Stereochemistry of an Agonist Determines Coupling Preference of β2-Adrenoceptor to Different G Proteins in Cardiomyocytes

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ABSTRACT

A fundamental question regarding receptor-G protein interaction is whether different agonists can lead a receptor to different intracellular signaling pathways. Our previous studies have demonstrated that although most β2-adrenoceptor agonists activate both Gα and Gβγ proteins, fenoterol, a full agonist of β2-adrenoceptor, selectively activates Gα protein. Fenoterol contains two chiral centers and may exist as four stereoisomers. We have synthesized a series of stereoisomers of fenoterol and its derivatives and characterized their receptor binding and pharmacological properties. We tested the hypothesis that the stereochemistry of an agonist determines selectivity of receptor coupling to different G protein(s). We found that the R,R isomers of fenoterol and methoxyfenoterol exhibited more potent effects to increase cardiomyocyte contraction than their S,R isomers. It is noteworthy that although (R,R)-fenoterol and (R,R)-methoxyfenoterol preferentially activate Gα signaling, their S,R isomers were able to activate both Gα and Gβγ proteins as evidenced by the robust pertussis toxin sensitivities of their effects on cardiomyocyte contraction and on phosphorylation of extracellular signal-regulated kinase 1/2. The differential G protein selectivities of the fenoterol stereoisomers were further confirmed by photoaffinity labeling studies on Gαs, Gαq, and Gα13 proteins. The inefficient Gα signaling with the R,R isomers is not caused by the inability of the R,R isomers to trigger the protein kinase A (PKA)-mediated phosphorylation of the β2-adrenoceptor, because the R,R isomers also markedly increased phosphorylation of the receptor at serine 262 by PKA. We conclude that in addition to receptor subtype and phosphorylation status, the stereochemistry of a given agonist plays an important role in determining receptor-G protein selectivity and downstream signaling events.

Differential activation of receptors to specific signaling pathways has evolved to be a paradigm in pharmacological theory that can be translated into clinical relevance (Kenakin, 2004, 2007; Mailman, 2007; Urban et al., 2007; Violin and Lefkowitz, 2007). As an archetypical member of the G protein-coupled receptor (GPCR) superfamily, the β2-adrenergic receptor (β2-AR) couples dually to Gα and Gβγ proteins, resulting in opposing effects on cardiac myocyte contractility and viability (Xiao et al., 1995, 1999; Zhu et al., 2001). In congestive heart failure, impaired β-AR response is often associated with increased Gα signaling (Feldman et al., 1988; Bohm et al., 1994) and selective down-regulation of β1-AR (higher β2/β1 ratio) (Bristow et al., 1986, 1993). Previous studies have demonstrated that disrupting Gα signaling with pertussis toxin (PTX) restores the markedly depressed β2-AR contractile response in two rat heart failure models, and that a full β2-AR agonist, fenoterol, which selectively activates β2-AR-coupled Gα signaling, reverses the diminished β2-AR inotropic effect in myocytes from failing spontaneously hypertensive rat hearts in the absence of PTX (Xiao et al., 2003). In vivo studies have further demonstrated that prolonged use of fenoterol not only improves cardiac function but also retards cardiac maladaptive remodeling, and that the overall beneficial effects of fenoterol with β1-AR blockade are greater than the salutary effects of β1-AR blockade alone in a rat chronic heart failure model-induced by myocardial infarction (Ahmet et al., 2004, 2005, 2008). These studies suggest that selective activation of the β2-AR-coupled Gα signaling may provide a useful therapeutic target for the treatment of congestive heart failure. Although the pharmaceutical preparation of fenoterol is a

ABBREVIATIONS: GPCR, G protein-coupled receptor; β2-AR, β2-adrenoceptor; ERK, extracellular signal-regulated kinase; HEK, human embryonic kidney; ISO, isoproterenol; PKA, protein kinase A; PTX, pertussis toxin; ICI 118,551, (+)-1-[2,3-(dihydro-7-methyl-1H-inden-4-yloxy)-3-[(1-methylethyl)amino]-2-butanol.

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racemic mixture of its \( R,R \) and \( S,S \) enantiomers (rac-fenoterol), our recent studies have shown that the \( R,R \) enantiomer is the only active isof orm in receptor binding and cardiom yocyte contraction assays (Beigi et al., 2006; Jozwiak et al., 2007). A cohort of fenoterol derivatives including the \( R,R, R,S, S,R \), and \( S,S \) isomers of fenoterol were synthesized (Beigi et al., 2006; Jozwiak et al., 2007). Using some of these compounds, we attempted to examine the hypothesis that the stereochemistry of an agonist determines functional selectivity of a given receptor coupling to different G protein(s) and resultant activation of subset(s) of downstream signaling pathways.

