Probing the $\beta_2$ adrenoceptor binding site with catechol reveals differences in binding and activation by agonists and partial agonists
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Running Title: Mechanistic Differences Between Agonists and Partial Agonists
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Abbreviations: $\beta_2$AR, $\beta_2$ adrenergic receptor; TMR-$\beta_2$AR, purified $\beta_2$AR labeled at Cys265 with tetramethylrhodamine maleimide; GPCR, G protein coupled receptor; ECL, extracellular loop; TM, transmembrane segment; DHA, dihydroalprenolol; ICL, intracellular loop; Tet-G$_{\alpha_s}$, membrane tethered G$_{\alpha_s}$.

The $\beta_2$ adrenergic receptor ($\beta_2$AR) is a prototypical Family A G protein coupled receptor (GPCR), and an excellent model system for studying the mechanism of GPCR activation. The $\beta_2$AR agonist binding site is well characterized and there is a wealth of structurally related ligands with functionally diverse properties. In the present study we use catechol (1,2-benzenediol, a structural component of catecholamine agonists) as a molecular probe to identify mechanistic differences between $\beta_2$AR activation by catecholamine agonists, such as isoproterenol, and by the structurally related non catechol partial agonist salbutamol. Using biophysical and pharmacologic approaches, we show that the aromatic ring of salbutamol binds to a different site on the $\beta_2$AR than the aromatic ring of catecholamines. This difference is important in receptor activation, as it has been hypothesized that the aromatic ring of catecholamines plays a role in triggering receptor activation through interactions with a conserved cluster of aromatic residues in the sixth transmembrane segment by a rotamer toggle switch mechanism. Our experiments indicate that the aromatic ring of salbutamol does not activate this mechanism either directly or indirectly. Moreover, the non catechol ring of partial agonists does not interact optimally with serine residues in the fifth transmembrane helix that have been shown to play an important role in activation by catecholamines. These results demonstrate unexpected differences in binding and activation by structurally similar agonists and partial agonists. Moreover, they provide evidence that activation of a GPCR is a multistep process that can be dissected into its component parts using agonist fragments.

G protein coupled receptors (GPCRs) are remarkably versatile signaling molecules. Many are capable of interacting with more than one G protein, and some have been observed to signal through non-G protein pathways (1,2). The activity of many GPCRs can be regulated by ligands having a spectrum of efficacies ranging from inverse agonists to agonists. Moreover, there is a growing body of evidence that GPCRs are conformationally complex, with different ligands inducing ligand-specific states (3,4). The $\beta_2$ adrenergic receptor ($\beta_2$AR) is one of the most extensively studied members of the Family A GPCRs. Its agonist binding site has been mapped in considerable detail using both site-directed mutagenesis and modified ligands (5-7) (Figure 1A).

Much of what is known about the structure and mechanism of activation of GPCRs comes from studies of rhodopsin. However rhodopsin is limited as an experimental system to investigate the mechanism of activation by diffusible ligands and the structural basis for ligand efficacy. We have used environmentally sensitive fluorophores including fluorescein (3,8) and tetramethylrhodamine (9) to monitor ligand-induced conformational changes in purified $\beta_2$AR. These studies provide evidence that, upon
activation, the $\beta_2$AR undergoes structural changes that are similar to those observed upon activation of rhodopsin (8). They also demonstrate that agonists and partial agonists induced distinguishable active states (3), and that the process of activation occurs through at least two kinetically distinguishable steps (9). Based on these results we proposed a model whereby agonist binding occurs through a series of discrete conformational intermediates as the receptor engages different components of the ligand.

In the present study we examine differences in the mechanism of activation of the $\beta_2$AR by catecholamine agonists and the non-catechol partial agonist salbutamol. We use catechol (1,2-benzenediol), a fragment of catecholamine agonists, as a molecular probe to characterize differences in binding and activation using biophysical and pharmacologic approaches. Our studies demonstrate that salbutamol binds to and activates the $\beta_2$AR in a manner different from catecholamine agonists. Moreover, they provide further evidence that activation occurs through a series of conformational intermediates having distinct functional properties.

