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Tyrosine 308 Is Necessary for Ligand-directed G_s Protein-biased Signaling of β_2-Adrenoceptor*

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Anthony Yiu-Ho Woo[‡§¶1], Krzysztof Jozwiak[‖], Lawrence Toll[**], Mary J. Tanga[‡‡], Joseph A. Kozocas[‡‡], Lucita Jimenez[‡‡], Ying Huang[‡§], Ying Song[‡§], Anita Plazinska[‖], Karolina Pajak[‖], Rajib K. Paul[§§], Michel Bernier[§§], Irving W. Wainer[§§], and Rui-Ping Xiao[§2]

*From the [‡]Key Laboratory of Chemical Genomics, School of Chemical Biology and Biotechnology, Peking University Shenzhen Graduate School, Shenzhen 518055, China, the [§]Institute of Molecular Medicine, Centers for Life Sciences, Peking University, Beijing 100871, China, the [¶]Laboratory of Cardiovascular Science and [§§]Laboratory of Clinical Investigation, NIA, National Institutes of Health, Baltimore, Maryland 21224, the [‖]Department of Chemistry, Medical University of Lublin, Lublin, Poland, the **Torrey Pines Institute for Molecular Studies, Port St. Lucie, Florida 34987, and [‡‡]SRI International, Menlo Park, California 94025*

Background: Ligand-specific receptor signaling is often referred to as functional selectivity or biased agonism.
Results: Single amino acid substitution on β_2-adrenoreceptor (Y308F) converts a ligand-specific signaling from G_s-biased to promiscuous G_s and G_i dual signaling.
Conclusion: Specific ligand-receptor interaction results in receptor conformation(s) sufficient to convey biased signaling.
Significance: Our work reveals a molecular mechanism for biased agonism.

Interaction of a given G protein-coupled receptor to multiple different G proteins is a widespread phenomenon. For instance, β_2-adrenoceptor (β_2-AR) couples dually to G_s and G_i proteins. Previous studies have shown that cAMP-dependent protein kinase (PKA)-mediated phosphorylation of β_2-AR causes a switch in receptor coupling from G_s to G_i. More recent studies have demonstrated that phosphorylation of β_2-AR by G protein-coupled receptor kinases, particularly GRK2, markedly enhances the G_i coupling. We have previously shown that although most β_2-AR agonists cause both G_s and G_i activation, (*R,R′*)-fenoterol preferentially activates β_2-AR-G_s signaling. However, the structural basis for this functional selectivity remains elusive. Here, using docking simulation and site-directed mutagenesis, we defined Tyr-308 as the key amino acid residue on β_2-AR essential for G_s-biased signaling. Following stimulation with a β_2-AR-G_s-biased agonist (*R,R′*)-4′-aminofenoterol, the G_i disruptor pertussis toxin produced no effects on the receptor-mediated ERK phosphorylation in HEK293 cells nor on the contractile response in cardiomyocytes expressing the wild-type β_2-AR. Interestingly, Y308F substitution on β_2-AR enabled (*R,R′*)-4′-aminofenoterol to activate G_i and to produce these responses in a pertussis toxin-sensitive manner without altering β_2-AR phosphorylation by PKA or G protein-coupled receptor kinases. These results indicate that, in addition to the phosphorylation status, the intrinsic structural feature of β_2-AR plays a crucial role in the receptor coupling selectivity to G proteins. We conclude that specific interactions between the ligand and the Tyr-308 residue of β_2-AR stabilize receptor conformations favoring the receptor-G_s protein coupling and subsequently result in G_s-biased agonism.

Increasing evidence has accumulated over the past decade indicating that a G protein-coupled receptor (GPCR)[3] does not respond similarly to all agonist ligands. Ligands can initiate multiple cascades of intracellular reactions that can be mediated by G proteins or be G protein-independent. The term biased agonism initially referred to the ability of a ligand to selectively activate either a G protein-mediated event, such as stimulation of adenylyl cyclase, or activation of the G protein-independent noncanonical β-arrestin-dependent signal transduction pathway (1). The emerging paradigm of biased agonism or functional selectivity suggests that binding of one ligand can stabilize receptor conformation(s) preferentially favoring recognition by a given set of signaling proteins or pathways on the intracellular side, although another ligand stabilizes a receptor state that is preferred by a different set of signaling proteins. In this manner, ligands can trigger qualitatively distinct signaling events in the cell (1–8).

β-Adrenergic receptors (β-AR) are G_s-coupled GPCRs and in fact β_1-AR couples only to G_s. For β_2-AR, the prototypical member of the GPCR family, studies (9, 10) have shown that

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[1] To whom correspondence may be addressed: School of Chemical Biology and Biotechnology, Peking University Shenzhen Graduate School, Shenzhen 518055, China. Tel.: 86-755-2603-2459; Fax: 86-755-2603-3174; E-mail: yiuhowoo@pkusz.edu.cn.
[2] To whom correspondence may be addressed: Institute of Molecular Medicine, Peking University, Beijing 100871, China. Tel.: 86-10-6275-7243; Fax: 86-10-6276-7143; E-mail: xiaor@pku.edu.cn.

[3] The abbreviations used are: GPCR, G protein-coupled receptor; adeno, adenovirus; aminoFen, 4′-aminofenoterol; β-AR, β-adrenoceptor; β_1-AR, β_1-adrenoceptor; β_2-AR, β_2-adrenoceptor; Fen, fenoterol; GRK, G protein-coupled receptor kinase; HB, hydrogen bond; ISO, (−)-isoproterenol; methoxyFen, 4′-methoxyfenoterol; MNFen, 4′-methoxy-1-naphthylfenoterol; PDB, Protein Data Bank; PhFen, phenylfenoterol; PKA, cyclic AMP-dependent protein kinase; PTX, pertussis toxin; TM, transmembrane.

agonist binding can activate both G_s and G_i. Our previous study has shown that fenoterol (Fen) is unique among the β_2-AR agonists in terms of ligand-induced receptor-G protein coupling selectivity (11). Although most β_2-AR agonists produce contractile responses in cardiomyocytes that can be sensitized by the G_i disruptor pertussis toxin (PTX), indicating dual G_s and G_i coupling, the inotropic effect of Fen is PTX-insensitive, suggesting that Fen preferentially promotes β_2-AR-G_s coupling. Fen contains two chiral centers in its molecule and may exist as four stereoisomers (Fig. 1). The stereoisomers of Fen and a series of Fen analogs have been synthesized (12–14). The role of ligand chirality in G protein-coupling selectivity has recently been demonstrated using these Fen derivatives (15). Specifically, we have shown that (*R,R′*)-Fen and (*R,R′*)-4′-methoxyfenoterol ((*R,R′*)-methoxyFen) preferentially activated G_s signaling, as evidenced by the lack of PTX sensitivity of their contractile responses in cardiomyocytes and their inability to activate G_i-dependent ERK signaling in HEK-293 cells. In contrast, the corresponding *(S,R′)*-isomers exhibited robust PTX sensitivity in these responses suggesting that they activated both G_s and G_i.

The mechanism for the differential G protein coupling of β-ARs has been the major focus of various studies. It has been suggested that phosphorylation of the β_2-AR by cAMP-dependent protein kinase (PKA) or G protein-coupled receptor kinases (GRKs) promotes the receptors to couple to G_i proteins (16–20). However, some evidence argues against this perception (21, 22). Thus, the molecular basis for inducing β_2-AR's coupling selectivity to different G proteins remains largely elusive.

Our recent simulation studies employing data obtained with [^{3}H]CGP-12177 as the marker ligand have identified hydrogen bond (HB) formation between the tyrosine 308 residue (Tyr-308 or $Y^{7.35}$ in Ballesteros-Weinstein numbering) in transmembrane (TM) 7 of β_2-AR and a HB acceptor at the 4′-position of (*R,R′*)-Fen and (*R,R′*)-methoxyFen (13, 23). These preliminary results suggest that the Tyr-308 residue is essential for agonist-induced β_2-AR preferential coupling to G_s protein. In this study, using site-directed mutagenesis, receptor pharmacology, and cardiomyocyte activation in conjunction with computer simulation, we demonstrated that (*R,R′*)-4′-aminofenoterol ((*R,R′*)-aminoFen) (Fig. 1) targets the WT β_2-AR but not the mutant receptor, β_2-AR Y308F, to G_s-biased signaling. These results experimentally verify our computation-based predictions, and here we present the first evidence that interactions with an individual residue in a conformation of the β_2-AR can induce or stabilize a conformation that leads to selective coupling to a G protein subunit.

EXPERIMENTAL PROCEDURES

β_2-AR Model Construction and Docking Methodology—Docking of ligands to β_2-AR models was performed as recently described (23). In brief, a crystallographic model of the β_2-AR co-crystallized with an inverse agonist carazolol (PDB entry 2RH1) was used as a docking target in simulations. The model was modified by swapping the tyrosine 308 residue into phenylalanine using Yasara to obtain the model representing the Y308F mutant. Molegro Virtual Docker software (MVD version 2010.4.0.0, Aarhus, Denmark) was used for docking simulations within the binding cavity of the target model using MolDock SE algorithm as a search engine.

