

# Recruitment of $\beta$ -Arrestin 1 and 2 to the $\beta_2$ -Adrenoceptor: Analysis of 65 Ligands<sup>§</sup>

Timo Littmann, Martin Göttle, Michael T. Reinartz, Solveig Kälble, Irving W. Wainer, Takeaki Ozawa, and Roland Seifert

*Institute of Pharmacology, Hannover Medical School, Hannover, Germany (T.L.; M.G.; M.T.R.; S.K., R.S.); Laboratory of Clinical Investigation, National Institute on Aging, Baltimore, Maryland (I.W.W.); and Department of Chemistry, School of Science, The University of Tokyo, Tokyo, Japan (T.O.)*

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## ABSTRACT

Beyond canonical signaling via  $G_{\alpha s}$  and cAMP, the concept of functional selectivity at  $\beta_2$ -adrenoceptors ( $\beta_2$ ARs) describes the ability of adrenergic drugs to stabilize ligand-specific receptor conformations to initiate further signaling cascades comprising additional G-protein classes or  $\beta$ -arrestins ( $\beta$ arr). A set of 65 adrenergic ligands including 40 agonists and 25 antagonists in either racemic or enantiopure forms was used for  $\beta$ arr recruitment experiments based on a split-luciferase assay in a cellular system expressing  $\beta_2$ AR. Many agonists showed only (weak) partial agonism regarding  $\beta$ arr recruitment. Potencies and/or efficacies increased depending on the

number of chirality centers in (*R*) configuration; no (*S*)-configured distomer was more effective at inducing  $\beta$ arr recruitment other than the eutomer.  $\beta$ arr2 was recruited more effectively than  $\beta$ arr1. The analysis of antagonists revealed no significant effects on  $\beta$ arr recruitment. Several agonists showed preference for activation of  $G_{\alpha s}$  GTPase relative to  $\beta$ arr recruitment, and no  $\beta$ arr-biased ligand was identified. In conclusion: 1) agonists show strong bias for  $G_{\alpha s}$  activation relative to  $\beta$ arr recruitment; 2) agonists recruit  $\beta$ arr1 and  $\beta$ arr2 with subtle differences; and 3) there is no evidence for  $\beta$ arr recruitment by antagonists.

## Introduction

According to the concept of canonical signaling,  $\beta_2$ -adrenoceptors ( $\beta_2$ ARs) induce bronchodilatory effects by activation of  $G_{\alpha s}$  and an increase in intracellular cAMP (Samama et al., 1993; Johnson, 1998).  $\beta_2$ -Sympathomimetics are agonists at the  $\beta_2$ ARs derived from the endogenous agonist epinephrine (EPI) (Supplemental Fig. 1) and constitute essential drugs in the treatment of bronchial asthma and chronic obstructive pulmonary disease. Representatives are the rapid-acting  $\beta_2$ AR agonists fenoterol (FEN) and albuterol (ALB) used to counter immediate asthmatic attacks, or the long-acting  $\beta_2$ AR agonists formoterol (FOR) and salmeterol (SAL) used for prolonged respiratory control (Hochhaus and Möllmann, 1992; Delmotte and Sanderson, 2010).

The concept of functional selectivity describes the ability of agonists to stabilize ligand-specific receptor conformations triggering the activation of multiple signaling cascades as well as the bias of ligands to preferentially activate certain cascades (Evans et al., 2010; Reiner et al., 2010; Seifert, 2013; van der Westhuizen et al., 2014). With regard to racemic sympathomimetic drug formulations consisting of (*R*)- and (*S*)-isoproterenol (ISOs), stereochemistry-related problems have been reported,

including paradoxical proinflammatory effects caused by the inactive (*S*)-distomer (Mazzoni et al., 1994; Mitra et al., 1998; Nelson et al., 1998; Templeton et al., 1998; Zhang et al., 1998; Gawchik et al., 1999; Handley et al., 2000, 2002; Baramki et al., 2002; Volcheck et al., 2005; Patel and Thomson, 2012). These side effects may be the consequence of yet unknown distomer-triggered activation of noncanonical signaling pathways. In addition to canonical signaling by  $G_{\alpha s}$ , the  $\beta_2$ AR couples to  $G_{\alpha i}$  proteins (Wenzel-Seifert and Seifert, 2000; Seifert et al., 2002; Birnbaumer, 2007; Magocsi et al., 2007) and  $\beta$ -arrestins ( $\beta$ arrs) (Oakley et al., 2000; Shenoy et al., 2006; Audet et al., 2010; Shukla et al., 2011; Reiter et al., 2012). Arrestins are responsible for desensitization of G-protein-coupled receptors upon prolonged stimulation and noncanonical signaling via mitogen-activated protein kinases (Freedman and Lefkowitz, 1996; Baillie et al., 2003; Beaulieu et al., 2005; Shenoy et al., 2006; Luttrell and Gesty-Palmer, 2010; Shukla et al., 2011). There are four known arrestin isoforms with arrestin1 and arrestin4 being restricted to visual sensory tissue and arrestin2 and arrestin3 [also referred to as  $\beta$ -arrestin1 ( $\beta$ arr1) and  $\beta$ -arrestin2 ( $\beta$ arr2), respectively] being ubiquitously expressed (Ferguson, 2001). In recent studies, we have shown that FEN stereoisomers stabilize ligand-specific  $\beta_2$ AR conformations and exhibit strong bias for  $G_{\alpha s}$  activation relative to  $G_{\alpha i}$  activation and  $\beta$ arr1 and  $\beta$ arr2 recruitment (Reinartz et al., 2015a,b).

Several experimental setups are available for the investigation of receptor-arrestin interactions. In the complementation