**EXPERIMENTAL PROCEDURES**

**Buffers**—The buffers used are as follows: Buffer A, 100 mM NaCl, 20 mM HEPES pH 7.5, 0.1% dodecylmaltoside (Anatrace); Buffer B, Buffer A, containing 0.02% cholesterolhemisuccinate (Steraloids,Inc), 200ug/ml Flag peptide and 1 mM EDTA; Buffer C, Buffer A with 300 µM alprenolol (Sigma) and 1 mM CaCl$_2$; Buffer D, Buffer A with 1 mM CaCl$_2$; Buffer E, Buffer A with 0.02% cholesterolhemisuccinate (Steraloids,Inc). Buffer F, 20 mM Hepes buffer pH 7.5, 100 mMNaCl and 1% octylglucoside.

**Receptor Purification and Labeling**—$\beta_2$AR was expressed in SF9 cells and solubilized using methods described previously (10) with modifications as follows. CaCl$_2$ was added to a final concentration of 1 mM, and the detergent-solubilized $\beta_2$AR was purified by M1-FLAG affinity chromatography (Sigma). The receptor was eluted from the M1-FLAG resin in Buffer B. Labeled receptor was then purified by alprenolol-Sepharose chromatography as described previously (10). The receptor was eluted from alprenolol-Sepharose with Buffer C and loaded directly onto M1-FLAG resin. The M1-FLAG resin was washed with Buffer D to remove free alprenolol and eluted with Buffer B. The concentration of functional, purified receptor was determined using a saturating concentration (10 nM) of $[^3]$Hdihydroalprenolol as described previously (10). Two liters of SF9 cells typically yield 500 µl of a 5 µM solution of $\beta_2$AR. The purified $\beta_2$AR was diluted to a concentration of 2 µM and labeled with tetramethylrhodamine-5-maleimide (Molecular Probes) at a final concentration of 2 µM for 30 min on ice. The labeling reaction was quenched with 1 mM cysteine and tetramethylrhodamine labeled $\beta_2$AR (TMR-$\beta_2$AR) was separated from unincorporated fluorophore by desalting on a 2 ml Sephadex G50 column equilibrated with Buffer E.

**Purification of Tethered $G_{\alpha}$**—Tethered $G_{\alpha}$ (Tet-$G_{\alpha}$) is a membrane tethered form of $G_{\alpha}$ that has been shown to couple more efficiently to the $\beta_2$AR than does unmodified $G_{\alpha}$ (11). The construction and characterization of Tet-$G_{\alpha}$ has previously been described (11). Briefly, the membrane tether of Tet-$G_{\alpha}$ consists of the FLAG epitope (Sigma) followed by amino acids 1-64 of the $\beta_2$AR (containing the amino terminus and first transmembrane domain) followed by amino acids 343-412 from the carboxyl terminus of the $\beta_2$AR. This $\beta_2$AR sequence is linked via a six-histidine sequence to the amino terminus of $G_{\alpha}$. SF9 cells expressing tethered $G_{\alpha}$ (Tet-$G_{\alpha}$) (11) were lysed in buffer containing 20mM Tris-HCL pH7.4, 1mM EDTA, 3mM MgCl$_2$, 100mM NaCl, 5mM NaF, 20uM AlCl$_3$, 10uM GDP, 10mM β-mercaptoethanol and a mixture of protease inhibitors. The lysate was dounced 20 times and centrifuged at 18,000 rpm for 20 min at 4°C. The pellet membranes were solubilized in buffer containing 1% n-dodecyl maltoside, 50mM Tris-HCL pH7.4, 3mM MgCl$_2$, 100mM NaCl, 5mM NaF, 20uM AlCl$_3$, 10uM GDP, 10mM β-mercaptoethanol and protease inhibitors for 1 hr at 4°C with gentle stirring, followed by centrifugation at 18,000 rpm for 20 min. Tet-$G_{\alpha}$ was purified from solubilized membrane proteins by successive chromatography on Chelating Sepharose Fast Flow resin (Pharmacia) charged with Ni followed by M1-Flag affinity resin (Sigma). The peak fractions
of pure Tet-Gas subunit were pooled and dialyzed against reconstituted buffer containing 20mM Tris-HCl pH7.4, 3mM MgCl$_2$, 100mM NaCl, 0.05%NDM, 10uM GDP and 10% glycerol for 3h. The purified Tet-Gas was frozen at −80°C.