Materials and Methods

Compounds and Reagents. A series of stereoisomers and derivatives of fenoterol, including the \( R,R \), \( R,S \), \( S,R \), and \( S,S \) isomers of fenoterol and methoxyfenoterol (see Fig. 1 for structures), were synthesized into enantiomeric purity. The detailed procedures of the chemical synthesis and the receptor binding affinities of the compounds have been reported previously (Beigi et al., 2006; Jozwiak et al., 2007). Zinterol was kindly supplied by Bristol-Myers (Evansville, IN). ICI 118,551, \((-\text{-isoproterenol (ISO), PTX, and other reagents were}

Cardiomyocyte Isolation, Cell Culture, and Adenoviral Infection. Cardiac myocytes were isolated from 2- to 4-month-old male Sprague-Dawley rats using a standard enzymatic technique and infected.

Fig. 1. Chemical structures of fenoterol and methoxyfenoterol. A, \((R,R)\)-fenoterol. B, \((S,R)\)-methoxyfenoterol. Chiral centers are indicated with asterisks.

Results

In our previous studies, we characterized the binding affinities to \( \beta \)-ARs for the 26 stereospecific fenoterol deriva-
The binding affinities reported previously to \( \beta_1 \)- and \( \beta_2 \)-AR for the \( R,R \) and \( S,R \) isomers of fenoterol and methoxyfenoterol are shown in Supplementary Table 1. Here, the specificity of these compounds was further assessed pharmacologically using cardiomyocyte contractility. Preliminary experiments have shown that near maximal contractile response could be achieved by the \( R,R \) isomers at 0.5 \( \mu \)M and by the \( S,R \) isomers at 10 \( \mu \)M. The selectivity of these compounds toward \( \beta_2 \)-AR was assessed by the inhibitory effect of ICI 118,551 (10\(^{-7}\) M). As shown in Fig. 2, all of these compounds could produce contractile responses that were blocked by ICI 118,551, suggesting that they are selective to \( \beta_2 \)-AR. These results are consistent with the binding affinity data.

The full concentration-response profiles for the fenoterol compounds stimulated contraction with or without PTX treatment are shown in Fig. 3. All four stereoisomers of fenoterol and methoxyfenoterol caused a concentration-dependent increase in the contraction amplitude of adult rat cardiomyocytes with similar maximal responses (~3-fold increase in cell contractility) (Fig. 3), whereas the \( S,S \) isomers had no detectable effect (data not shown), consistent with our previous report (Beigi et al., 2006). Disruption of \( G_s \) signaling by PTX had only minor effects on the contractility profiles of the \( R,R \) isomers (Fig. 3, A and B), as indicated by the insignificant changes in the EC\(_{50} \) values (in terms of log [M]: \(-7.11 \pm 0.17 \) for the control versus \(-7.44 \pm 0.07 \) for the PTX-treated group in \( R,R \)-fenoterol, \( P = 0.085 \); and \(-6.76 \pm 0.15 \) versus \(-6.84 \pm 0.10 \) in \( R,R \)-methoxyfenoterol, \( P = 0.579 \)). On the contrary, PTX treatment caused a clear leftward shift of the concentration-response curves of the \( S,R \) isomers (Fig. 3, C and D) and significantly decreased the EC\(_{50} \) values (from \(-5.63 \pm 0.21 \) to \(-6.08 \pm 0.03 \) for \( S,R \)-fenoterol, \( P < 0.01 \); and from \(-5.50 \pm 0.03 \) to \(-5.96 \pm 0.09 \) for \( S,R \)-methoxyfenoterol, \( P < 0.05 \)). These results suggest that the \( R,R \) isomers evoke \( G_s \)-selective \( \beta_2 \)-AR signaling, whereas the \( S,R \) isomers allow the receptor to activate both \( G_s \) and \( G_i \) pathways.