Compounds and Reagents—Fen analogs used in this study (Fig. 1) were synthesized as described previously (13, 14). Cell culture reagents were purchased from Invitrogen. Zinterol was obtained from Tocris Bioscience (Bristol, UK). Forskolin, ICI-118,551, 3-isobutyl-1-methylxanthine, (−)-isoproterenol (ISO), PTX, and other reagents were purchased from Sigma.

Animals—Male Sprague-Dawley rats (200–250 g) were purchased from Charles River. β_2-AR knock-out mice were generous gifts from Dr. Brian Kobilka (Stanford University Medical Center, Palo Alto, CA). Animals were housed and studied in conformance with the "Guide for the Care and Use of Laboratory Animals, Eighth Edition" from the National Institutes of Health (NOT-OD-12–020, released 2011), with institutional Animal Care and Use Committee approval.

Generation of Stable Cell Lines and Recombinant Adenoviruses—HEK293A cells were obtained from Invitrogen and maintained in DMEM supplemented with 10% FBS at 37 °C in a humidified 5% CO_2 incubator. The plasmids encoding for the human β_2-AR and β_2-AR Y308F mutant were kindly provided by Dr. Brian Kobilka and Dr. Hitoshi Kurose (Kyushu University, Fukuoka, Japan), respectively. Each of these coding sequences of β_2-AR was subcloned into pcDNA3.1− vector (Invitrogen). HEK cells were transfected with the resultant plasmids using Lipofectamine 2000 reagent (Invitrogen), and stably transfected clones were selected against G418 (0.8 mg/ml). When stable expression was achieved, the cells were cultured in the presence of 0.3 mg/ml G418. Adenoviruses for human β_2-AR (adeno-β_2-AR) and green fluorescent protein (adeno-GFP) have been described previously (24, 25). Adenoviral expression vector carrying the β_2-AR Y308F coding sequence was generated by subcloning. Viral particles were purified from transfected HEK cells using standard viral amplification and CsCl purification methods. Viral titers were determined in dilution assays by an immunocytochemical technique using an antibody raised against β_2-AR (sc-569, Santa Cruz Biotechnology, Santa Cruz, CA).

Cardiomyocyte Isolation, Adenoviral Gene Transfer, and Contractility Measurement—Cardiomyocytes were isolated from male β_2-AR knock-out mice (2–4 months old) or Sprague-Dawley rats using a standard enzymatic technique (9, 24). Mouse cardiomyocytes were seeded on laminin-coated coverslips and infected with the adenoviruses at a multiplicity of infection of 100 (24). The cells were subsequently cultured for 24 h in minimal essential medium (M1018, Sigma) supplemented with forskolin (1 μM) and 2,3-butanedione monoxime (10 mM). Contractility of single cardiomyocytes was measured as described previously (9). In brief, cardiomyocytes were perfused with a buffer containing (in mM) 137 NaCl, 4.9 KCl, 1.2 $MgCl_2$, 1 NaH_2PO_4, 1 $CaCl_2$, 20 glucose, and 20 HEPES (pH 7.4) and electrically paced (0.5 Hz for rat cardiomyocytes or 1 Hz for mouse cardiomyocytes) at ambient temperature on a microscopic stage. Cell length was monitored by an optical edge-tracking method using an instrument setup manufactured by IonOptix (Milton, MA). Measurements were made under steady-state conditions before and after exposure of the myo-

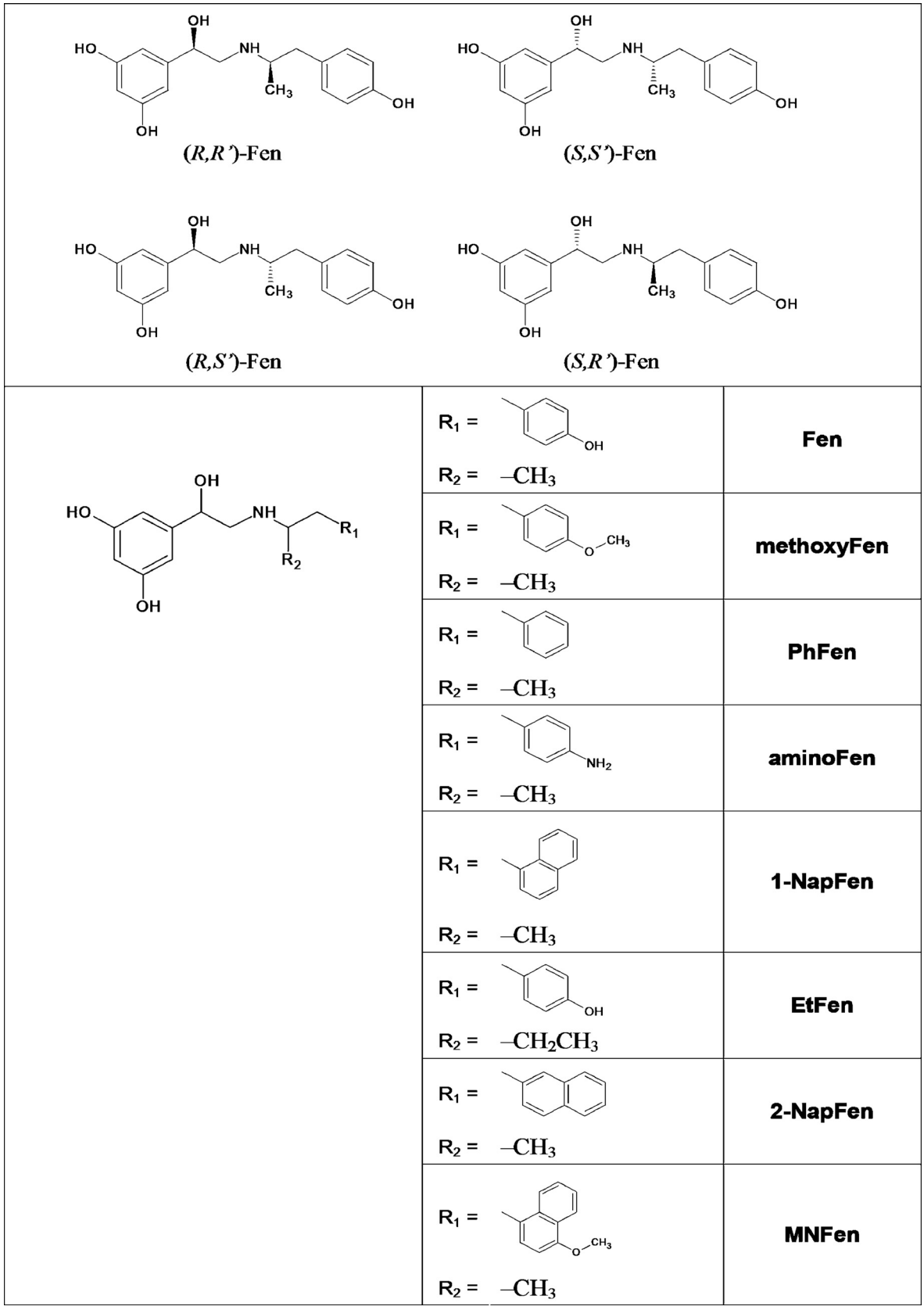

FIGURE 1. **Structures of Fen and its derivatives used in the study.** The following terms are used: fenoterol (*Fen*), 4′-methoxyfenoterol (*methoxyFen*); 4′-aminofenoterol (*aminoFen*); phenylfenoterol (*PhFen*); 1-naphthylfenoterol (*1-NapFen*); ethylfenoterol (*EtFen*); 2-naphthylfenoterol (*2-NapFen*); and 4′-methoxy-1-naphthylfenoterol (*MNFen*).

TABLE 1

Effect of PTX treatment on the contractile responses to the Fen derivatives

EC_{50} ± S.E. (nM) for PTX-treated and control groups determined based on concentration-response profiles for the compound-stimulated cardiomyocyte contraction.