**Preparation of lipids**—Phospholipid 18:1 DOPC was purchased from Avanti Polar Lipids Inc. and their purity was ascertained by high performance liquid chromatography. Cholesterolhemisuccinate was purchased from steraloids Inc. A stock solution of 20mg/ml of DOPC and 10mg/ml of cholesterolhemisuccinate was made in chloroform. 3mg of DOPC and 0.3mg of cholesterolhemisuccinate were aliquoted into a glass vial from respective stock solutions. The chloroform was evaporated under a stream of argon and then vacuum dried for 1h to remove any residual chloroform. The lipid mixture was then hydrated in Buffer F. Then the lipid mixture was vortexed vigorously and sonicated for over two hours with 10 min intervals in an ice/water bath. This mixture was stored in −80°C and used for reconstitution.

**Preparation of reconstituted receptor**—TMR-β2AR (100ul of 2μM solution) was mixed with 75μl of lipid mixture (3mg DOPC+0.3mg CHS) and diluted with 25 μl of Buffer F. The receptor-lipid mixture was mixed well and allowed to reconstitute on ice for 2 h. The vesicles formed by removing detergent on a 25x0.8 cm SephadexG-50 (fine).

**Fluorescence Spectroscopy**—Experiments were performed on a SPEX FluoroMax-3 spectrofluorometer with photon counting mode using an excitation and emission bandpass of 3.2 nm. For time course experiments, excitation was at 541 nm, and emission was monitored at 571 nm. Unless otherwise indicated, all experiments were performed at 25°C, and the sample underwent constant stirring. Fluorescence intensity was corrected for dilution by ligands in all experiments and normalized to the initial value. All of the compounds tested had an absorbance of less than 0.01 at 541 and 571 nm at the concentrations used, excluding any inner filter effect in the fluorescence experiments.

**Ligand Binding assays**—All the binding assays were performed on purified, reconstitute β2AR. All of the assays were performed for 1 h at room temperature with shaking at 230 rpm. Competition assays were carried out with 1nM [3H]DHA and the indicated concentration of competing ligand. Binding data were analyzed by nonlinear regression analysis using Prism from GraphPad Software, San Diego.

[35S]GTPγS Binding—Purified β2AR and Tet-Gas protein were mixed in a molar ratio of 1:5 and reconstituted as described above. Reconstituted receptor and Tet-Gas were suspended in 500 μl of cold binding buffer (75 mM Tris-HCl pH7.4, 12.5 mM MgCl2 and 1 mM EDTA) supplemented with 0.05% (w/v) BSA, 0.4 nM [35S]GTPγS, GDP (1 μM) with or without β2AR ligands. Incubations were performed for 30 min at 25°C with shaking at 230 rpm. Nonspecific binding was determined in the presence of 100 μM GTPγS, and was always less than 0.2% of total binding. Bound [35S]GTPγS was separated from free [35S]GTPγS by filtration through GF/C filters, followed by three washes with 3 ml of cold binding buffer. Filter-bound radioactivity was determined by liquid scintillation counting.

**Molecular modeling of the β2AR**—The residues in the transmembrane (TM) segments of β2AR are numbered according to their position in the β2AR human sequence and using the Ballesteros general numbering scheme (12). A model of the TM domain and the cytoplasmic helix 8 of β2AR was built by homology modeling using the crystal structure of bovine rhodopsin (PDB ID code 1L9H (13)) as a template. SCWRL-3.0 (14) was employed to add the side chains to the non conserved amino acids, while the conformation of conserved residues was adjusted to reproduce key local interactions which are likely to be preserved in Family A GPCRs. Subsequently, loops were added to the transmembrane bundle, using the Modeler (15) and SYBYL 6.9.2 (Tripos, Inc.) loop-building routines. The volume of the binding pocket was calculated using Grasp (16). Isoproterenol, salbutamol and catechol were parameterized with the Antechamber program using the GAFF force field (17) and HF/6-31G*-derived RESP atomic charges. These ligands were
docked in the binding pocket of the β2AR according to the experimentally inferred interactions with Asp113, Ser203, Ser204, Ser207, and Asn293. The receptor models with the docked ligands were immersed in a patch of pre-equilibrated palmitoyloleoylphosphatidyl-choline lipidic bilayer solvated with water. These systems were energy-minimized and then equilibrated (500 ps) and simulated (500 ps) using molecular dynamics. The simulations were carried out with the Sander module of AMBER 8 (Case et al., AMBER 8 (2004); University of California, San Francisco) at constant pressure, using the particle mesh Ewald method and the ff2002EP polarizable force field, SHAKE bond constraints in all bonds, a 2-fs integration time step, and constant temperature coupled to a heat bath. Figures were created using MolScript 2.1.2 (20) and Raster3D 2.7 (21).

