK-β2AR WT or pBK-β2AR Y219A using Fugene 6 transfection reagent. Twenty-four hours after transfection, the cells were plated at 2×10⁶ cells per well in six-well plates. Forty-eight hours after transfection, the cells were starved in serum-free medium for 4.5 hours before stimulation. The cells were then treated with carvedilol or ISO (each at 10 μM) at 37°C for the times indicated in the figure. The stimulation was quenched by the addition of 2×Laemmli sample buffer (250 μl per well), which was followed by sonication. Total ERK2 and pERK2 were detected by Western blotting.

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analysis with anti-MAPK 1/2 (1:10,000; EMD Millipore) and anti-p44/42 MAPK (1:4000; Cell Signaling), respectively. Western blots were quantified by densitometry with ImageJ software [National Institutes of Health (NIH)], and GraphPad Prism was used for data analyses. The densitometry values for pERK were divided by their respective total ERK densitometry values and presented as a percentage of the average maximum β2AR WT signals (5 min after stimulation). The statistical significance of ERK activation (phosphorylation) in response to carvedilol was verified by setting either β2AR WT (at 0 min) or β2AR Y219A (at 0 min) as a point of comparison in Dunnett’s multiple comparisons test after one-way ANOVA analysis. The difference in ERK phosphorylation between the maximal signals of β2AR WT (at 5 min) and β2AR Y219A (at 5 min) in response to ISO (Fig. S3B) was verified by Tukey’s multiple comparisons test after two-way ANOVA analysis.

**Assay of receptor phosphorylation in cells**

The agonist-dependent phosphorylation of β2AR WT and β2AR Y219A in Expi293f cells was measured with overexpressed receptors and endogenously expressed kinases. Exp293f cells (2.9 × 10⁵ cells/ml in 8.5 ml) were transiently transfected with 10 μg of pBK-β2AR WT or pBK-β2AR Y219A and the ExpiFectamine 293 transfection reagent (Gibco). Forty-eight hours after transfection, 2.5 ml of cells per condition was resuspended in 1 ml of hypotonic lysis buffer [10 mM tris-HCl (pH 7.4), 2 mM EDTA, Halt Protease Inhibitor Cocktail, and Halt Phosphatase Inhibitor]. Receptors were extracted in 300 μl of solubilization buffer [20 mM Hepes (pH 7.4), 100 mM NaCl, 1% dodecylmaltoside (DDM), 0.1% cholesteryl hemisuccinate (CHS), Halt Protease Inhibitor Cocktail, and Halt Phosphatase Inhibitor] for 1 hour at 4°C. Equal amounts of protein from clarified lysates were loaded onto M1 anti–FLAG resin (resin volume, 20 μl) to immunoprecipitate N-terminal FLAG-tagged β2AR WT or β2AR Y219A. The eluted samples in 20 mM Hepes (pH 7.4), 100 mM NaCl, 0.1% maltose-neopentyl glycol, Halt Protease Inhibitor Cocktail, and Halt Phosphatase Inhibitor were subjected to Western blot analysis using phospho-specific antibodies. The phosphorylation at β2AR at Ser355 and Ser356 was detected by commercially available anti-β2AR pS355 and pS356 antibodies (1:1000; Santa Cruz Biotechnology), and phosphorylation of β2AR at Ser356 and Ser262 (1:500) and Ser461 and Ser411 (1:500) was measured with phospho-specific antibodies that were developed previously (20). The total amount of β2AR was measured with an anti–FLAG–horseradish peroxidase (HRP) antibody (1:1000; Sigma). Western blots were quantified by densitometry with ImageJ software (NIH), and GraphPad Prism was used for data analyses. Densitometry values of bands corresponding to phosphorylated receptors on Western blots were divided by their respective densitometry values of total receptors and presented as a percentage of the average maximum β2AR WT signals. The statistical significance of differences between phosphorylation of β2AR WT in response to ISO (Fig. 3F, second black bar) and all other conditions was verified by Tukey’s multiple comparisons test after two-way ANOVA analysis.

**Expression and purification of the β2AR**

Full-length, N-terminal FLAG-tagged β2AR WT and β2AR Y219A were expressed in SF9 insect cells and purified with M1 anti–FLAG affinity and alprenolol-ligand affinity chromatography as previously described (45). The β2AR-LPETGGH WT or β2AR-LPETGHH Y219A (LPETGHH is a sortase recognition sequence inserted after residue 365) used for enzymatic (sortase) ligation of V2Rpp (sequence derived from a previous study (27)) was expressed and purified from a tetracycline-inducible Expi293f cell system. The pcDNA3.1-Zeo plasmids containing the cytomegalovirus promoter with two tet operator sequences in tandem, followed by sequences encoding β2AR-LPETGHH WT or β2AR-LPETGHH Y219A, were used to transiently transfect tetracycline-inducible Expi293f cells (26) using the ExpiFectamine 293 transfection reagent (Gibco). Forty-eight hours after transfection, the cells were induced with doxycycline (4 g/ml) and sodium butyrate (5 μM) and then were harvested 24 hours later. The cells were then lysed in a buffer containing 10 mM tris-HCl (pH 7.4), 2 mM EDTA, 10 mM MgCl₂, and Benzonase. The receptors were then extracted from the cell membranes into a solubilization buffer containing 1% DDM, 0.1% CHS, 20 mM Hepes (pH 7.4), 100 mM NaCl, 10 mM MgCl₂, and Benzonase at 4°C for 1 hour. The solubilized lysate was loaded onto the M1 anti–FLAG resin and washed with a buffer containing 20 mM Hepes (pH 7.4), 100 mM NaCl, 2 mM CaCl₂, 0.1% DDM, and 0.01% CHS. The receptors were then eluted with FLAG peptide (0.2 mg/ml), 5 mM EDTA, 20 mM Hepes (pH 7.4), 100 mM NaCl, 0.1% DDM, and 0.01% CHS. All buffers listed earlier contained 1 μM alprenolol and the protease inhibitors benzamidine and leupeptin. M1-purified receptors were subjected to size-exclusion chromatography with a Superdex200 Increase 10/300 GL column (GE Healthcare) in 20 mM Hepes (pH 7.4), 100 mM NaCl, 0.1% DDM, and 0.01% CHS.