Compound	EC_{50} (nM) −PTX	EC_{50} (nM) +PTX	*p* value	PTX sensitivity
(*R,R′*)-Fen	73 ± 18[a,b]	37 ± 10[a]	0.085[c]	No
(*R,S′*)-Fen	575 ± 122[b]	270 ± 100	0.034	Yes
(*S,R′*)-Fen	3451 ± 3788[a]	830 ± 234[a]	0.003	Yes
(*R,R′*)-MethoxyFen	186 ± 50[a,b]	144 ± 50[a]	0.579[c]	No
(*R,S′*)-MethoxyFen	506 ± 107[b]	182 ± 53	0.022	Yes
(*S,R′*)-MethoxyFen	3184 ± 1160[a]	1161 ± 336[a]	0.018	Yes
(*R,R′*)-PhFen	547 ± 167	124 ± 52	0.015	Yes
(*R,R′*)-AminoFen	176 ± 78	145 ± 64	0.641	No
(*R,S′*)-AminoFen	605 ± 225	311 ± 93	0.071	No
(*S,R′*)-AminoFen	3083 ± 1286	778 ± 169	0.004	Yes
(*R,R′*)-1-NapFen	82 ± 2	12 ± 2	0.004	Yes
(*R,S′*)-1-NapFen	111 ± 43	16 ± 6	<0.001	Yes
(*R,R′*)-EtFen	8551 ± 4992[b]	1330 ± 443	0.004	Yes
(*R,R′*)-2-NapFen	133 ± 25[b]	27 ± 7	<0.001	Yes
(*R,R′*)-MNFen	16 ± 7[b]	3 ± 2	0.028	Yes

[a] EC_{50} values were recalculated from Ref. 15.
[b] EC_{50} values have been reported in Ref. 14 as partial data. Complete sets of data are presented here. Comparisons between the −PTX and +PTX groups and the calculation of the *p* values were performed in experiments with a parallel design.
[c] *p* values were adopted from Ref. 15.

cytes to a single dose of the agonist. In a subset of experiments, aliquots of cells were incubated with PTX (0.75 μg/ml at 37 °C for >3 h) to block G_i signaling, as described previously (9).

cAMP Accumulation Assay—Cells cultured on poly-D-lysine-coated 12-well plates were treated with PTX (0.3 μg/ml) or vehicle overnight and incubated for 10 min with the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (1 mM) in HEPES-buffered Hanks' balanced salt solution prior to stimulation. Cells were then treated with the agonist or control vehicle for 10 min. Reactions were stopped by the addition of HCl. cAMP contents in the clarified cellular extracts were determined with an enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI) according to the manufacturer's protocol. cAMP contents were normalized with total cellular protein. Protein contents were determined by the method of Lowry (Bio-Rad).

Immunoblotting—Whole-cell lysates in lysis buffer (Cell Signaling Technology, Danvers, MA) containing protease inhibitor mixture (Roche Diagnostics) and phosphatase inhibitor mixture (EMD Millipore, Billerica, MA) were centrifuged at 15,000 × *g* for 10 min. Clarified cell lysates (20 μg) for β_2-AR detection were further denatured and treated with 125 units of peptide:*N*-glycosidase (New England Biolabs) for 2 h at 37 °C. The samples were denatured in Laemmli sample buffer and resolved by SDS-PAGE. Phosphorylation of ERK and β_2-AR was detected by immunoblotting using the same antibodies as described previously (15).

Radioligand Binding Assay—Receptor density was determined on membranes derived from HEK stable cell lines expressing WT or mutant forms of β_2-AR as described previously (14). The B_{max} and K_d values were determined by nonlinear regression analysis using Prism 4 (GraphPad Software, San Diego).

Statistical Analysis—Results are expressed as means ± S.E. Unless described otherwise, unpaired Student's *t* test was performed to compare the means between two groups and one-way analysis of variance for multiple group comparison followed by post hoc analysis with Bonferroni's *t* test. Statistical analysis and curve-fitting of the concentration-response curves were conducted using Prism 4. The curves of the cAMP assays were fitted to the sigmoid curves by nonlinear regression analysis using the four-parameter logistic model without giving any constraints. Curve-fitting of the cardiomyocyte contractility data was conducted using the same algorithms and constraints laid out in our previous study (15).

RESULTS

Role of the Aminoalkyl Substituent of (R,R′)-Fen on Preferential β_2-AR-G_s Coupling—To define the structural features of Fen compounds contributing to selective β_2-AR-G_s signaling, we have undertaken a structure-activity relationship approach. In this campaign, PTX was used to distinguish the contribution of β_2-AR-G_i signaling in the agonist-stimulated inotropic effects of a collection of Fen derivatives (Fig. 1) on a cardiomyocyte contractility model. By inhibiting the G_i signaling with PTX, the regulatory inhibition of adenylyl cyclase on cAMP synthesis would be decreased, and as a result the G_s-stimulated contractile response would be enhanced. Four Fen derivatives ((*R,R′*)-Fen, (*R,R′*)-methoxyFen, (*R,R′*)-aminoFen, and (*R,S′*)-aminoFen) eliciting a G_i-independent activation of β_2-AR (thus PTX-insensitive) were identified (Table 1 and Fig. 2*B*) with (*R,R′*)-aminoFen demonstrating the highest G_s selectivity (as assessed by the small difference between the EC_{50} values of the −PTX and the +PTX groups, Table 1). Their contractility stimulatory effects were mediated through β_2-AR because these effects could be antagonized by ICI-118,551, a specific β_2-AR antagonist (Fig. 3). (*R,R′*)-AminoFen was subsequently used as the typical G_s-selective β_2-AR agonist in the rest of this investigation.

(R,R′)-AminoFen Selectively Activates β_2-AR-G_s Signaling in Cardiomyocytes Expressing WT β_2-AR but Activates Both G_s and G_i in Cardiomyocytes Expressing the β_2-AR Y308F Mutant—Cardiomyocytes express both β_1-AR and β_2-AR, and robust β_2-AR-G_i coupling has been demonstrated in freshly isolated

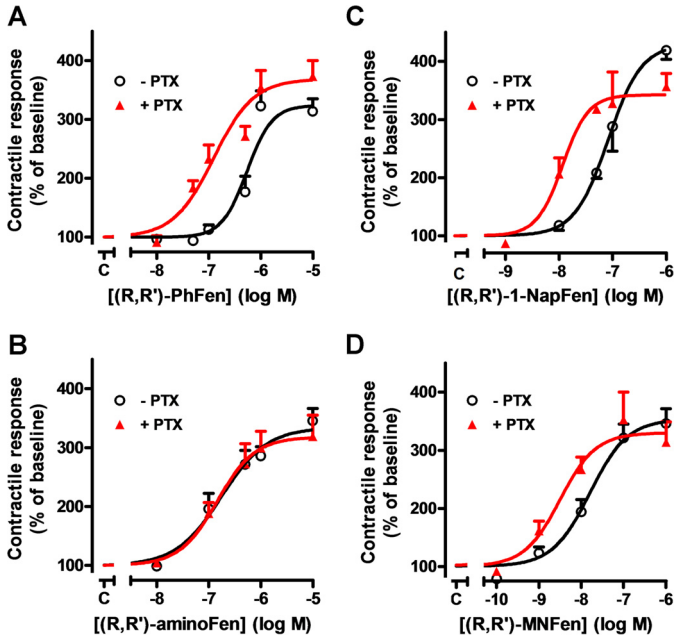

FIGURE 2. **Substitution on the aminoalkyl portion of (*R,R′*)-Fen determines the PTX sensitivity of the agonist-stimulated contractile response in rat cardiomyocytes.** Concentration-response profiles of cardiomyocyte contractility in cells subjected to (*R,R′*)-PhFen (*A*), (*R,R′*)-aminoFen (*B*), (*R,R′*)-1-NapFen (*C*), and (*R,R′*)-MNFen (*D*) with (▲) and without (○) PTX treatment (0.75 μg/ml at 37 °C for >3 h). Contractile response to the agonist is expressed as a percentage of the basal contractility (mean ± S.E., $n = 9–11$ cells from 5 to 9 hearts for each data point).

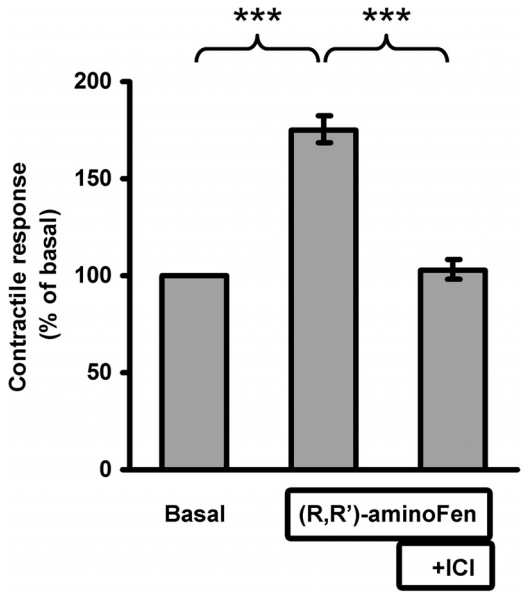

FIGURE 3. **(*R,R′*)-AminoFen exhibits β_2-AR subtype selectivity in cardiomyocyte contractile response.** Single ventricular myocytes from rats were set to pace under perfusion. The contraction amplitude of a cell in response to (*R,R′*)-aminoFen (0.1 μM) followed by ICI-118,551 (*ICI*, 0.1 μM) was monitored. Steady-state contractility was recorded. Contractile response is expressed as a percentage of the basal contractility. Data are means ± S.E., $n = 4$ cells from four hearts. ***, $p < 0.001$ (by paired *t* test).

adult mouse cardiomyocytes expressing endogenous β_2-AR or human β_2-AR at 200-fold over basal level (10). Hence, we employed cardiomyocytes from β_2-AR knock-out mice transduced with exogenous β_2-AR or its mutants as a physiological model to investigate the role of the β_2-AR Tyr-308 residue on ligand-directed G protein selectivity. In our recent study, we have shown that β_2-AR in adult rodent cardiomyocytes lost its coupling to G_i after overnight culture, and addition of forskolin in the culture medium could maintain functional dual coupling of β_2-AR to G_s and G_i proteins (26). In this investigation, we first confirmed the presence of functional β_2-AR-G_i coupling in β_2-AR knock-out mouse cardiomyocytes reconstituted with human β_2-AR using zinterol, a selective β_2-AR agonist (Fig. 4). In another control experiment, cultured cardiomyocytes from β_2-AR knock-out mice were infected with adeno-GFP and then subjected to (*R,R′*)-aminoFen stimulation to study the effect of this compound on stimulating β_1-AR. The results in Fig. 5*A* show that the β_1-AR stimulatory effect of (*R,R′*)-aminoFen was undetectable at 100 nM, was minor at 500 nM (175 ± 26%), and became very substantial (about 350%) at 1 μM. In subsequent contractility studies, we only tested (*R,R′*)-aminoFen up to 500 nM.