RESULTS
Non-catechol partial agonists induce a slow, monophasic conformational change in TMR-β2AR.

To monitor ligand-induced conformational changes, we labeled purified β2AR at Cys265 (Figure 1A) with tetramethylrhodamine maleimide (TMR-β2AR), as previously described (9). Ligand-induced conformational changes lead to a change in the molecular environment of the covalently bound tetramethylrhodamine that results in a change in emission intensity. We followed fluorescence intensity as a function of time before and after the addition of a saturating concentration of ligand. Figure 2A shows the biphasic response of TMR-β2AR to a saturating concentration of norepinephrine. The dotted black lines indicate the magnitudes of the rapid and slow components. We have previously shown that interactions between the catechol ring and the receptor are necessary for the rapid component of the conformational change, while interactions between the receptor and the chiral β-hydroxyl are required for the slow phase (9). Biphasic conformational changes are also observed upon binding to epinephrine and isoproterenol (9).

Figure 2B shows the fluorescence response of TMR-β2AR as a function of time following the addition of a saturating concentration of the non-catechol partial agonist salbutamol. This ligand shares similar structural features with catecholamines, and it is likely that, as with catecholamines, the amine of these ligands interacts with Asp113. However, salbutamol differs from catecholamines in the structure of the aromatic component. In the meta position of the aromatic ring salbutamol has a hydroxymethyl instead of a hydroxyl group (Fig. 2B). When a saturating concentration of salbutamol is added to TMR-β2AR, we observe only a slow phase, which is comparable to the slow component of the norepinephrine response (Fig. 2A). The results are consistent with our previous studies suggesting that the catechol ring is required for the rapid conformational change observed in TMR-β2AR (9).

Catechol can bind to β2AR occupied by non-catechol partial agonists and antagonists. Catechol is a structural component of catecholamine agonists, but not of the non-catechol partial agonist salbutamol or of antagonists (Fig. 1B). Catechol alone can induce a rapid, monophasic conformational change in TMR-β2AR (Figure 2C) (9). The fluorescence response to catechol is saturable with an apparent KD of 160 µM (data not shown). The location of the binding site for catechol in the β2AR appears to overlap with the binding site for catecholamines such as norepinephrine, epinephrine and isoproterenol. This can be shown by the inability of catechol to induce a change in fluorescence in TMR-β2AR occupied by catecholamine agonists (Figure 3A and C). In contrast, catechol produces a response in TMR-β2AR bound to a saturating concentration of salbutamol (Figure 3B) that is comparable to the response observed in unliganded TMR-β2AR (Figure 3C). This indicates that the binding sites for catechol and salbutamol do not overlap.

Antagonists are believed to share a common interaction with agonists and partial agonists at Asp113. Unlike agonists and partial agonists, βAR antagonists cause little or no change in the fluorescence intensity of TMR-β2AR. However, as with partial agonists, catechol induces an increase in fluorescence in β2AR bound to the antagonist alprenolol as well as timolol and ICI118,551 (Figure 3C). Therefore, the binding sites for these antagonists do not overlap with the binding site for catechol.

To confirm the results of the biophysical studies, we performed more conventional
equilibrium competition binding assays (Figure 4A). As expected, isoproterenol, norepinephrine and salbutamol all compete with the antagonist [3H]DHA for binding sites on the β2AR. In contrast, catechol does not displace [3H]DHA even at concentrations up to 10 mM. This is consistent with the ability of catechol to induce a response in TMR-β2AR occupied by the antagonist alprenolol (Figure 3C). To determine the ability of catechol to compete with agonists and partial agonists, we performed competition binding studies in the absence and the presence of 1 and 10 mM catechol. In the presence of catechol, the apparent EC50 for isoproterenol is shifted to the right (Figure 4B), demonstrating that they compete for a common binding site. In contrast, catechol has no effect on the ability of salbutamol to compete for [3H]DHA binding sites (Figure 4C).