**Enzymatic ligation of the synthetic phosphopeptide V2Rpp to β2AR**

Purified β2AR-LPETGHH WT or β2AR-LPETGHH Y219A (10 μM or higher final concentration) was incubated with sortase (1:5 sortase/receptor
mass/mass ratio), 5 mM CaCl₂, 200 µM NiSO₄, and the synthetic phosphopeptide V₂Rpp-GGG [fivefold molar excess of receptor on ice overnight as previously described (26)]. The ligation efficiency of V₂Rpp to the receptors using sortase under this condition was close to 100%, as assessed by Coomassie staining (fig. S4A). The ligated receptors were reconstituted into HDL particles as previously described (46). The excess V₂Rpp-GGG was removed by dialysis overnight at 4°C.

**Coomunoprecipitation assay**

The physical interaction of purified β-arrestin1 with FLAG-β₂ARpp WT or FLAG-β₂ARpp Y219A was validated by an in vitro coimmunoprecipitation experiment. ISO (10 µM), 6 µg of FLAG receptor, and molar equivalent amounts of β-arrestin1 and Fab30 were incubated in an assay buffer containing 20 mM Hepes (pH 7.4), 100 mM NaCl, 2 mM CaCl₂, 0.1% DDM, and 0.01% CHS for 30 min at room temperature. Next, 20 µl of M1 anti-FLAG resin was added and incubated for 30 min at room temperature with rotation. Subsequently, the resin was rapidly washed three times with assay buffer and eluted with FLAG peptide (1 mg/ml), 5 mM EDTA, 20 mM Hepes (pH 7.4), and 100 mM NaCl. Coimmunoprecipitated β-arrestin1 was visualized by Coomassie staining.

**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 GTase-Glo assay (Promega). β₂AR WT (4 nM) or β₂ARpp Y219A (4 nM) reconstituted in HDL particles was incubated with 10 µM ISO, 2.5 µM ribonucleotide GTP, 5 µM β-arrestin1, 10 µM Fab30, or 5 µM Nb80 for 10 min at room temperature in a 96-well, clear-bottom white plate. The GTP hydrolysis reaction was initiated by the addition of 443 nM Gs protein and 1 mM dithiothreitol to the preincubated receptor mixture at a total volume of 25 µl 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 2 hours. The reaction was quenched by the addition of the reconstituted GTase-Glo reagent (25 µl per well), which was followed by incubation at room temperature for 30 min with shaking. The GTase-Glo detection reagent (50 µl per well) was then applied and incubated at room temperature for 10 min in the dark. The luminescence signal was detected by a SpectraMax M5 plate reader (Molecular Devices). Control experiments with unligated receptors (β₂AR-LPETGGH WT and β₂AR-LPETGGH Y219A reconstituted in HDL particles) were performed in parallel under identical conditions within the same 96-well plates. The luminescence values from the individual conditions were normalized to the values of the respective total GTP input and presented as a percentage of the total GTP hydrolyzed. The desensitization due to β-arrestin1 was verified by calculating the statistical significance between the indicated pairs in Fig. 4 (E and F) with Tukey’s multiple comparisons test after one-way ANOVA analysis.

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**REFERENCES AND NOTES**

**Fig. S5. β-Arrestin–mediated desensitization does not occur for nonphosphorylated β₂AR WT or β₂AR Y219A.**

**Table S1.** Cell signaling and competition radioligand binding log (IC₅₀) values of β₂AR WT and β₂AR Y219A.

**SUPPLEMENTARY MATERIALS**

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Fig. S1. Analysis of β-arrestin recruitment to β₂AR Y219A.

Fig. S2. Analysis of β₂AR Y219A internalization.

Fig. S3. Impaired β₂-arrestin–mediated activation of ERK2 downstream of β₂AR Y219A.

Fig. S4. β₂-arrestin1 does not alter the affinity of ISO for nonphosphorylated β₂AR WT or β₂AR Y219A.

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Keywords: G protein-coupled receptors; β-arrestins; dual agonism; biased agonism; G protein–coupled receptor kinases; endocytosis; lipoprotein

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G protein–coupled receptor kinases (GRKs) orchestrate biased agonism at the β2-adrenergic receptor

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Determining bias
Biased signaling by GPCRs results in their preferential use of either G proteins or β-arrestins to transduce signals. Drugs that selectively activate one of these pathways provide effective treatment without side effects. Noting that GPCRs must undergo GRK-mediated phosphorylation to recruit β-arrestins, Choi et al. characterized a mutant GPCR that exhibits G protein–biased signaling and found that it could not be phosphorylated by GRKs. When a phosphorylated peptide was fused to the C-terminal region of the mutant receptor, mimicking GRK-mediated phosphorylation, β-arrestin protein recruitment and biased signaling were recovered. These data suggest that G protein–biased signaling is driven more by an inability to recruit GRKs than by an inability to couple to β-arrestins, which may have implications for the design of biased drugs.