Next, we investigated the positive inotropic effects of (*R,R′*)-aminoFen on β_2-AR knock-out mouse cardiomyocytes infected with adeno-β_2-AR or adeno-β_2-AR Y308F and the sensitivities of these responses toward PTX. In cardiomyocytes transduced with the WT β_2-AR, the positive inotropic effect of (*R,R′*)-aminoFen was insensitive to PTX treatment (Fig. 5*B*). Notably, the (*R,R′*)-aminoFen-stimulated positive inotropic effect was markedly enhanced by PTX treatment in cells transduced with the β_2-AR Y308F mutant (Fig. 5*C*).

Residue Tyr-308 of β_2-AR Is Necessary for Ligand-directed G_s-biased β_2-AR Signaling—It has been demonstrated in HEK cells that β_2-AR agonists trigger an acute increase in ERK phosphorylation, which peaks at 5 min, and this effect is mediated in part by a G_i-dependent mechanism (16, 27). Furthermore, both G_s- and G_i-mediated β_2-AR activation can lead to ERK phosphorylation (16, 22, 27). Cell lines stably expressing WT β_2-AR (HEK-β_2-AR cells) and β_2-AR Y308F mutant (HEK-β_2-AR Y308F cells) were established from HEK293A cells. The levels of β_2-AR in these cell lines were 4033 ± 826 and 2300 ± 80 fmol/mg protein, respectively, as assayed by radioligand binding, whereas the level of β_2-AR in the parental cells was 30–40 fmol/mg (21). Next, we studied the G protein pathways responsible for phospho-ERK (p-ERK) induction by (*R,R′*)-aminoFen and ISO in these cell lines. The sensitivity of agonist-induced ERK phosphorylation toward PTX was used to indicate G_i activation.

Stimulation with ISO increased p-ERK by about 6-fold in HEK-β_2-AR cells (Fig. 6, *A* and *B*) and HEK-β_2-AR Y308F cells (Fig. 6, *C* and *D*). This activation of p-ERK was mediated by a combination of G_s- and G_i-dependent pathways as demonstrated by the decreases in maximal ERK phosphorylation in the PTX-treated groups. In contrast, in HEK-β_2-AR cells, (*R,R′*)-aminoFen induced ERK phosphorylation in a PTX-insensitive manner (Fig. 6, *A* and *B*). Importantly, the increase in phosphorylation of ERK in response to (*R,R′*)-aminoFen exhibited a robust PTX sensitivity in HEK-β_2-AR Y308F cells, and this G_i-dependent effect appeared to be positively correlated with the concentration of (*R,R′*)-aminoFen (Fig. 6, *C* and *D*).

We also measured cAMP accumulation in HEK cells stably expressing β_2-AR and its Y308F mutant. Although (*R,R′*)-

aminoFen produced a PTX-insensitive cAMP response in HEK-β_2-AR cells (Fig. 7*A*), the concentration-response profile shifted upwards in response to PTX treatment in HEK-β_2-AR Y308F cells (Fig. 7*B*). Efficacy data (Table 2) show that PTX significantly increased the E_{max} value (from 89 ± 1 to 117 ± 2, $p < 0.01$) without altering the $logEC_{50}$ value of the cAMP response of (*R,R′*)-aminoFen in HEK-β_2-AR Y308F cells (from −8.05 ± 0.01 to −8.03 ± 0.02, $p = 0.75$). We did not observe sensitivity of cAMP response to PTX when HEK-β_2-AR cells were stimulated with ISO (data not shown), thus corroborating a previous experiment (17). This may be due to an inherent limitation of the assay. In contrast, PTX caused an increase in the E_{max} of the ISO-stimulated cAMP response (from 109 ± 4 to 126 ± 5, $p < 0.05$, Table 2) in HEK-β_2-AR Y308F cells, mirroring a similar observation in cells expressing the β_2-AR D-4 mutant, a receptor phenotype having a reduced G_s-coupling, and an increased G_i-coupling (17). Taken together, these results demonstrate that the β_2-AR-Y308 residue is necessary for the G_s-biased β_2-AR signaling and that Y308F mutation fully restored β_2-AR-G_i signaling in response to (*R,R′*)-aminoFen, a G_s-selective β_2-AR agonist, in cardiomyocytes and in HEK cells.

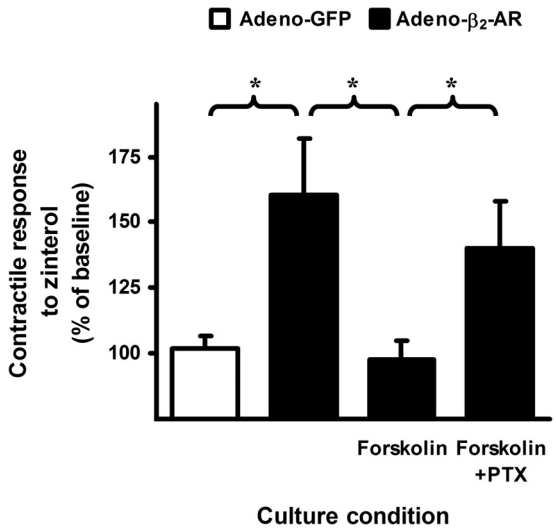

FIGURE 4. **Addition of forskolin reconstitutes functional coupling of β_2-AR to G_i protein in cultured β_2-AR knock-out mouse cardiomyocytes induced with human β_2-AR.** Cardiomyocytes from β_2-AR knock-out mice were infected with adeno-GFP (*white bar*) or adeno-β_2-AR (*black bars*) and cultured for 24 h in the presence or absence of forskolin (1 μM) and/or PTX (0.75 μg/ml) as indicated. Cells were transferred to a perfusion chamber, electrically paced, and subjected to stimulation with zinterol (0.2 μM, a concentration without an inotropic effect in freshly isolated cardiomyocytes from WT mice, see Fig. 1*A* in Ref. 26). Steady-state contractility was measured. Data (mean ± S.E., $n = 10–15$ cells from 5 to 9 hearts for each data point) are expressed as percentages of the basal contractility. *, $p < 0.05$. Zinterol (0.2 μM) did not increase contractility in cells infected with adeno-GFP demonstrating no β_1-AR stimulatory effect at this concentration. In cells infected with adeno-β_2-AR and cultured in the absence of forskolin, the inotropic response produced by zinterol stimulation was the result of a pure β_2-AR-G_s-mediated effect because β_2-AR and G_i proteins were functionally uncoupled. In cells infected with adeno-β_2-AR in the presence of forskolin, the coupling of β_2-AR to G_i protein was reestablished. Therefore, the cardiomyocytes were unresponsive to zinterol as if they were freshly isolated WT β_2-AR^+ cells when β_2-AR-G_i coupling was intact. In cells infected with adeno-β_2-AR in the presence of forskolin and PTX, the coupling of β_2-AR to G_i protein still occurred, but G_i had lost its function and could no longer negatively regulate β_2-AR-G_s activation by zinterol.

Effects of Agonist-induced Receptor Phosphorylation on G Protein-coupling Selectivity of β_2-AR—It is well established that agonist stimulation of β_2-AR leads to phosphorylation of the receptor by PKA and GRK, with important implications on receptor desensitization (28). In addition, phosphorylation of β_2-AR at the GRK or the PKA sites has been suggested to be necessary for β_2-AR-G_i protein coupling in naive cells (16, 17), cardiomyocytes (18–20), and *in vivo* hearts (20). Therefore, we investigated the agonist-stimulated receptor phosphorylation in HEK cells expressing either the WT β_2-AR or the β_2-AR Y308F mutant using phosphosite-specific antibodies (29). Stimulation with ISO (1 μM) for 5 min, a treatment time period reportedly leading to near-maximal receptor phosphorylation responses (30–33), increased the phosphorylation of β_2-AR and β_2-AR Y308F at Ser-262 (PKA-site) to about 5-fold of basal (Fig. 8, *A* and *B*). Similarly, (*R,R′*)-aminoFen (1 μM) produced the same maximal responses in both HEK-β_2-AR cells and HEK-β_2-AR Y308F cells (Fig. 8, *A* and *B*). Treatment with ISO