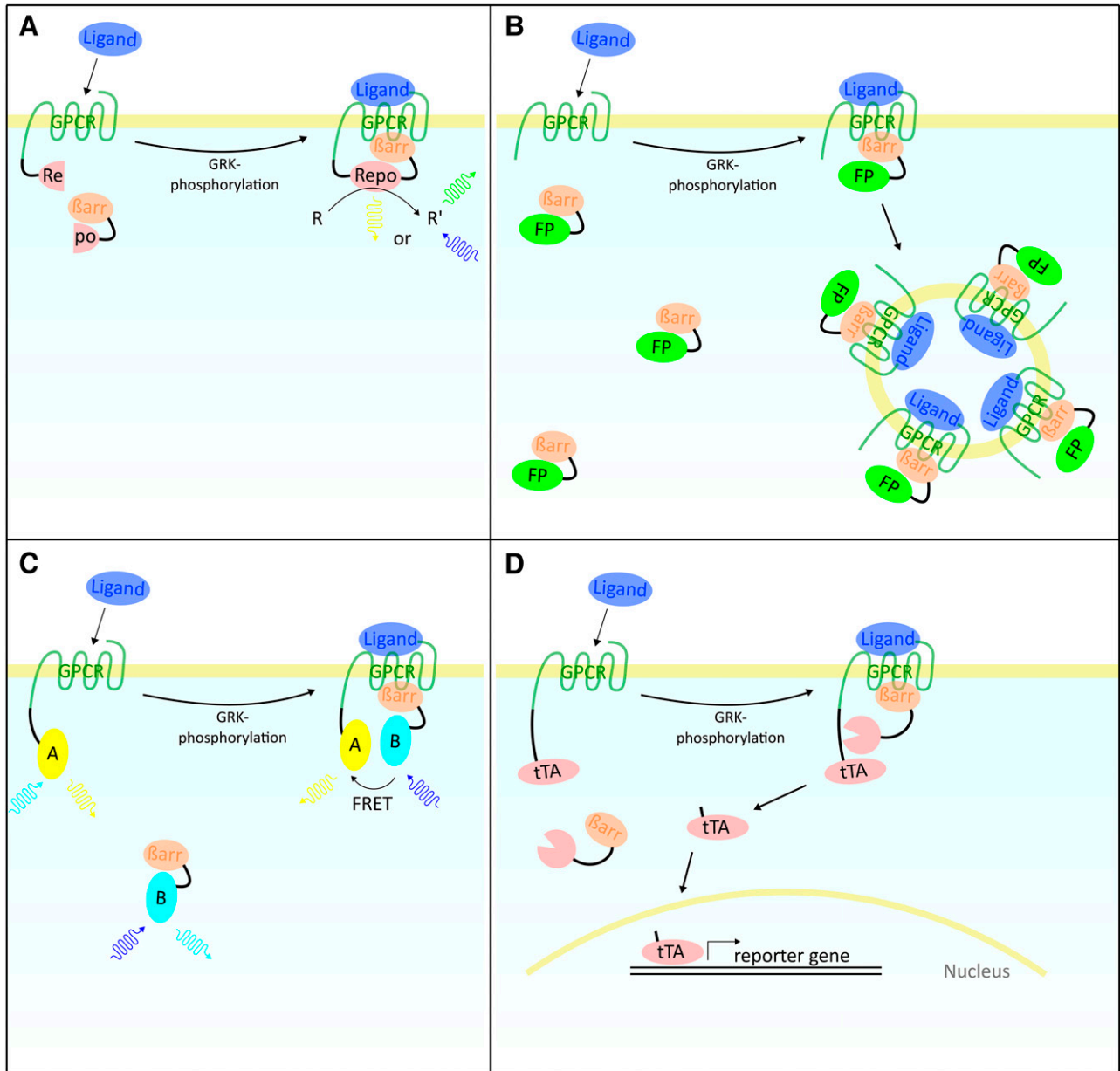
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**ABBREVIATIONS:** ALB, albuterol;  $\beta_2$ AR,  $\beta_2$ -adrenoceptor;  $\beta$ arr,  $\beta$ -arrestin; CAR, carvedilol; EPI, epinephrine; FEN, fenoterol; FOR, formoterol; ISO, isomer; MNF, 4'-methoxy-1-naphthylfenoterol; SAL, salmeterol.

assay, a split reporter protein such as luciferase becomes functional upon recruitment of  $\beta$ arr to the G-protein-coupled receptor (Fig. 1A). One example is the split-luciferase assay system used in this study, which is based on cells expressing two fusion proteins: The first protein consists of the receptor and the N-terminal fragment of a luciferase from the click beetle

*Photinus pyralis* and the second protein consists of the  $\beta$ arr fused to the C-terminal fragment of the luciferase. Upon recruitment of  $\beta$ arr to the  $\beta_2$ AR, the two fragments of the luciferase complement each other and light at  $\lambda = 558$  nm is emitted (Takakura et al., 2012). In this study, a cell line expressing the  $\beta_2$ AR-luciferase protein and the  $\beta$ arr1-luciferase



**Fig. 1.** Assay setups for the investigation of arrestin recruitment. (A) Complementation assay. Fusion proteins of G-protein-coupled receptor (GPCR) and  $\beta$ arr are each used with one part of a trenched reporter protein (Repo). Upon recruitment of  $\beta$ arr to the GPCR the two parts of the reporter protein complement each other and it becomes functional. Reporter proteins are luciferases,  $\beta$ -lactamase, or  $\beta$ -galactosidase that process specific substrates (R). Their activity can be detected, either by measuring luminescence (luciferase,  $\beta$ -galactosidase), or fluorescence ( $\beta$ -lactamase). (B) GFP (YFP)-distribution assays. The distribution of fluorescent proteins (FPs) (e.g., GFP, YFP) is analyzed. Without the influence of ligands the FP- $\beta$ arr fusion proteins are uniformly distributed in the cell. Upon recruitment of the FP- $\beta$ arr fusion proteins to the GPCR, internalization of the receptors in vesicles is mediated. GPCRs and bound FP- $\beta$ arr are colocalizing, leading to the formation of grains within the cells, which is quantified using fluorescence microscopy and specific software. (C) CFP/YFP Förster resonance energy transfer (FRET) assay. These methods use FRET for quantification. Two fusion proteins of the GPCR and  $\beta$ arr are used. Each protein is fused to a fluorescent protein of which the emission spectrum of the first overlaps the excitation spectrum of the second (e.g., YFP and CFP). When both proteins come in close proximity to each other FRET occurs and is measured. Another possibility is to use a luciferase as a FRET donor instead of a fluorescent protein. (D) Tango assay. The  $\beta$ arr is fused to a tobacco etch virus (TEV) protease. The GPCR is fused to a linker region containing a cleavage site for the TEV protease and the tTA transcription factor. Upon recruitment of  $\beta$ arr to the GPCR, the protease fusion protein is close enough to the GPCR fusion protein to cut off the tTA, which is now able to translocate to the nucleus where it induces transcription of a reporter gene (e.g., luciferase).

protein ( $\beta_2$ AR- $\beta$ arr1) as well as a cell line expressing the  $\beta_2$ AR-luciferase protein and the  $\beta$ arr2-luciferase protein ( $\beta_2$ AR- $\beta$ arr2) were analyzed. In the DiscoverX (Fremont, CA) PathHunter  $\beta$ arr assay, a  $\beta$ -galactosidase enzyme is complemented upon  $\beta$ arr recruitment (Yin et al., 2009). Alternative experimental approaches are the GFP (YFP)-distribution assays (Fig. 1B), the CFP/YFP Förster resonance energy transfer assay technique (Fig. 1C), and the Tango assay format (Fig. 1D).

To this end, the effects of  $\beta_2$ AR ligands on  $\beta$ arr1 and  $\beta$ arr2 recruitment have not yet been systematically analyzed. Therefore, the aim of this study was to fill this gap in our knowledge and to investigate the recruitment of  $\beta$ arr1 and  $\beta$ arr2 to the  $\beta_2$ AR using 65 adrenergic ligands either as racemic forms or, if available, as pure enantiomers. Ligands were chosen from different structural classes (Supplemental Figs. 1 and 2) and included antagonists because several studies have shown that  $\beta$ arr recruitment can be induced by antagonists as well (Wisler et al., 2007; Erickson et al., 2013).