In HEK293 cells, treatment with agonists triggers the phosphorylation of ERK1/2, which peaks at 5 min (Daaka et al., 1997). Based on the concentration-response relationships obtained above for fenoterol and its derivatives to induce cardiomyocyte contraction, preliminary experiments were conducted to determine the concentrations of the compounds to be used for agonist-induced ERK1/2 activation in HEK293 cells. We found that treatment of cells with a nonselective \( \beta \)-AR agonist, ISO (10\(^{-6}\) M), resulted in a 5-fold increase in phosphorylation of ERK1/2 over the control and that treatment of cells with PTX reduced ERK1/2 activation to approximately 2-fold of the control level (Fig. 4, A and C), which is similar to the previous notion (Daaka et al., 1997). It is interesting that the \( S,R \) compounds at 10\(^{-6}\) M, a concentration with minimal effects on myocyte contraction (Fig. 3, C and D), were able to induce a full activation of ERK1/2, an effect comparable with that induced by ISO treatment (Fig. 4, C and D) and also completely inhabitable by ICI 118,551 (data not shown). The \( R,R \) isomers at this concentration (10\(^{-6}\) M) also increased ERK1/2 activation to a similar extent (Fig. 4, C and D). It is noteworthy that treatment of cells with PTX largely abrogated ERK1/2 activation induced by the \( S,R \) isomers but had minimal effects on ERK1/2 activation induced by the \( R,R \) isomers (Fig. 4).
The lack of PTX-sensitivity in the myocyte contractile responses and in ERK1/2 activation in cells stimulated with (R,R)-fenoterol and (R,R)-methoxyfenoterol suggests that the (R,R) isomers selectively stimulate β2-AR-coupled Gs signaling. To directly assay G protein activation, we measured photoaffinity labeling of the α subunits of G proteins with

Fig. 3. Effect of PTX treatment on the contractile responses to the fenoterol compounds. Concentration-response profiles of cardiomyocyte contractility subjected to (R,R)-fenoterol (A), (R,R)-methoxyfenoterol (B), (S,R)-fenoterol (C), and (S,R)-methoxyfenoterol (D) with and without PTX treatment. Contractile response to the agonist is expressed as a percentage of the basal contractility (n = 9–14 cells from 7–9 hearts for each data point). Basal contraction amplitude is 5.46 ± 0.14% (n = 138 cells). PTX did not alter basal contraction (5.33 ± 0.14%, n = 130 cells).

Fig. 4. Chirality determines the PTX sensitivities of fenoterol derivatives in activating ERK1/2. HEK293 cells were grown to confluence in growth medium in six-well plates before deprivation of the serum for 7 h. Treatment with PTX (0.5 μg/ml) was implemented during serum-starvation. The cultured cells were then stimulated with agonists for 5 min followed by cell lysis. After adjusting the protein concentration of the resultant cell lysates, the extent of ERK1/2 phosphorylation was analyzed by Western blotting. A and B, representative Western blots of phospho-ERK1/2 and total ERK1/2 (as protein loading control) after stimulation with ISO (10⁻⁶ M) or the fenoterol compounds (10⁻⁶ M) with or without PTX as indicated. C and D, averaged data from three to four independent experiments. The data are presented as the fold increase over the −PTX control. #, P < 0.01 compared with the −PTX control; +, P < 0.05; ++, P < 0.01 compared with the −PTX group in the same agonist treatment group.
the photoreactive GTP analog \([\gamma^{32}\text{P}]{\text{GTP}}-\text{azidoanilide}\) in response to the fenoterol derivatives. Subsequent immunoprecipitation with specific antisera was carried out to determine the amount of the activated G protein(s). The top of Fig. 5A shows that at the same concentration (10^{-6} M), fenoterol compounds and the nonspecific \(\beta\)-AR agonist ISO increased the incorporation of \([\gamma^{32}\text{P}]{\text{GTP}}-\text{azidoanilide}\) in G\(_s\). Both the short and long isoforms of G\(_s\) (which have approximate molecular masses of 45 and 47 kDa, respectively) are activated similarly in response to the stimuli. Moreover, the G\(_s\) labeling induced by (R,R)-fenoterol is significantly greater than that induced by (S,R)-fenoterol (\(P < 0.05\), Fig. 5B). As a positive control, zinterol (10^{-5} M), a selective \(\beta\)-AR partial agonist, was able to activate both G\(_{i2}\) and G\(_{i3}\) (Fig. 5A, bottom), consistent with our previous notion (Xiao et al., 1999). It is interesting that the fenoterol compounds (10^{-6} M) exhibited diverse effects on G\(_i\) proteins (Fig. 5A, bottom). In particular, activation of G\(_{i2}\), the predominant G\(_i\) protein in the heart, was observed only if \(\beta\)-AR was stimulated with (S,R)-fenoterol, but not by (R,R)-fenoterol, (R,R)-methoxyfenoterol, or (S,R)-methoxyfenoterol (Fig. 5C). The activation effects of (S,R)-fenoterol and (R,R)-fenoterol on G\(_{i2}\) are also statistically different (\(P < 0.01\); Fig. 5C). In addition, activation of G\(_{i3}\) in response to S,R isomers was significantly greater than that induced by R,R isomers of the fenoterol derivatives (Fig. 5D; \(P < 0.01\) for fenoterol and \(P < 0.05\) for methoxyfenoterol).