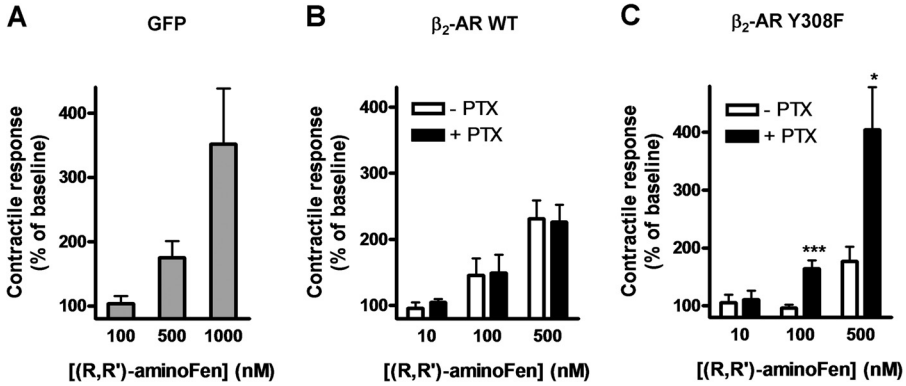

FIGURE 5. **PTX increases the inotropic effect of (*R,R′*)-aminoFen in cardiomyocytes expressing β_2-AR Y308F mutant but not in cardiomyocytes expressing WT human β_2-AR.** *A*, (*R,R′*)-aminoFen-stimulated contractile responses in β_2-AR knock-out mouse cardiomyocyte adenoviral gene transfer GFP. Cardiomyocytes from β_2-AR knock-out mice were infected with adeno-GFP and cultured for 24 h. Cells were paced under perfusion and subjected to (*R,R′*)-aminoFen (100, 500, or 1000 nM). Steady-state contractility before and after agonist stimulation was measured. *B*, (*R,R′*)-aminoFen-stimulated contractile responses in β_2-AR knock-out mouse cardiomyocytes adenoviral gene transfer WT β_2-AR in the presence or absence of PTX treatment. Cardiomyocytes from β_2-AR knock-out mice were infected with adeno-β_2-AR and cultured with or without PTX (0.75 μg/ml) for 24 h. Cells were paced under perfusion and subjected to (*R,R′*)-aminoFen (10, 100, or 500 nM). *C*, (*R,R′*)-aminoFen-stimulated contractile responses in β_2-AR knock-out mouse cardiomyocyte adenoviral gene transfer β_2-AR Y308F in the presence or absence of PTX treatment. Contractile responses are expressed as percentages of the basal contractility (mean ± S.E., $n = 9–14$ cells from 4 to 8 hearts for each data point). Concentration dependence of the (*R,R′*)-aminoFen-stimulated responses was verified (by two-way analysis of variance, $p < 0.0001$) for all datasets. *, $p < 0.05$; ***, $p < 0.001$ *versus* corresponding −PTX group.

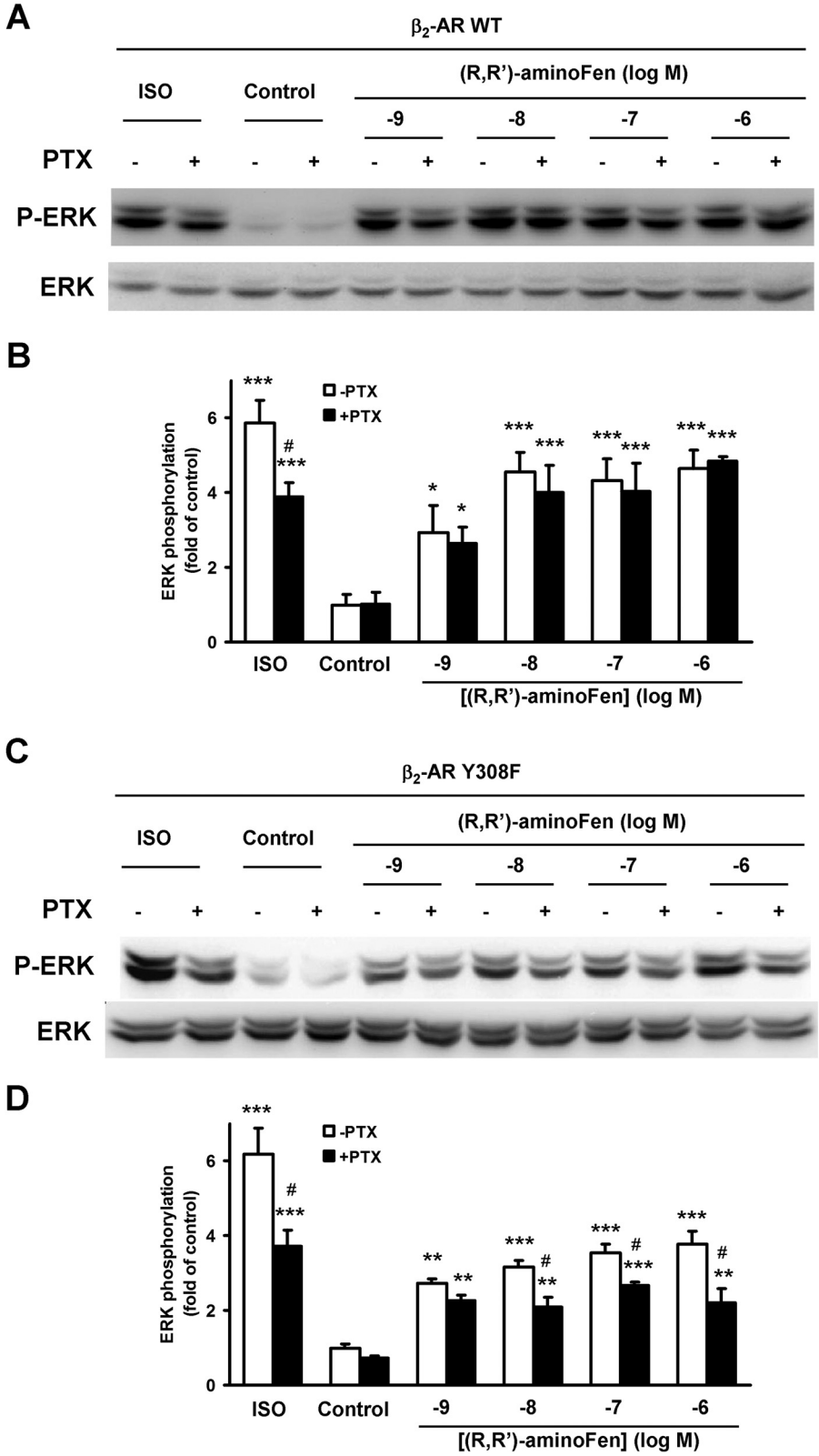

FIGURE 6. **Y308F substitution on β_2-AR increases the PTX sensitivity of (*R,R'*)-aminoFen-induced ERK phosphorylation in HEK stable cell lines.** Confluent cultures of HEK-β_2-AR cells and HEK-β_2-AR Y308F cells were deprived of serum overnight. Treatment with PTX (0.3 μg/ml, +) or vehicle (−) was implemented during serum starvation. Cells were then stimulated with ISO (1 μM) or (*R,R'*)-aminoFen (10^{-9} to 10^{-6} M) for 5 min at 37 °C as indicated. ERK phosphorylation was determined by immunoblotting. *A,* immunoblots of p-ERK and total ERK (as protein loading control) in response to agonist stimulation in HEK-β_2-AR cells, and *B,* averaged data. *C,* immunoblots of p-ERK and total ERK in response to agonist stimulation in HEK-β_2-AR Y308F cells, and *D,* averaged data. Data are presented as fold increase over −PTX control (means ± S.E. in 3–4 independent experiments). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ *versus* vehicle controls; #, $p < 0.05$ *versus* −PTX group.

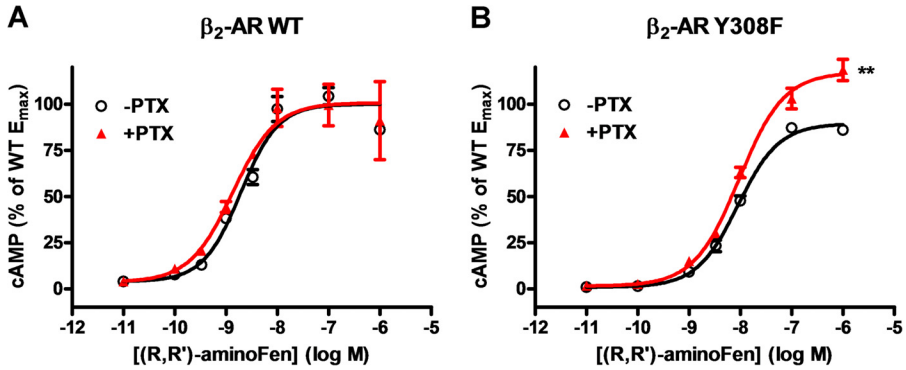

FIGURE 7. **Y308F substitution on β_2-AR increases the PTX sensitivity of (*R,R′*)-aminoFen-stimulated cAMP production in HEK stable cell lines.** HEK-β_2-AR cells and HEK-β_2-AR Y308F cells were cultured in 12-well plates in parallel, and subsets of the cells were treated with PTX (0.3 μg/ml) or vehicle overnight. Agonist stimulation was allowed to proceed for 10 min at 25 °C in the presence of 3-isobutyl-1-methylxanthine (1 mM). Cellular cAMP contents were determined by enzyme immunoassay. HEK-β_2-AR cells were subjected to (*R,R′*)-aminoFen (10^{-11} to 10^{-6} M) (*A*), and HEK-β_2-AR Y308F cells were subjected to (*R,R′*)-aminoFen (*B*), with (▲) and without (○) PTX treatment. Data (means ± S.E. in three independent experiments performed in triplicate) are expressed as percentages of the E_{max} response of the β_2-AR WT −PTX group. Curve-fitting analysis of the concentration-response curves were conducted using Prism. **, $p < 0.01$ *versus* −PTX group.