## Materials and Methods

**Materials.** The following ligands and reagents were obtained from Sigma (Steinheim, Germany): alprenolol [racemic, (*R*)-, 1-(2-allylphenoxy)-3-(isopropylamino)propan-2-ol]; atenolol (racemic, 2-[4-[2-hydroxy-3-(propan-2-ylamino)propoxy]phenyl]acetamide); CGP 20712A (2-hydroxy-5-[2-[[2-hydroxy-3-[4-[1-methyl-4-(trifluoromethyl)-1*H*-imidazol-2-yl]phenoxy]propyl]amino]ethoxy]-benzamide methanesulfonate salt); ephedrine (enantiopure, (1*R*,2*S*)-2-(methylamino)-1-phenylpropan-1-ol); EPI (enantiopure, (*R*)-, [4-(1-hydroxy-2-(methylamino)ethyl)benzene-1,2-diol]; FEN (racemic, enantiopure, (*R,R'*)-, (*S,S'*)-, (*S,R'*)-, (*S,S'*)-, [5-(1-hydroxy-2-[[2-(4-hydroxyphenyl)-1-methylethyl]amino]ethyl)benzene-1,3-diol]; isoproterenol (enantiopure, (*R*)-, (*S*)-, 4-[1-hydroxy-2-(isopropylamino)ethyl]benzene-1,2-diol); labetalol (racemic, 2-hydroxy-5-[1-hydroxy-2-[[1-methyl-3-phenylpropyl]amino]ethyl]benzamide); metoprolol (racemic, 1-(isopropylamino)-3-[4-(2-methoxyethyl)phenoxy]propan-2-ol); nadolol (racemic, (2*R*\*,3*S*\*)-5-[(2*R*\*)-3-(tert-butylamino)-2-hydroxypropyl]oxy]-1,2,3,4-tetrahydronaphthalene-2,3-diol); norepinephrine (enantiopure, (*R*)-, (*S*)-, 4-[(1*R*)-2-amino-1-hydroxyethyl]benzene-1,2-diol); propranolol (enantiopure, (*R*)-, (*S*)-, 1-(1-methylethylamino)-3-(1-naphthoxy)propan-2-ol); salbutamol (racemic, 4-[2-(tert-butylamino)-1-hydroxyethyl]-2-(hydroxymethyl)phenol]; sotalol (racemic, *N*-[4-[1-hydroxy-2-(propan-2-ylamino)ethyl]phenyl]methanesulfonamide); timolol [(*S*)-1-(tert-butylamino)-3-[[4-morpholin-4-yl-1,2,5-thiadiazol-3-yl]oxy]propan-2-ol]; Dulbecco's modified Eagle's medium; and fetal bovine serum. Dulbecco's modified Eagle's medium without phenol red was obtained from GE Healthcare (Pasching, Austria). The following ligands were obtained from Tocris (Avonmouth, United Kingdom): betaxolol (racemic, 1-[4-[2-(cyclopropylmethoxy)ethyl]phenoxy]-3-(isopropylamino)propan-2-ol); bisoprolol (racemic, 1-[4-[(2-isopropoxyethoxy)methyl]phenoxy]-3-(isopropylamino)propan-2-ol); BRL 37344 [(*R*\*,*R*\*)-( $\pm$ )-4-[2-[(3-chlorophenyl)-2-hydroxyethyl]amino]propyl]phenoxyacetic acid, sodium salt]; CGP 12177 (4-[3-[(1,1-dimethylethyl)amino]-2-hydroxypropyl]-1,3-dihydro-2*H*-benzimidazol-2-one hydrochloride); CGP 20712 (1-[2-[(3-carbamoyl-4-hydroxy)phenoxy]ethylamino]-3-[4-(1-methyl-4-trifluoromethyl-2-imidazolyl)phenoxy]-2-propanol dihydrochloride); ICI 118,551 (racemic, [erythro-(*S*\*,*S*\*)-1-[2,3-(dihydro-7-methyl-1*H*-inden-4-yl)oxy]-3-[(1-methylethyl)amino]-2-butanol hydrochloride)]; ICI 215,001 [(*S*)-4-[2-hydroxy-3-phenoxypropylamino]ethoxy]phenoxyacetic acid hydrochloride]; pindolol (racemic, [1-(1*H*-indol-4-yl)oxy]-3-[(1-methylethyl)amino]-2-propanol]; nebivolol (racemic, ( $\pm$ )-[2*R*\*(1*S*\*5*S*\*(*S*\*))]- $\alpha$ , $\alpha'$ -[Iminobis(methylene)bis(6-fluoro-3,4-dihydro-2*H*-1-benzopyran-2-methanol)]; practolol (racemic, *N*-[4-[2-hydroxy-3-(isopropylamino)propoxy]phenyl]acetamide); xamoterol

(racemic, 1-(4-hydroxyphenoxy)-3-[2-(4-morpholinocarboxamido)ethylamino]-2-propanol hemifumarate); and zinterol (racemic, *N*-[5-[2-[(1,1-dimethyl-2-phenylethyl)amino]-1-hydroxyethyl]-2-hydroxyphenyl]methanesulfonamide hydrochloride). The following ligands were obtained from Boehringer-Ingelheim (Biberach, Germany): carvedilol (CAR) (racemic, [3-(9*H*-carbazol-4-yl)oxy]-2-hydroxypropyl]-[2-(2-methoxyphenoxy)ethyl]amine); FOR (racemic, (*R,R*)-, (*S,S*)-, *N*-[2-hydroxy-5-[1-hydroxy-2-[1-(4-methoxyphenyl)propan-2-ylamino]ethyl]phenyl]formamide); olodaterol (enantiopure, (*R*)-, (*S*)-, 6-hydroxy-8-[(1*R*)-1-hydroxy-2-[[1-(4-methoxyphenyl)-2-methylpropan-2-yl]amino]ethyl]-4*H*-1,4-benzoxazin-3-one); salbutamol (racemic, enantiopure, (*R*)-, (*S*)-, 4-[2-(tert-butylamino)-1-hydroxyethyl]-2-(hydroxymethyl)phenol); and salmeterol (racemic, enantiopure, (*R*)-, (*S*)-, 2-(hydroxymethyl)-4-[1-hydroxy-2-[6-(4-phenylbutoxy)hexylamino]ethyl]phenol). CAR (enantiopure, (*R*)-, (*S*)-, [3-(9*H*-carbazol-4-yl)oxy]-2-hydroxypropyl]-[2-(2-methoxyphenoxy)ethyl]amine) was obtained from Dr. Peter Gmeiner (University of Erlangen, Erlangen, Germany). Cyanopindolol (racemic, 4-[3-(tert-butylamino)-2-hydroxypropoxy]-1*H*-indole-2-carbonitrile) was obtained from Biotrend (Cologne, Germany). Racemic FEN [5-(1-hydroxy-2-[[2-(4-hydroxyphenyl)-1-methylethyl]amino]ethyl)benzene-1,3-diol] and derivatives [enantiopure, (*R,R'*)-, (*R,S'*)-, (*S,R'*)-, (*S,S'*)-] were obtained from SRI (Menlo Park, CA). The Bright-Glo luciferase reagent was obtained from Promega (Mannheim, Germany).

**Cells and Cultures.** Cells were maintained and used in the experiments as described previously (Takakura et al., 2012). HEK293 cells transfected with the  $\beta_2$ AR fused to one fragment of split luciferase derived from the click beetle *P. pyralis* and either  $\beta$ arr1 or  $\beta$ arr2 linked to a second fragment of split luciferase were grown in Dulbecco's modified Eagle's medium (high glucose, free of phenol red) containing 10% (v/v) fetal bovine serum, 1% (v/v) penicillin/streptomycin, 1% (v/v) L-glutamine, 0.8 mg/ml G418, and 0.04 mg/ml zeocin at 37°C in the presence of 5% (v/v) CO<sub>2</sub>.