It has been proposed previously that PKA-mediated phosphorylation of \(\beta\)-AR is necessary and sufficient for the switch of the receptor coupling from G\(_s\) to G\(_i\) (Daaka et al., 1997). To determine whether PKA-mediated phosphorylation of \(\beta\)-AR is affected by the chirality of the fenoterol compounds, cultured rat cardiomyocytes overexpressing human \(\beta\)-AR were stimulated with ISO (10^{-6} M) and the four fenoterol compounds (10^{-6} M), and the extent of PKA-mediated receptor phosphorylation was then detected by Western blotting using the anti-p-Ser262 antibody (Tran et al., 2004). The levels of the total \(\beta\)-AR in the cell lysates were also determined in parallel using an anti-C-tail antibody. As expected, treatment with ISO increased the PKA-mediated phosphorylation of \(\beta\)-AR by 9-fold after adjusting for the amount of total \(\beta\)-AR (Fig. 6), a result confirming those obtained from another study (Iyer et al., 2006). The effects of the S,R isomers on receptor phosphorylation at Ser262 are comparable with that of ISO in magnitude. It is interesting that the R,R isomers also evoked an approximately 12-fold increase in phosphorylation of the receptor at Ser262. The differences in the potencies of these agonists are not significant. These results suggest that the degree of \(\beta\)-AR-G\(_i\) coupling in response to the fenoterol isomers is not proportional to the

Fig. 5. Differential activation of G\(_s\) and G\(_i\) proteins by fenoterol stereoisomers. Rat heart membranes were labeled with \([\gamma^{32}\text{P}]{\text{GTP}}-\text{azidoanilide}\) in the presence of ISO (10^{-6} M), zinterol (10^{-5} M), or fenoterol compounds (10^{-6} M). The radiolabeled G protein \(\alpha\) subunits G\(_{\alpha s}\), G\(_{\alpha i2}\), and G\(_{\alpha i3}\) were immunoprecipitated with the corresponding subunit-specific rabbit polyclonal antibodies and then subjected to electrophoresis. A, representative autoradiographs of the resolved G proteins. B to D, averaged data of the densitometric analysis of the labeled G protein bands from three experiments. Data are presented as percentages of the control. *, \(P < 0.05\); **, \(P < 0.01\) compared with the control; †, \(P < 0.05\); ‡, \(P < 0.01\) compared with the corresponding diastereomer.
phosphorylation status of β2-AR by PKA. Thus, PKA-mediated phosphorylation of β2-AR at Ser262 is insufficient for β2-AR-Gi coupling.

Discussion

As a prototypical Gs-coupled GPCR, β-AR stimulation activates the well established Gs-adenylyl cyclase-cAMP-PKA signaling cascade, which increases cardiac contractility via PKA-mediated phosphorylation of a panel of proteins involved in cardiac excitation-contraction coupling. Whereas β1-AR couples only to the Gs signaling pathway, β2-AR couples dually to Gs and G proteins (Xiao et al., 1995, 1999). As a result, stimulation of the β2-AR by most β2-AR agonists results in increased cardiomyocyte contractility that can be augmented by the inhibition of Gs signaling by PTX (Xiao et al., 1995, 2003). A major finding of the present study is that the positive inotropic effects and activation of ERK1/2 in HEK293 cells expressing endogenous β2-AR are selectively direct β2-AR to the Gs, bypassing the Gi coupling. It is interesting that alteration of their stereochemistry from (R,R)-methoxyfenoterol to (S,R)-methoxyfenoterol selectively activate the β2-AR-coupled Gs pathway, whereas (S,R)-fenoterol and (S,R)-methoxyfenoterol can activate both the Gs and G1 proteins, as manifested by the robust PTX sensitivities in their responses.

Substitution and Chirality Confer the Fenoterol Compounds Different Effectiveness in Activating Gs and Gi Proteins. In the present study, we compared different fenoterol isomers on their effectiveness in activating Gs and Gi proteins by means of direct labeling of the G proteins on isolated heart membranes with a radioactive GTP analog (Fig. 5). We found that substituting the hydroxyl group with the methoxy group in fenoterol is PTX-sensitive (Fig. 4), suggesting the involvement of a G1-independent mechanism. On the other hand, activation of ERK1/2 by (S,R)-fenoterol and (S,R)-methoxyfenoterol is PTX-sensitive (Fig. 4), suggesting the Gi-dependence of these effects. The lack of PTX-sensitivity in their effects on ERK1/2 activation further suggests that (R,R)-fenoterol and (R,R)-methoxyfenoterol selectively activate the β2-AR-coupled Gs pathway, whereas (S,R)-fenoterol and (S,R)-methoxyfenoterol can activate both the Gs and G1 proteins.