TABLE 2

E_{max} % and logEC_{50} values of the (*R,R′*)-aminoFen- and ISO-induced cAMP responses in HEK cell lines stably expressing β_2-AR WT or β_2-AR Y308F

Calculations of logEC_{50} and E_{max} values were based on concentration-response profiles for the compound-stimulated cAMP production. E_{max} values are expressed as percentages of the E_{max} response of the β_2-AR WT −PTX group. Means ± S.E., $n = 3$. *, $p < 0.05$; **, $p < 0.01$ *versus* −PTX group.

	(*R,R′*)-AminoFen				ISO			
	−PTX		+PTX		−PTX		+PTX	
Cell lines	E_{max} %	log EC_{50}	E_{max} %	log EC_{50}	E_{max} %	log EC_{50}	E_{max} %	log EC_{50}
β_2-AR WT	100 ± 3	−8.71 ± 0.05	101 ± 2	−8.87 ± 0.03	100 ± 4	−10.14 ± 0.10	97 ± 3	−10.25 ± 0.07
β_2-AR Y308F	89 ± 1	−8.05 ± 0.01	117 ± 2**	−8.03 ± 0.02	109 ± 4	−9.36 ± 0.06	126 ± 5*	−9.33 ± 0.07

also increased the phosphorylation of β_2-AR at Ser-355,356 (GRK sites) by about 17-fold in these cell lines (Fig. 8, *C* and *D*). Because it has been reported that phosphorylation of the β_2-AR mediated by GRK depends on a high concentration of agonists (27–31), we subsequently performed the receptor phosphorylation assay at higher concentrations of (*R,R′*)-aminoFen (namely 100- and 1000-fold of $EC_{50\ cAMP}$ concentrations, corresponding to a near-saturating and a saturating concentration of the agonist for receptor stimulation, respectively, refer to Fig. 7, *A* and *B*). As shown in Fig. 8, *C* and *D*, (*R,R′*)-aminoFen at the 100-fold $EC_{50\ cAMP}$ concentration (R100) produced a significant increase in the phosphorylation of β_2-AR at Ser-355,356 (GRK sites) as compared with the vehicle control in HEK cell lines expressing either the WT β_2-AR or the β_2-AR Y308F mutant. Treatment with (*R,R′*)-aminoFen at the 1000-fold $EC_{50\ cAMP}$ concentration (R1000) caused β_2-AR-Ser-355,356 phosphorylation in both HEK-β_2-AR cells and HEK-β_2-AR Y308F cells indistinguishable in magnitude as compared with the stimulation with ISO (1 μM, a saturating concentration, refer to Table 2) (Fig. 8, *C* and *D*).

In HEK-β_2-AR cells, increased phosphorylation of the β_2-AR at the PKA site could be observed 5 min after stimulation with ISO or (*R,R′*)-aminoFen at 1 μM (Fig. 8, *A* and *B*). Similarly, (*R,R′*)-aminoFen (0.2 and 2 μM) and ISO (1 μM) also increased the phosphorylation of β_2-AR at the GRK sites (Fig. 8, *C* and *D*). The same treatment with (*R,R′*)-aminoFen (10^{-8} to 10^{-6} M) increased the p-ERK level by about 4-fold via activating G_s but not G_i (Fig. 6, *A* and *B*). Treatment with ISO after disrupting the activity of G_i with PTX also increased the p-ERK level by about 4-fold, which could be further increased to 6-fold in the absence of PTX (Fig. 6, *A* and *B*). Thus, both (*R,R′*)-aminoFen and ISO can induce the phosphorylation of ERK and β_2-AR via a G_s-dependent pathway, but only ISO can activate ERK through a β_2-AR-G_i signaling pathway. These results clearly demonstrate that (*R,R′*)-aminoFen and ISO produced similar effects in triggering phosphorylation of β_2-AR at the PKA sites or the GRK sites, although they exhibited diverse G protein selectivity in HEK stable cell lines expressing the WT β_2-AR. In addition, Y308F substitution on the β_2-AR caused a qualitative change in the G protein selectivity of (*R,R′*)-aminoFen from being exclusively G_s-activating (Fig. 6, *A* and *B*) to dually G_s/G_i-activating (Fig. 6, *C* and *D*) without significantly affecting its activities in eliciting receptor phosphorylation by both PKA and GRKs ($p > 0.05$, Fig. 8, *B* and *D*). Thus, phosphorylation of β_2-AR at its PKA or GRK sites is insufficient to trigger the receptor coupling to G_i proteins.

Docking Simulation on β_2-AR—To reveal the molecular interactions important for the ligand-directed G_s-biased agonism of β_2-AR, a molecular model of (*R,R′*)-aminoFen was docked to the crystal model of the β_2-AR-binding site as well as to a model representing the Y308F mutant receptor using the same procedures as described previously (23). As shown in Fig. 9, *A* and *C*, the ligand molecule can be fitted reasonably well into the binding sites of both β_2-AR conformations (PDB entry 2RH1 for carazolol-bound and PDB entry 3SN6 for BI-167107-and G_s protein-bound, respectively) with the resorcinol ring of the ligand pointed in the direction of $S203^{5.42}$ and $S207^{5.46}$, the β-OH and the secondary amine groups interacted with $D113^{3.32}$ and $N312^{7.39}$ residues. Notably, a HB interaction between the 4′-amino group of the ligand and the hydroxyl