**Arrestin Recruitment Experiments.** Experiments for the investigation of  $\beta$ arr1 or  $\beta$ arr2 recruitment to the  $\beta_2$ AR were conducted as described previously (Takakura et al., 2012). In brief, 24 hours prior to the experiments, 100,000 cells in 90  $\mu$ l of growth medium were seeded in 96-well microtiter plates suitable for cell culture and luminescence detection (Corning, Kaiserslautern, Germany). Cells were incubated in triplicate with 10  $\mu$ l of ligand for 10 minutes at 37°C in a 96-well microtiter plate reader (Synergy 4, BioTek, Winooski, VT). When antagonism was analyzed, another 10 minute incubation period with 10  $\mu$ l of antagonist solution was conducted prior to agonist incubation. Next, 50  $\mu$ l/well of medium was removed and 50  $\mu$ l/well of Bright-Glo luciferase reagent (Promega) was added. After shaking for 2 minutes, luminescence was measured at  $\lambda = 558$  nm for 2 seconds/well. Raw data were normalized to a solvent control (baseline signal) as well as to data obtained from incubation with 10  $\mu$ M (*R*)-ISO (maximal response of the system, 100%). The time courses of  $\beta$ arr1 or  $\beta$ arr2 recruitment for various selected ligands were similar (Supplemental Figs. 3 and 4). Hence, in the following experiments, to ensure high signal intensities and for practical reasons, all experiments were conducted for 10 minutes. Data were fitted, plotted, and statistically analyzed with Graph Pad Prism 5 (Graph Pad Software, La Jolla, CA). To further validate the results obtained using the previously described method, some  $\beta_2$ AR- $\beta$ arr2 recruitment experiments were replicated with the commercially available PathHunter system, PathHunter eXpress (DiscoverX, Fremont, CA) according to the manufacturer's instructions. Confluent Chinese hamster ovary cell layers in 96-well plates were inoculated with different concentrations of the ligands in duplicate for 90 minutes at 37°C. Reactions were stopped by the addition of the detection reagent. Luminescence was measured after 60 minute incubation at room temperature. Readings were normalized between the basal signal without ligand and maximal stimulation by 10  $\mu$ M (*R*)-ISO.

**Bias Quantification.** Bias quantification was performed according to van der Westhuizen et al. (2014). In brief, the transduction coefficients [log( $\tau/K_A$ )] were obtained by fitting the concentration-response

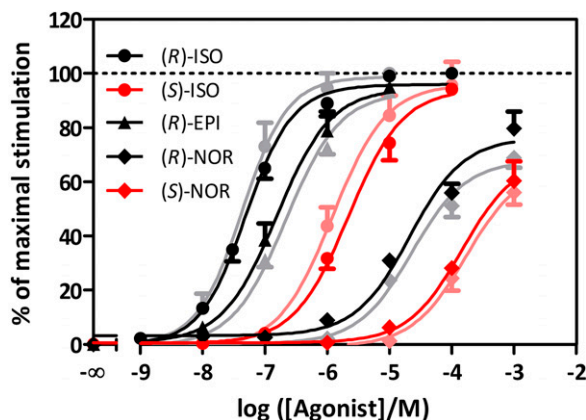
data to the operational model for agonism by Black and Leff (1983). For each ligand the transduction coefficients were compared by subtraction to the reference ligand (*R*)-EPI to correct for system bias [ $\Delta\log(\tau/K_A)$ ]. The comparison between two pathways was performed by subtracting the  $\Delta\log(\tau/K_A)$  values for each individual ligand for one pathway by those from another one. This subtraction yielded the  $\Delta\Delta\log(\tau/K_A)$  values, which represent the bias of a ligand toward a certain pathway. A bias was defined as significant if the 95% interval of confidence of the investigated ligand did not overlap that of the reference ligand (*R*)-EPI. Several ligands were defined as extremely biased because they were either giving no signal in one of the two compared pathways, or the signal in one of the two pathways was too low to get a robust fit with the operational model.

## Results

### Endogenous Catecholamines and Isoproterenol.

Representative concentration-response curves for  $\beta$ arr1 or  $\beta$ arr2 recruitment by agonists are shown in Fig. 2. (*R*)-Stereoisomers generally showed higher potencies compared with (*S*)-ISOs. Moreover, efficacies for (*R*)-stereoisomers were higher compared with (*S*)-isomers for most agonists (Table 1). (*S*)-ISO and (*R*)-ISO exhibited similar efficacies. With respect to biased recruitment of  $\beta$ arr1 or  $\beta$ arr2 subtypes, neither ISO nor endogenous catecholamines showed significant selectivity with respect to potency and efficacy. Comparison of the split-luciferase assay and the DiscoverX PathHunter assay revealed similar pharmacological properties of (*R*)-ISO and (*R*)-EPI in both assay systems (Supplemental Fig. 5). In contrast to the endogenous catecholamines, a series of antagonists showed no or only minimal stimulatory effects on  $\beta$ arr1 or  $\beta$ arr2 recruitment (Supplemental Figs. 4, 6, and 7), pointing to the specificity of the assay for agonists.

**Rapid-Acting  $\beta_2$ AR Agonists.** FEN possesses two stereocenters yielding four stereoisomers (Supplemental Fig. 1). The most potent and efficacious FEN isomer was (*R,R'*), followed by (*R,S'*), (*S,S'*), and (*S,R'*) (Supplemental Figs. 8 and 9; Table 1). The racemic mixture exhibited similar pharmacological properties as the (*R,R'*)-isomer. (*S,R'*)-FEN recruited  $\beta$ arr2 with higher potency and efficacy compared with  $\beta$ arr1, and (*R,R'*)-FEN induced  $\beta$ arr2 recruitment with higher efficacy. (*R*)-ALB showed higher potency and efficacy than



**Fig. 2.** Concentration-response curves for  $\beta_2$ AR agonists in relation to  $\beta$ arr recruitment. Concentration-response curves for pairs of enantiomers of ISO and norepinephrine (NOR), and (*R*)-EPI. Cells were treated as described in *Materials and Methods*. Curves and symbols for  $\beta$ arr1 are given in lighter colors than those for  $\beta$ arr2. ( $N \geq 3$ ; data  $\pm$  S.D.).

the racemic mixture and (*S*)-ALB. In-depth analysis of (*R*)-ALB confirmed the small bias toward  $\beta$ arr2 recruitment since the differences in potencies of  $\beta$ arr subtype recruitment were significant (Fig. 3).

**Long-Acting  $\beta_2$ AR Agonists.** Racemic SAL and (*R*)-SAL showed similar potencies and efficacies of about 15% for  $\beta$ arr2 and 10% for  $\beta$ arr1 normalized to (*R*)-ISO. Again, the (*S*)-isomer showed reduced potency and efficacy. In-depth analysis of (*S*)-SAL yielded significantly increased potency and efficacy for  $\beta$ arr2 compared with  $\beta$ arr1 recruitment (Fig. 3; Table 1). The racemic mixture of FOR and (*R,R*)-FOR exhibited similar pharmacological properties, whereas the concentration-response curve of (*S,S*)-FOR showed a right shift as well as a slight reduction in maximal response. All three substances showed higher efficacies for  $\beta$ arr2 recruitment. Olodaterol isomers showed no bias between  $\beta$ arr1 and  $\beta$ arr2.