PKA-Mediated Phosphorylation of β2-AR Is Insufficient to Cause Receptor Coupling to Gi Protein. Desensitization of β2-AR is triggered by the phosphorylation of the receptor by a combination of actions of PKA and G protein-coupled receptor kinases. In particular, phosphorylation of β2-AR at the PKA sites is suggested to switch the receptor coupling from Gs to Gi (Daaka et al., 1997), although alternative G1-independent mechanisms (Schmitt and Stork, 2000; Friedman et al., 2002) have been proposed. Some G protein-independent mechanisms have been described previously (Shenoy et al., 2006; Sun et al., 2007). We have demonstrated here that activation of ERK1/2 by (R,R)-fenoterol and (R,R)-methoxyfenoterol is insensitive to PTX treatment (Fig. 4), suggesting the involvement of a G1-independent mechanism. On the other hand, activation of ERK1/2 by (S,R)-fenoterol and (S,R)-methoxyfenoterol is PTX-sensitive (Fig. 4), suggesting the Gi-dependence of these effects. The lack of PTX-sensitivity in their effects on ERK1/2 activation further suggests that (R,R)-fenoterol and (R,R)-methoxyfenoterol selectively activate the β2-AR-coupled Gs pathway, whereas (S,R)-fenoterol and (S,R)-methoxyfenoterol can activate both the Gs and G1 proteins.
portional to their ability to induce G$_1$ coupling. Thus, PKA-dependent phosphorylation of $\beta_2$-AR is insufficient to trigger the receptor coupling to G$_i$ proteins in the physiologically relevant setting, adult rat cardiac myocytes.

**Molecular Nature of (R,R)-Fenoterol-Activated G$_s$ Signaling.** Ligand-induced different G protein coupling has been described in various GPCRs (Akam et al., 2001; Cordeaux et al., 2001, 2004; Gazi et al., 2003; Beyermann et al., 2007). The present study is the first to suggest the role of chirality in the functional selectivity of an agonist. Differential activation of receptors to specific signaling pathways might be explained by the fact that agonists can stabilize a receptor into multiple conformational states (Gether et al., 1995; Ghanouni et al., 2001; Swaminath et al., 2004; Granier et al., 2007), and each of which can trigger a distinct pluridimensional functional outcomes, including G protein coupling, receptor phosphorylation, receptor dimerization/oligomerization, receptor desensitization, receptor internalization, and/or $\beta$-arrestin-dependent ERK activation (Kenakin, 2007; Kobilka and Deupi, 2007; Urban et al., 2007). Further studies using these compounds to characterize the receptor conformation that confers selectivity to G protein coupling are highly warranted. The data thus obtained could be applied to the development of assay systems for future drug-screening.

**Potential Clinical Implications of G$_s$-Signaling Selective $\beta_2$AR Activation.** Because enhanced G$_s$ signaling is involved in the dysfunction of both $\beta_1$-AR and $\beta_2$-AR in the failing heart (Sato et al., 2004; Xiao and Balke, 2004; He et al., 2005; Zhu et al., 2005), selective activation of the $\beta_2$-AR-G$_s$ coupling may provide an effective means to improve contractile function of the failing heart without $\beta_1$-AR detrimental effects (Zeng et al., 2005). We have reported previously the contractility stimuliatory effects of a number of $\beta_2$ agonists on rat cardiomyocytes (Xiao et al., 2003). Although most $\beta_2$-agonists increased myocyte contraction in a PTX-sensitive manner, the contractility stimuliatory effect of rac-fenoterol was insensitive to PTX (Xiao et al., 2003). We have concluded that rac-fenoterol selectively activates the $\beta_2$-AR-G$_s$ pathway and further postulated that this special property of rac-fenoterol might have potential therapeutic implications in heart failure. This idea is supported by recent in vivo studies in an ischemic rat heart failure model (Ahmet et al., 2005; Zhu et al., 2005), selective activation of the human A1 adenosine receptor to different heterotrimetric G proteins: evidence for agonist-specific G protein activation. Br J Pharmacol 143:765–714.

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**References**


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