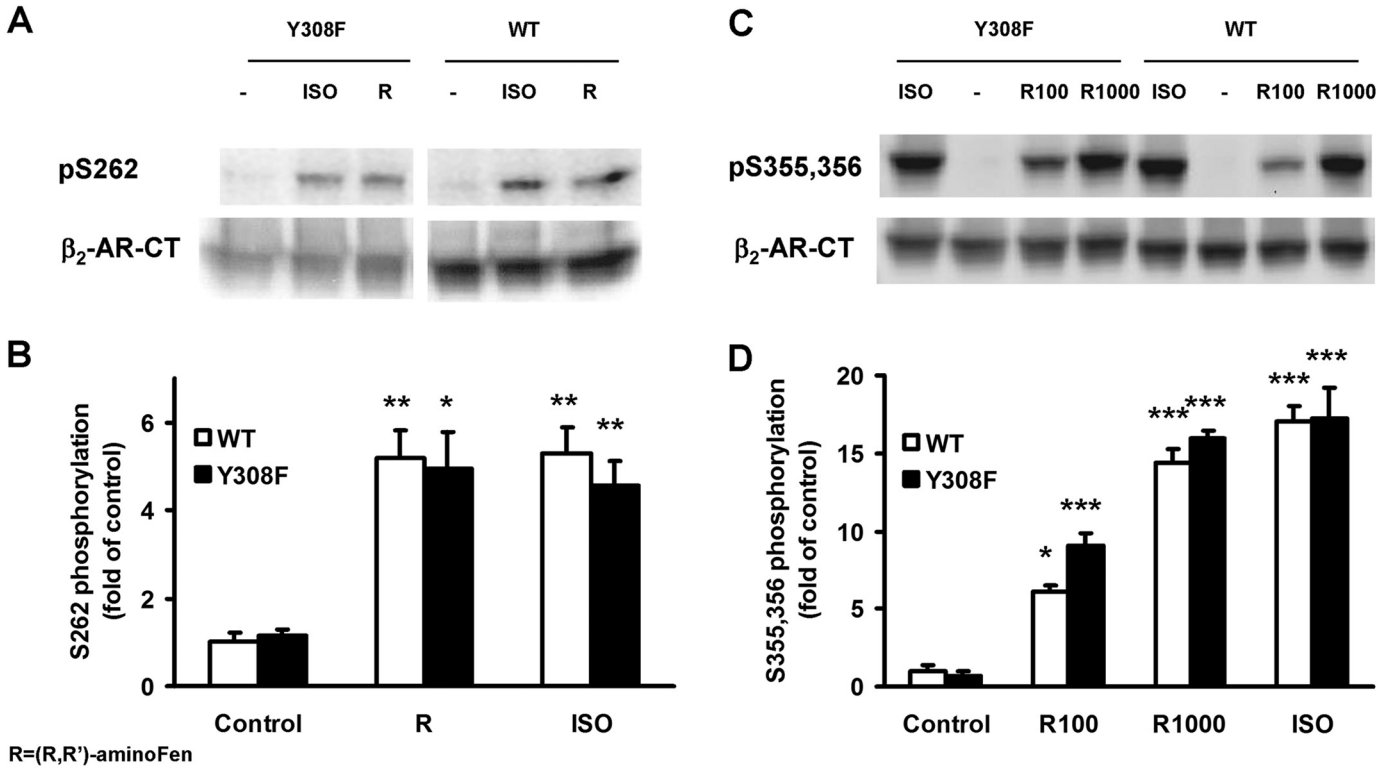

FIGURE 8. **(*R,R'*)-AminoFen induces phosphorylation of β_2-AR and β_2-AR Y308F mutant at the GRK and the PKA sites in HEK stable cell lines.** Confluent cultures of HEK-β_2-AR cells and HEK-β_2-AR Y308F cells were incubated in serum-free medium for 3 h and then stimulated with vehicle control (−), ISO (1 μM), or (*R,R'*)-aminoFen (R, 1 μM; R100 for WT, 0.2 μM; R100 for Y308F, 1 μM; R1000 for WT, 2 μM; R1000 for Y308F, 10 μM) for 5 min at 37 °C. Phosphorylated β_2-AR was detected by phosphosite-specific antibodies against Ser(P)-262 for PKA sites and Ser(P)-355,356 for GRK sites. Total β_2-AR was detected after stripping and reprobing the membrane with the β_2-AR-CT antibody. *A,* immunoblots of Ser(P)-262-β_2-AR and total β_2-AR in response to agonist stimulation, and *B,* averaged data (normalized to total β_2-AR). *C,* immunoblots of Ser(P)-355,356-β_2-AR and total β_2-AR in response to agonist stimulation, and *D,* averaged data. Data are expressed as fold increase over control (means ± S.E. in at least three independent experiments). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ *versus* vehicle controls (two-way analysis of variance with post hoc *t* test). No significant differences were found for all within-group comparisons between WT and Y308F, $p > 0.05$.

group of $Y308^{7.35}$ was found probable in the carazolol-bound β_2-AR model (Fig. 9*A*). When (*R,R'*)-aminoFen was docked to the modified model of the Y308F mutant receptor (Fig. 9*B*), the position of the molecule within the binding site was very similar, and the above-mentioned interactions still occurred with the exception of the HB created by the 4′-amino moiety. In effect, the 4′-aminobenzyl ring assumed a slightly different position than during docking to the WT receptor model.

DISCUSSION

The current data (Figs. 5–8) suggest that the G_s-selective signaling depends on specific interactions between the agonist and the β_2-AR-Y308 residue, and induction of receptor phosphorylation alone does not necessarily lead to a switching of the receptor coupling from G_s to G_i as once proposed (16). These results are consistent with those reported in our previous study on cardiomyocytes (15) in which the stereoisomers of Fen and methoxyFen possessing different G protein selectivity induced similar phosphorylation of β_2-AR at the PKA sites. Because (*R,R'*)-Fen, (*R,R'*)-methoxyFen, and (*R,R'*)-aminoFen are full agonists of β_2-AR capable of inducing receptor phosphorylation just as ISO (Fig. 8) (15), the only explanation for their preferential G_s selectivity would be their exceptional abilities in stabilizing a receptor conformation favoring receptor-G_s protein interaction. As the emerging paradigm of functional selectivity suggests, ligands can perturb a GPCR to attain "ensembles" of multiple conformations, and each of these conformations is capable of activating a distinct set of signaling events (1–8). Therefore, one possibility is that the binding of the G_s-selective agonists causes the β_2-AR to assume or stabilize conformations leading to G_s protein coupling. β_2-AR in such conformation(s) regardless of its phosphorylation status interacts strongly with G_s protein, prohibiting receptor-G_i protein interaction from taking place. Furthermore, if the β_2-AR-Y308 residue is mutated, the G_s selectivity of these agonists will be lost (Figs. 5 and 6), suggesting that mutation of this residue affects the receptor conformations stabilized by these agonists. The latter conformations, possibly resembling the ISO-bound receptor conformations, are G_i protein-permissive.

$Y308^{7.35}$ has been implicated for the high affinity binding of β_2-AR-selective agonists (34). Importantly, our recent docking simulation study (23) supported the initial determination (13, 14) that in inverse agonist-associated conformations, such as the S_1 and S_2 conformations described by Kim *et al.* (35), $Y308^{7.35}$ may interact directly with the 4′-hydroxyl, 4′-methoxy, or 4′-amino group of the (*R,R'*)-Fen derivatives through hydrogen bonding. This hypothesis was confirmed in this study (Fig. 9*A*).

Because the human β_2-AR and the rat β_2-AR share a high sequence homology particularly at the TM regions encompassing the ligand binding pocket (data not shown), the data derived from the rat cardiomyocyte study (Fig. 2 and Table 1) could be

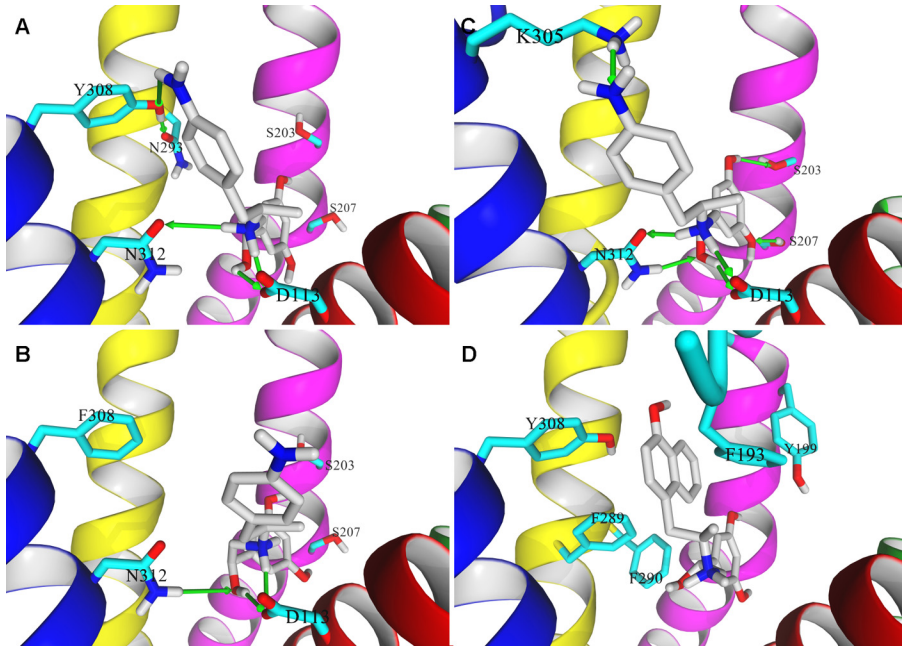

FIGURE 9. **Binding poses of (*R,R′*)-aminoFen and (*R,R′*)-MNFen docked to the β_2-AR models.** *A,* docking of (*R,R′*)-aminoFen to the carazolol-bound β_2-AR model (PDB entry 2RH1), and *B,* to the β_2-AR Y308F mutant. The 4′-amino group of (*R,R′*)-aminoFen forms a HB with the Tyr-308 residue of β_2-AR (*green arrow*), and the interaction is lost in the β_2-AR Y308F mutant. *C,* docking of (*R,R′*)-aminoFen to a BI-167107 and G_s protein-bound conformation of β_2-AR (PDB entry 3SN6). The location of the docked molecule highly resembles the orientation of co-crystallized agonist, BI-167107, as both molecules share significant structural similarities. The agonists form a network of analogous HB interactions with the receptor residues Ser-203, Ser-207, Asn-312, and Asp-113. An additional interaction can be observed between the 4′-amino moiety of the ligand and Lys-305 residue. *D,* docking of (*R,R′*)-MNFen to a carazolol-bound conformation of β_2-AR (PDB entry 2RH1). Aromatic residues in the ligand binding pocket able to form π-π interactions with the naphthyl moiety of the ligand are shown. Ligand molecule is rendered in atom-type color-coded stick mode, and five essential TM helices of target β_2-AR model are shown and colored as follows: TM3, *magenta*; TM4, *green*; TM5, *red*; TM6, *yellow*; and TM7, *blue*.

interpreted in the light of the structural insight gained from the human β_2-AR models. As shown in Fig. 2, *A*, *C*, and *D*, and Table 1, the *R,R′*-isomers of Fen derivatives containing the following substituents on the aminoalkyl portion, phenyl (PhFen), 1-naphthylfenoterol, 2-naphthylfenoterol, ethylfenoterol, and 4′-methoxy-1-naphthyl (MNFen), produced PTX-sensitive contractile responses in cardiomyocytes. These results suggest that these compounds activate both G_s and G_i pathways of β_2-AR. In contrast, the positive inotropic effects of (*R,R′*)Fen, (*R,R′*)-methoxyFen, (*R,R′*)-aminoFen, and (*R,S′*)-aminoFen were PTX-insensitive (Table 1 and Fig. 2*B*), indicating that they selectively activate β_2-AR-G_s signaling. Together, these data illustrate the structural features of a Fen compound for ligand-directed selective β_2-AR-G_s signaling as follows: (i) a benzyl rather than a naphthyl moiety on its aminoalkyl substituent; (ii) a 4′-oxygen or a 4′-nitrogen moiety on this aromatic substituent; and (iii) a mandatory *R*-configuration on the chiral center of the β-OH group and a preferred *R*-configuration on the second chiral center.