**Derivatives of FEN.** 4'-Methoxy-desmethylfenoterol showed a similar pattern as the SAL and FOR isomers. (*S*)-isomer exhibited lower potency and efficacy than the (*R*)-isomer. Moreover, (*R*)-4'-methoxy-desmethylfenoterol showed higher potency at inducing recruitment of  $\beta$ arr2 and (*S*)-4'-methoxy-desmethylfenoterol showed higher efficacy at inducing recruitment of  $\beta$ arr2 compared with  $\beta$ arr1. In the case of 4'-methoxyfenoterol, potencies and efficacies increased depending on the number of chirality centers in the (*R*) configuration, attributing major importance the center closest to the catechol moiety. (*S,R'*)-Methoxyfenoterol and (*S,S'*)-4'-methoxyfenoterol were more effective in inducing  $\beta$ arr2 than  $\beta$ arr1 recruitment. Regarding 4'-methoxy-1-naphthylfenoterol (MNF), (*S,R'*)-MNF was the second efficacious isomer after (*R,R'*)-MNF. The lowest efficacy was again found for (*S,S'*)-ISO. (*R,S'*)-MNF, (*S,R'*)-MNF, and (*S,S'*)-MNF preferred  $\beta$ arr2 over  $\beta$ arr1 recruitment. (*R,R'*)-MNF preferred  $\beta$ arr2 concerning potency. Interestingly, (*R,S'*)-MNF and (*S,S'*)-MNF showed slight inverse agonism concerning  $\beta$ arr1 recruitment. The (*R,R'*) configuration of 4'-methoxy-ethylfenoterol showed considerably higher potency and efficacy relative to (*R,S'*)-4'-methoxy-ethylfenoterol regardless of the  $\beta$ arr subtype. (*R,S'*)-4'-Methoxy-ethylfenoterol effected weak  $\beta$ arr2 recruitment and was incapable of recruiting  $\beta$ arr1. In the case of 4'-methoxy-*n*-propylfenoterol, the (*R,R'*) and (*R,S'*) configurations both revealed a bias toward  $\beta$ arr2. No activity was detected for the induction of  $\beta$ arr1 recruitment by (*R,S'*)-4'-methoxy-*n*-propylfenoterol. In contrast to all other FEN derivatives yielding higher potencies and efficacies for the (*R,R'*) configuration, (*R,R'*)-4'-methoxy-*i*-propylfenoterol did not induce recruitment of either of the analyzed  $\beta$ arr subtypes. For this compound the only effect that could be detected was  $\beta$ arr2 recruitment by (*R,S'*)-4'-methoxy-*i*-propylfenoterol.

**Other Ligands.** While racemic zinterol showed no bias toward a given arrestin isoform, racemic BRL 37344 was only capable of inducing  $\beta$ arr2 recruitment.

**Antagonist Effects.** As exemplarily shown in Supplemental Fig. 10 for (*R*)-CAR and (*S*)-CAR,  $\beta$ -adrenergic antagonists inhibited signals induced by (*R*)-ISO. However, when  $\beta$ -adrenergic antagonists and partial agonists were examined in the absence of ISO at a fixed concentration of 10  $\mu$ M, only one ligand was found to significantly influence  $\beta$ arr recruitment. Specifically, the  $\beta_3$ AR agonist ICI 215,001 showed very weak but significant inverse agonism concerning  $\beta$ arr1 recruitment (Fig. 4; Table 2). Even when tested at different time points and at a broad range of concentrations, antagonists were virtually

TABLE 1

Potencies and efficacies of agonists at inducing  $\beta$ arr1 or  $\beta$ arr2 recruitment

When concentration-response curves did not reach saturation, the activation at the highest measured concentration is given as  $E_{\max}$ . To analyze the data for preferred activation of  $\beta$ arr1 versus  $\beta$ arr2, two-way analysis of variance and the Bonferroni post-test were performed. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ ; data  $\pm$  S.D.;  $N \geq 4$ .

Ligand	Stereo-Configuration	pEC <sub>50</sub> $\beta$ arr1	pEC <sub>50</sub> $\beta$ arr2	Statistical Significance	$E_{\max}$ $\beta$ arr1	$E_{\max}$ $\beta$ arr2	Statistical Significance
					%	%	
ISO	(R)	7.38 $\pm$ 0.05	7.24 $\pm$ 0.03	ns	100	100	ns
	(S)	5.92 $\pm$ 0.07	5.66 $\pm$ 0.05	*	96.07 $\pm$ 2.54	94.22 $\pm$ 1.96	ns
EPI	(R)	6.66 $\pm$ 0.05	6.81 $\pm$ 0.05	ns	93.10 $\pm$ 1.78	94.42 $\pm$ 1.82	ns
NOR	(R)	4.64 $\pm$ 0.08	4.68 $\pm$ 0.10	ns	67.79 $\pm$ 2.08	76.20 $\pm$ 3.13	***
	(S)	No saturation	No saturation		56.01 $\pm$ 4.35	60.36 $\pm$ 7.21	*
DCI	Racemic	No signal	No signal		No signal	No signal	
ALB	Racemic	6.32 $\pm$ 0.05	6.71 $\pm$ 0.07	***	16.58 $\pm$ 0.42	19.79 $\pm$ 0.51	ns
	(R)	6.55 $\pm$ 0.05	6.82 $\pm$ 0.09	***	19.59 $\pm$ 3.32	19.82 $\pm$ 1.92	ns
	(S)	4.77 $\pm$ 0.34	4.65 $\pm$ 0.10	ns	0.36 $\pm$ 0.05	3.45 $\pm$ 0.13	ns
FEN	Racemic	7.02 $\pm$ 0.06	7.04 $\pm$ 0.06	ns	63.94 $\pm$ 1.45	61.48 $\pm$ 1.51	ns
	(R,R')	7.28 $\pm$ 0.07	7.20 $\pm$ 0.06	ns	70.56 $\pm$ 1.63	64.69 $\pm$ 1.48	***
	(R,S')	5.78 $\pm$ 0.09	5.85 $\pm$ 0.05	ns	31.69 $\pm$ 1.17	29.27 $\pm$ 0.61	ns
	(S,R')	4.48 $\pm$ 0.07	4.76 $\pm$ 0.07	***	11.20 $\pm$ 0.34	17.32 $\pm$ 0.48	***
	(S,S')	4.49 $\pm$ 0.09	4.62 $\pm$ 0.06	ns	25.69 $\pm$ 1.02	28.71 $\pm$ 0.79	ns
SAL	Racemic	7.76 $\pm$ 0.09	7.83 $\pm$ 0.08	ns	10.35 $\pm$ 0.39	14.69 $\pm$ 0.46	**
	(R)	7.44 $\pm$ 0.07	7.42 $\pm$ 0.11	ns	10.90 $\pm$ 0.36	14.26 $\pm$ 0.69	ns
	(S)	6.79 $\pm$ 0.14	6.99 $\pm$ 0.11	***	3.90 $\pm$ 1.24	7.39 $\pm$ 1.88	***
FOR	Racemic	7.69 $\pm$ 0.06	7.63 $\pm$ 0.05	ns	76.80 $\pm$ 1.77	84.86 $\pm$ 1.78	***
	(R,R')	7.95 $\pm$ 0.03	8.03 $\pm$ 0.08	ns	73.29 $\pm$ 0.96	80.33 $\pm$ 2.45	***
	(S,S')	5.91 $\pm$ 0.07	5.82 $\pm$ 0.07	ns	65.13 $\pm$ 1.79	73.12 $\pm$ 2.03	***
OLO	(R)	8.11 $\pm$ 0.04	8.11 $\pm$ 0.08	ns	34.43 $\pm$ 0.58	34.49 $\pm$ 1.11	ns
	(S)	6.06 $\pm$ 0.07	6.00 $\pm$ 0.08	ns	31.15 $\pm$ 0.81	32.11 $\pm$ 1.08	ns
MDF	(R)	5.47 $\pm$ 0.08	5.89 $\pm$ 0.10	***	26.51 $\pm$ 0.94	26.35 $\pm$ 1.02	ns
MEF	(S)	No saturation	No saturation		7.02 $\pm$ 0.23	14.52 $\pm$ 2.00	***
	(R,R')	7.00 $\pm$ 0.05	7.10 $\pm$ 0.05	ns	63.14 $\pm$ 1.31	61.18 $\pm$ 1.23	ns
MEtF	(R,S')	6.03 $\pm$ 0.07	5.91 $\pm$ 0.06	ns	39.45 $\pm$ 1.02	42.48 $\pm$ 0.97	ns
	(S,R')	No saturation	4.92 $\pm$ 0.07		3.17 $\pm$ 0.53	10.09 $\pm$ 0.32	***
	(S,S')	No saturation	4.76 $\pm$ 0.08		3.64 $\pm$ 1.25	9.43 $\pm$ 0.39	***
	(R,R')	6.36 $\pm$ 0.08	6.47 $\pm$ 0.05	ns	47.24 $\pm$ 1.67	40.37 $\pm$ 0.83	***
MnF	(R,S')	No saturation	No saturation		-0.04 $\pm$ 0.14	2.69 $\pm$ 0.21	ns
	(R,R')	No saturation	No saturation		5.75 $\pm$ 1.34	12.83 $\pm$ 0.98	***
MiF	(R,S')	No saturation	No saturation		-0.14 $\pm$ 0.13	3.15 $\pm$ 0.36	ns
	(R,R')	No signal	No signal		No signal	No signal	
MNF	(R,S')	No saturation	No saturation		-0.01 $\pm$ 0.19	3.61 $\pm$ 0.26	ns
	(R,R')	6.6 $\pm$ 0.12	6.84 $\pm$ 0.06	**	34.04 $\pm$ 1.67	35.96 $\pm$ 0.88	ns
	(R,S')	No saturation	6.39 $\pm$ 0.11		0.07 $\pm$ 0.17	2.17 $\pm$ 0.11	ns
	(S,R')	No saturation	5.71 $\pm$ 0.07		9.42 $\pm$ 1.47	17.15 $\pm$ 0.47	***
ZIN	(S,S')	No saturation	6.02 $\pm$ 0.39		-0.06 $\pm$ 0.11	0.75 $\pm$ 0.16	ns
	Racemic	7.62 $\pm$ 0.10	7.65 $\pm$ 0.07	ns	26.38 $\pm$ 1.07	28.22 $\pm$ 0.77	ns
BRL	Racemic	No saturation	6.26 $\pm$ 0.19		0.02 $\pm$ 0.23	1.52 $\pm$ 0.13	ns

BRL, BRL 37344; DCI, sodium dichloroisoproterenol; MDF, 4'-Methoxy-desmethylfenoterol; MEF, 4'-methoxyfenoterol; MEtF, 4'-methoxy-ethylfenoterol; MiF, 4'-methoxy-*i*-propylfenoterol; MnF, 4'-methoxy-*n*-propylfenoterol; NOR, norepinephrine; ns, not significant; OLO, olodaterol; ZIN, zinterol.

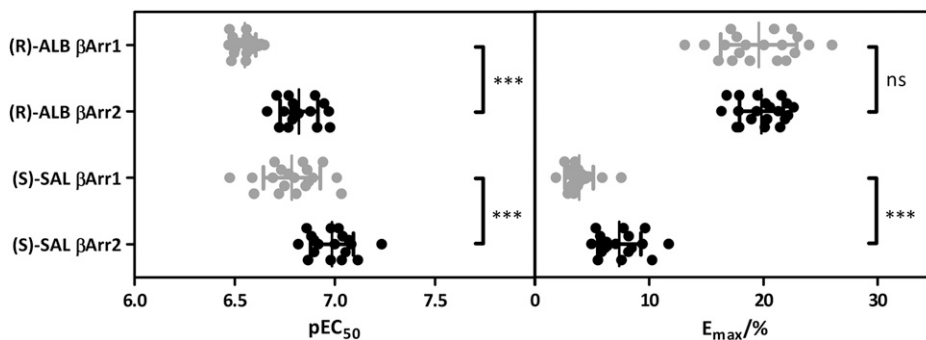
devoid of stimulatory effects on  $\beta$ arr recruitment (Supplemental Figs. 4, 6, and 7).

**GTPase Data.** For comparison with  $\beta$ arr recruitment, we also examined activation of  $G_{\alpha s}$  by selected ligands. These data are listed in Supplemental Tables 1 and 2. In general, agonists were more potent and efficacious at stimulating  $G_{\alpha s}$  than at recruiting  $\beta$ arr. The corresponding

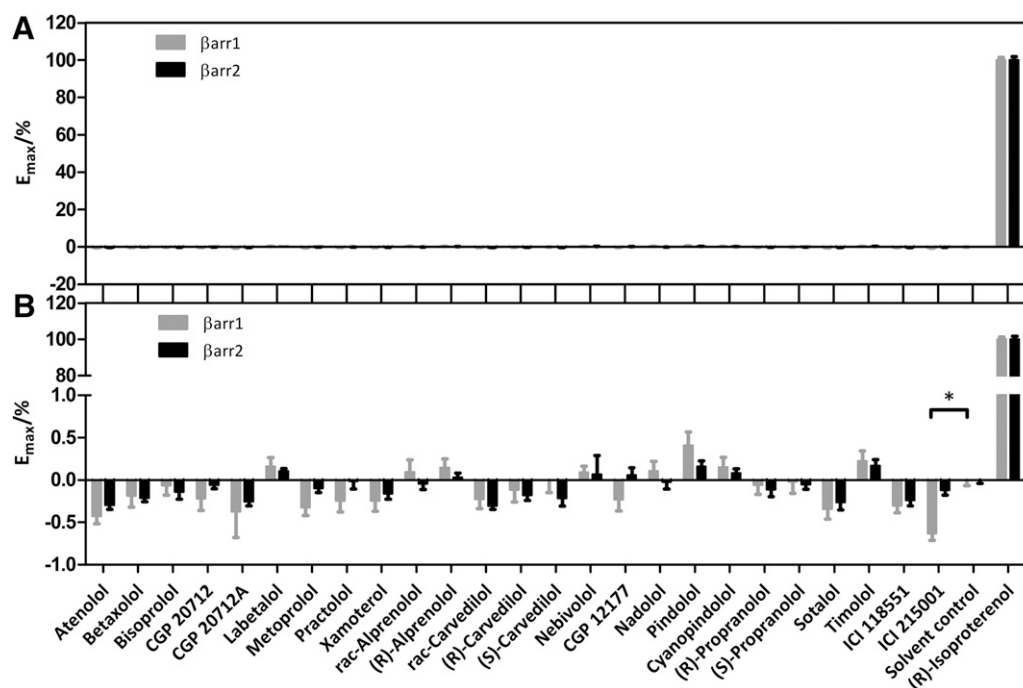
bias analyses are shown in Supplemental Tables 3 and 4 and Fig. 5.