The fact that (*R,R′*)-PhFen, but not (*R,R′*)-Fen, (*R,R′*)-methoxyFen, and (*R,R′*)-aminoFen, produced a PTX-sensitive inotropic effect (Fig. 2, *A* and *B*, and Table 1) suggests that the 4′-(N/O) moieties in these (*R,R′*)-Fen derivatives are indispensable for agonist-induced preferential G_s activation. If either the phenyl hydroxyl group of Tyr-308 or the 4′-(N/O) moiety is lost, the HB between the ligand and the 7.35 residue (Fig. 9*A*) will not exist, and promiscuous G_s and G_i dual signaling rather than G_s-biased signaling will be induced (Figs. 5*C*, 6*C*, and 7*B*). This indicates that specific interaction between Tyr-308 and the 4′-(N/O)-benzyl moiety promotes preferential receptor-G_s protein coupling. Our simulation study (23) has also shown that (*R,R′*)-Fen derivatives with naphthyl moieties interact not by hydrogen bonding with Tyr-308 but rather by π-π interactions with the other aromatic residues in the ligand binding pocket (Fig. 9*D*). The opposite is true for compounds with 4′-(N/O)-benzyl moieties (23). The dominance of the π-π interactions with (*R,R′*)-MNFen binding, irrespective of the presence of a potentially hydrogen bonding 4′-methoxy moiety, is associated with dual G_s and G_i protein coupling of β_2-AR (Fig. 2*D*). These key features in the ligand-receptor interaction make (*R,R′*)-MNFen a superior negative model compound as compared with ISO or the (*S,R′*)-isomers in the study of G_s-biased signaling, and the functional data with (*R,R′*)-MNFen stimulation (Fig. 2*D*) also point to the same conclusion. Thus, based on our simulated docking study and experimental evidence, we conclude that HB interactions between the 4′-(N/O)-benzyl moiety of the (*R,R′*)Fen derivatives and the β_2-AR-Y308 residue play an important role on ligand-directed β_2-AR-G_s signaling.

The structural features of Fen derivatives for preferential G_s selectivity and receptor subtype selectivity have both similarities and differences. Our initial studies (13, 14) have shown that both stereochemistry and the aminoalkyl substituent play essential roles on the β_2-AR subtype selectivity of the Fen compounds. Although an *R*-configuration, hydrogen bonding with Tyr-308, and π-π interactions with aromatic residues in the ligand binding pocket can all contribute to high ligand binding affinity and increased selectivity to β_2-AR (14), only the *R*-configuration and hydrogen bonding with Tyr-308 correlate with

G_s selectivity. The π-π interactions, however, likely have a detrimental effect on G_s selectivity of the Fen derivatives. It is conceivable that the above-mentioned molecular interactions would impact the receptor conformational ensembles stabilized by different β_2-AR agonists and subsequently result in the differential G protein-coupling selectivity.

Recent studies have identified $H^{6.55}$ to be a major determinant of ligand-biased signaling in dopaminergic D_{2L} receptors (36). A follow-up study has further characterized the receptor conformations involved in D_{2L} receptor functional selectivity (37). Briefly, a model of functional selectivity for D_{2L} receptor has been proposed in which TM6 represents a rotatory switch in response to the binding of different functionally selective agonists. In this model, if $H^{6.55}$ in TM6 rotates toward $S^{5.43}$ on TM5, the resultant ligand-stimulated receptor conformation will favor the activation of the arachidonic acid pathway. Conversely, if $H^{6.55}$ rotates toward TM7 and interacts with $Y^{7.35}$, the ligand-stimulated receptor conformation will lead to a signaling bias toward cAMP/MAPK activation.

A very similar mechanism occurs in β_2-AR. $Y308^{7.35}$ is known to form a HB with a neighboring residue $N293^{6.55}$ in TM6, and this specific interaction (additionally shown on Fig. 9*A*) remains intact in all reported crystallographic structures of the receptor. Because the 4′-(N/O) moiety of the G_s-selective ligand involves the hydroxyl group of Tyr-308 in another HB interaction, a competition occurs between the Tyr-308–Asn-293 interaction and Tyr-308–ligand interaction. It is therefore postulated that the naturally occurring Tyr-308–Asn-293 HB is disrupted when the G_s-selective ligand binds to the receptor. The Tyr-308–Asn-293 interaction bridges the upper parts of TM6 and TM7. Breaking this interaction during the receptor conformational transition might be a key phenomenon leading to specific activation of the β_2-AR to a form favoring selective G_s protein coupling.

Using acetylcholine M_2 receptor as a model, Bock *et al.* (38) have designed "dualsteric" agonists to study the role of allosteric vestibules on G protein activation. The allosteric vestibule is located at the entrance of the orthosteric binding cavity of many class A GPCRs and has been implicated for ligand binding (39). Acetylcholine is known to activate G_i and G_s signaling of the M_2 receptor. The authors have found that dualsteric agonists (such as iper-6-phth and iper-6-naph) exhibited a G_i over G_s signaling bias compared with acetylcholine and their parent compound Iperoxo, an orthosteric muscarinic agonist. Mutagenic studies have identified the M_2-$W422^{7.35}$ residue located at the allosteric vestibule to be critical for both G_s and G_i protein activation, with the gain in dualsteric probe efficacy for G_i activation in the allosteric mutant. Interestingly, $W422^{7.35}$ and $Y177^{5.32}$ in extracellular loop 2 of the M_2 receptor and the analogous $Y308^{7.35}$ and $F193^{5.32}$ in β_2-AR that line the passage to the orthosteric binding cavity have been suggested to undergo a conformational rearrangement during receptor activation (39, 40). The authors implied from their findings that spatial rearrangement of this passage is critical for receptor movements required for appropriate unfolding of the intracellular domain region for G protein coupling.

The two previous studies and this study indicate the important role of the 7.35 residue on GPCR conformational transition leading to G protein activation. Notably, here we provide the direct evidence to pinpoint the role of this residue on functional selectivity and illustrate this point with an "extreme" form of signaling bias (in nominal terms of with or without PTX sensitivity rather than in ratiometric terms of a biased factor) in a physiological context of adult cardiomyocytes. Further studies are needed to determine whether this deduction could be generalized in a broader sense, such as to other class A aminergic GPCRs.

Our study design necessitates the investigation of a single aspect of the β_2-AR agonists, specifically their differential selectivity to G_s and G_i proteins. From a chemical biology perspective, however, the β_2-AR is only one of the many possible *in vivo* targets of the Fen compounds. Indeed, in complex biological systems such as the adult cardiomyocytes, a compound is likely to produce its effects via interactions with multiple cellular proteins. Therefore, it is unsurprising to find that high concentrations of (*R,R′*)-aminoFen also stimulate β_1-AR (Fig. 5*A*) given that only 1 out of the 15 amino acids that constitute the ligand binding pocket differs between β_1-AR and β_2-AR (41). Consistently, our previous binding affinity data (13) have also shown that the subtype selectivity of β_2-AR relative to β_1-AR in terms of $K_i\beta_1$-AR/$K_i\beta_2$-AR ratio was 9 for (*R,R′*)-aminoFen. Interestingly, it is the 7.35 residue (β_2-AR-Y308) that is different, and the corresponding residue is a phenylalanine or Phe-359 in β_1-AR, incidentally the same mutation characterized in this study. However, mutation on β_2-AR to convert the amino acid residues in its ligand binding pocket to that resembling β_1-AR produced a dissimilar function of increased receptor-G_i protein coupling in the β_2-AR Y308F mutant, yet it is known that β_1-AR does not normally couple to G_i (10). Thus, it is not the amino acid residues themselves but rather their different interactions with the ligand and the resultant conformational changes (42) that determine the diverse selectivity of the β-AR subtypes or mutants to different G proteins. As a cautionary note, this interpretation is only confined to the very first step of G protein coupling at the receptor level, without taking into account other intracellular mechanisms such as phosphorylation, internalization, G protein abundance, and subcellular compartmentation.

In addition, cross-talk between different receptor-mediated signaling pathways is very common when a given ligand simultaneously stimulates two or more receptors. We observed this cross-talk of signals between β_1-AR and β_2-AR in cardiomyocytes stimulated by 500 nM (*R,R′*)-aminoFen as exemplified by a higher contractile response in the β_2-AR Y308F mutant-expressing cells (Fig. 5*C*) *versus* the WT β_2-AR-expressing cells (Fig. 5*B*) in the PTX-treated groups. The detailed mechanism of the cross-talk between the β_1- and β_2-AR signals is beyond the scope of this study, although an elaborated discussion can be found in Zhang *et al.* (43). This example illustrates that no single assay or approach can adequately elucidate the complex pharmacology of a compound.

In conclusion, this study has identified an amino acid residue in β_2-AR necessary for functional selectivity. Mutation of this residue causes a G_s-selective agonist to gain the ability to activate G_i when it binds to the β_2-AR. We also provide, for the first time, functional data confirming the identification of the ligand-receptor interactions important for G_s-biased signaling in β_2-AR. Advances in structural biological techniques (42,

44–48) will ultimately unravel how these interactions during ligand binding translate into receptor conformation(s) for selective coupling to different G proteins. This investigation has elucidated the molecular basis of G_s-biased agonism in β_2-AR, and this is one step closer to structure-based design of signaling pathway-specific drugs.

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