## Discussion

**$\beta$ arr Recruitment by  $\beta_2$ AR ligands: Comparison with Literature Data.** Potency and efficacy data differ depending



**Fig. 3.** Scatter-plot of pEC<sub>50</sub> and  $E_{\max}$  values for (R)-ALB and (S)-SAL. Cells were treated as described in *Materials and Methods*. While (S)-SAL exhibited significantly different pEC<sub>50</sub> and  $E_{\max}$  values comparing  $\beta$ arr1 (gray) and  $\beta$ arr2 (black), (R)-ALB only showed significant differences with regard to potency. Detailed values can be found in Table 1. ( $N \geq 18$ ; data  $\pm$  S.D.; unpaired *t* test with two-tailed *P* values; ns, not significant; \*\*\* $P < 0.001$ ).



**Fig. 4.** Effect of antagonists (10  $\mu$ M each) on the recruitment of  $\beta$ arr. Cells were treated as described in *Materials and Methods*. Each analyzed ligand was examined for the induction of  $\beta$ arr1 (gray) and  $\beta$ arr2 (black) recruitment. Unless otherwise noted, racemic mixtures were analyzed. (A) Ligands are presented with an uninterrupted y-axis to show relative differences from the reference ligand (*R*)-ISO. (B) The y-axis is interrupted to show ligand effects in detail. Statistical analysis of antagonist effects compared with solvent control yielded no significance except for the  $\beta_3$ AR agonist ICI 215,001, which is a very weak inverse agonist concerning  $\beta$ arr2 recruitment. ( $N = 4$ ; data  $\pm$  S.D.; two-way analysis of variance with Bonferroni post-test;  $*P < 0.05$ ).

on the experimental readout system as well as the cellular system used (Supplemental Table 5). Strikingly, values determined in the Tango assay systemically showed higher potencies but lower efficacies, which may be associated with the normalization of ligand parameters to those of the partial

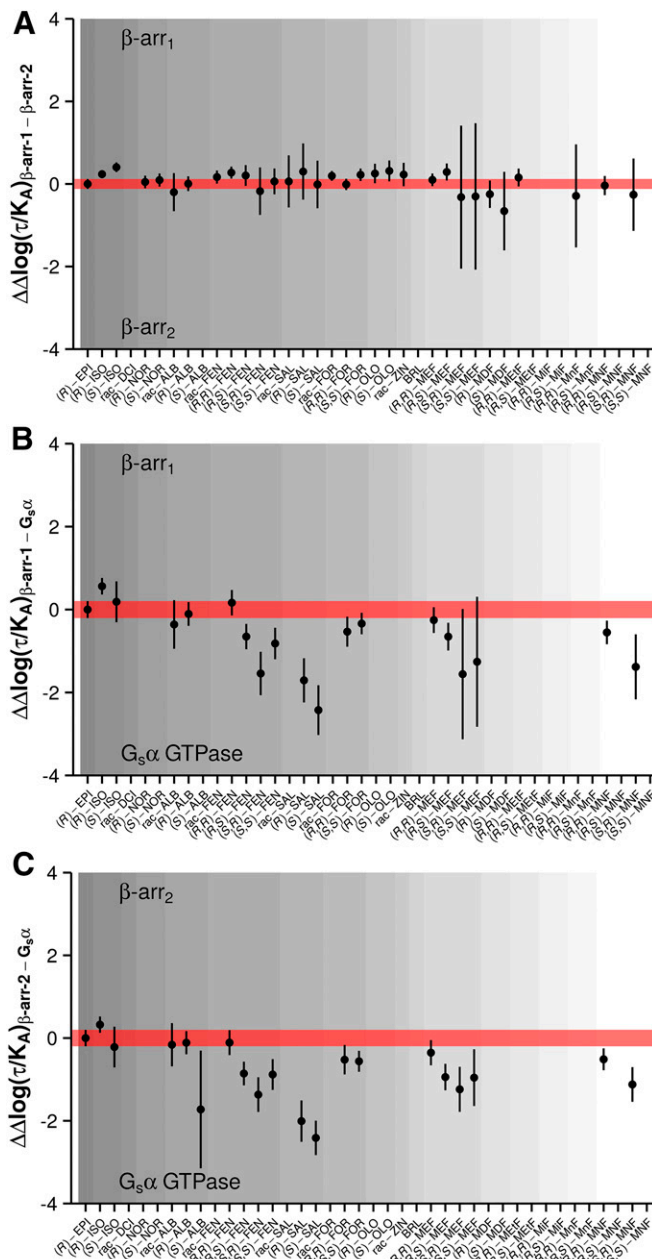
agonist FOR instead of the endogenous ligand EPI or its substitute ISO (Rajagopal et al., 2011). Considering results from fluorescent protein-distribution assays, differences in comparison with results from this study were rather small and not as systemic (Oakley et al., 2002; Reiner et al., 2010).

TABLE 2

Stimulation of  $\beta$ arr1 or  $\beta$ arr2 recruitment by antagonists and partial agonists analyzed in the split-luciferase assay

Ligand activities were normalized to (*R*)-ISO (100%), and statistical significance was determined compared with values from the solvent control (two-way analysis of variance with the Bonferroni post-test).

Ligand	Stimulation $\beta$ arr1	Stimulation $\beta$ arr2	Significance $\beta$ arr1	Significance $\beta$ arr2
	%	%		
( <i>R</i> )-ISO	100.0	100.0	$P < 0.001$	$P < 0.001$
Timolol	0.22	0.17	$P > 0.05$	$P > 0.05$
Nadolol	0.10	-0.02	$P > 0.05$	$P > 0.05$
Labetalol	0.16	0.10	$P > 0.05$	$P > 0.05$
Metoprolol	-0.33	-0.10	$P > 0.05$	$P > 0.05$
Nebivolol	0.09	0.06	$P > 0.05$	$P > 0.05$
Alprenolol	0.09	-0.04	$P > 0.05$	$P > 0.05$
Atenolol	-0.43	-0.30	$P > 0.05$	$P > 0.05$
Sotalol	-0.34	-0.26	$P > 0.05$	$P > 0.05$
Practolol	-0.25	-0.01	$P > 0.05$	$P > 0.05$
Bisoprolol	-0.07	-0.14	$P > 0.05$	$P > 0.05$
Xamoterol	-0.25	-0.16	$P > 0.05$	$P > 0.05$
Betaxolol	-0.19	-0.21	$P > 0.05$	$P > 0.05$
( <i>R</i> )-Alprenolol	0.14	0.03	$P > 0.05$	$P > 0.05$
( <i>R</i> )-Propranolol	-0.06	-0.11	$P > 0.05$	$P > 0.05$
( <i>S</i> )-Propranolol	-0.02	-0.05	$P > 0.05$	$P > 0.05$
CGP 20712A	-0.37	-0.25	$P > 0.05$	$P > 0.05$
CGP 20712	-0.22	-0.06	$P > 0.05$	$P > 0.05$
Pindolol	0.40	0.16	$P > 0.05$	$P > 0.05$
Cyanopindolol	0.15	0.08	$P > 0.05$	$P > 0.05$
Rac-CAR	-0.22	-0.31	$P > 0.05$	$P > 0.05$
( <i>R</i> )-CAR	-0.12	-0.18	$P > 0.05$	$P > 0.05$
( <i>S</i> )-CAR	-0.001	-0.22	$P > 0.05$	$P > 0.05$
ICI 118,551	-0.30	-0.24	$P > 0.05$	$P > 0.05$
CGP 12177	-0.23	0.06	$P > 0.05$	$P > 0.05$
ICI 215,001	-0.63	-0.12	$P < 0.05$	$P > 0.05$



**Fig. 5.** The  $\Delta\Delta\log(\tau/K_A)$  values for three different pathways. The  $\Delta\Delta\log(\tau/K_A)$  values were obtained by subtracting the system bias-corrected  $\Delta\log(\tau/K_A)$  values for each ligand for one pathway from those of another pathway. Shown are the values with their corresponding 95% confidence interval (CI). The 95% CI of the reference ligand (*R*)-EPI is shown as a red bar. A ligand is defined as biased if its 95% CI does not overlap with the one from the reference.

These differences may result from the rather challenging quantification of changes in fluorescence distribution within cells. Data from  $\beta$ -galactosidase complementation assays yielded different ligand parameters depending on the cell type used. The results obtained by Kopra et al. (2013) using Chinese hamster ovary cells are in good agreement with the results from this study, whereas Yaffe and Saxel (1977) and Carter and Hill (2005) obtained higher efficacies on myocytic, polynuclear C2C12 cells. It is difficult to provide a definitive explanation for these differences.

**Partial Agonism.** Many agonists analyzed in this study are full agonists with respect to G-protein activation and cAMP production (Toll et al., 2011; Brunskole Hummel et al., 2013);

however, they turned out to be only (weak) partial agonists regarding  $\beta$ arr recruitment, indicating a strong bias toward  $G_{\alpha_s}$ -mediated cAMP formation. Compounds showing low efficacies for  $\beta$ arr recruitment were ALB, FOR, SAL, zinterol, and in particular the FEN derivatives (Baker, 2010; Brunskole Hummel et al., 2013; Plazinska et al., 2014). Reduced efficacies may result from modification of the two hydroxyl groups in the *m* and *p* positions of the catechol moiety of the endogenous agonists and ISO. The replacement of these hydroxyl groups by chlorine moieties in sodium dichloroisocyanurate and BRL 37344 is associated with the complete loss of  $\beta$ arr recruitment. In addition, secondary amines appeared to effect full activation of the system, while the primary amine norepinephrine only exhibits an  $E_{\max}$  value of about 80%.

**Signaling Bias.**  $\beta$ arr1 and  $\beta$ arr2 function in similar ways within the cell, for example, in the case of knockout of one subtype, the other  $\beta$ arr isoform can rescue the phenotype nearly completely (Conner et al., 1997; Bohn et al., 2002). Moreover,  $\beta_2$ AR exhibits higher affinities for  $\beta$ arr2 than for  $\beta$ arr1 (Oakley et al., 2000). Few ligands exhibited biased  $\beta$ arr signaling (Fig. 5), and if so,  $\beta$ arr2 was recruited more effectively and potently than  $\beta$ arr1 (Table 1).

**Stereochemistry.** (*R*)-Configured agonists activated  $\beta$ arr more effectively than (*S*)-stereoisomers. Presumably, the stereocenter next to the catechol moiety significantly contributes to receptor activation by the formation of specific hydrogen bonds. MNF is an exception to the rule because only a very small signal could be detected for the (*R,S'*)-enantiomer, which may be a result from the size of the naphthyl moiety and the resulting steric hindrance. No distomer was more potent and effective at inducing  $\beta$ arr recruitment than its eutomer, which could have explained some of the reported paradoxical proinflammatory adverse effects of clinically administered racemic drugs (Templeton et al., 1998; Volcheck et al., 2005; Patel and Thomson, 2012). Further research that focuses on downstream signaling is required to determine whether the two different subtypes of  $\beta$ arr differ in their ability to activate signaling pathways within cells apart from receptor internalization.

**Antagonists.** Several groups have reported  $\beta$ arr recruitment as a result from antagonist binding to G-protein-coupled receptors. Antagonists induced receptor internalization and G-protein-independent (but  $\beta$ arr-dependent) activation of the mitogen-activated protein kinase ERK1/2 (Wisler et al., 2007; Erickson et al., 2013). Normalized to the effect of 10  $\mu$ M (*R*)-ISO, CAR induced about 14% of receptor internalization and generated about 40% of ERK1/2 activation, which was reduced to about 15% by knocking down  $\beta$ arr2 with siRNA (Wisler et al., 2007). Similar characteristics have also been described for the  $\beta$ AR antagonist nebivolol (Erickson et al., 2013). In contrast, the analysis of antagonists with the split-luciferase assay revealed no significant effects on the recruitment of  $\beta$ arr, although each ligand was used at a concentration of 10  $\mu$ M (Fig. 4). Several explanations are possible. First, experiments by Wisler et al. (2007) were performed with  $\beta_2$ AR/vasopressin V2 receptor chimeras, which may have altered receptor characteristics. Second, CAR effects may also result from antagonizing  $\alpha_1$ ARs and not only  $\beta$ ARs (Pedersen and Cockcroft, 2007). Third, the high concentrations of antagonists used may have caused off-target effects (Hagelüken et al., 1994). In conclusion,  $\beta$ AR agonists show strong bias for  $G_{\alpha_s}$  activation relative to  $\beta$ arr activation, agonists activate  $\beta$ arr1 and  $\beta$ arr2 signaling with subtle differences, and there is no evidence for  $\beta$ arr recruitment by  $\beta$ AR antagonists.

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## Authorship Contributions

*Participated in research design:* Littmann, Göttle, Seifert.  
*Conducted experiments:* Littmann, Göttle, Reinartz, Kälble.  
*Contributed new reagents or analytic tools:* Wainer, Ozawa.  
*Performed data analysis:* Littmann, Göttle, Reinartz, Seifert.  
*Wrote or contributed the writing of the manuscript:* Göttle, Littmann, Seifert.

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**Address correspondence to:** Dr. Roland Seifert, Institute of Pharmacology, Hannover Medical School, Carl-Neuberg-Strasse 1, D-30625 Hannover, Germany. E-mail: seifert.roland@mh-hannover.de