Taken together, the results from conformational studies using TMR-β2AR and conventional ligand binding assays using purified, labeled receptor demonstrate that the aromatic rings of the non-catechol partial agonist salbutamol and the antagonists alprenolol, timolol and ICI-118,551 do not occupy the same space in the β2AR binding pocket as does the catechol ring of catecholamines.

Functional effects of catechol-induced conformational changes. To determine the functional consequence of ligand-induced conformational changes in the β2AR, we reconstituted purified receptor with purified, membrane tethered Gαs (Tet-Gαs) (11), and monitored the effect of ligands on [35S]GTPγS binding. We have previously shown that Tet-Gαs couples more efficiently to β2AR than does wild type Gαs when expressed in insect cells (11). Figure 5A shows the maximum response to a saturating concentration of isoproterenol, dopamine and catechol. The response to catechol is small, but significantly greater than no drug (Fig. 5C). Moreover, the catechol response is not due to a non-specific effect on Tet-Gαs, as no response was observed in the absence of receptor (data not shown). Figure 5B shows the effect of 100 µM catechol on dose response curves for salbutamol and isoproterenol. Catechol has no significant effect on isoproterenol. In contrast, catechol reduced the maximal response to salbutamol without significantly altering the EC50.

Catechol activates β2AR occupied by an inverse agonist. As shown in Figure 3C, catechol can induce a conformational change in β2AR occupied by a saturating concentration of the inverse agonist ICI-118,551. Figure 5C shows that this conformational change is associated with an enhanced coupling to Tet-Gαs.

Molecular modeling of the salbutamol binding site. Figure 6A shows isoproterenol in the ligand binding space defined by TM3, TM5, TM6 and the second extracellular loop (ECL2) in a model of the β2AR based on the structure of rhodopsin (see Methods). The docking of isoproterenol in the β2AR is based on interactions with the receptor that have been identified by mutagenesis studies summarized in Figure 1. Isoproterenol occupies the bottom of the binding pocket interacting with the aromatic residues Phe2895.11 and Phe2905.52 in TM6 (22,23). It also interacts with Ser2037.42, Ser2047.43 and Ser2077.46 in TM5 (7,24). The fact that isoproterenol and catechol compete with each other in binding (Figure 4B) and conformational assays (Figure 3C) suggests that catechol occupies the same space in the β2AR as that occupied by the aromatic ring of isoproterenol. In contrast, our experimental evidence suggests that the aromatic ring of salbutamol does not occupy the same binding site as catechol. It is therefore unlikely that the binding site for salbutamol is identical to that for isoproterenol. Examination of the β2AR binding space identified aromatic amino acids Tyr174 and Phe193 in ECL2, and Tyr1995.38 in TM5 that may interact with salbutamol. Photoaffinity labeling studies suggest a role for Tyr1995.38 in antagonist binding (25). Of interest, our results demonstrate that catechol can bind to an antagonist occupied receptor (Figure 3C and 4A), suggesting that, like salbutamol, the binding site for the aromatic ring of antagonists does not overlap the binding site for the aromatic ring of catecholamines. In the model shown in Figure 6B, the aromatic ring of salbutamol is shifted to the top of the binding pocket close to Tyr174, Phe193 and Tyr199. In this position, there is room for the simultaneous binding of catechol in the lower part of the binding pocket.

We used molecular dynamics simulations to examine the stability of interactions between
ligand and receptor when isoproterenol and salbutamol are docked in the lower binding pocket (Fig. 6A). Analysis of multiple trajectories and their energies shows that isoproterenol remains stable in the lower binding pocket [RMS (isoproterenol) < 1.5 Å], interacting strongly with residues in the lower region of the binding site, mainly Ser203<sup>5,42</sup>, Ser204<sup>5,43</sup>, Ser207<sup>5,46</sup>, Trp286<sup>6,48</sup> and Phe289<sup>6,51</sup>. On the other hand, salbutamol is less stable in the binding pocket [RMS (salbutamol) ≈ 2.5 Å], and adopts different orientations during the trajectories. This ligand is able to interact with the aromatic residues facing the upper region of the binding pocket, mainly with Phe193 in ECL2. Interestingly, the non-catechol aromatic ring of salbutamol interacts only weakly with the serine residues in TM5, which may explain why salbutamol has a weaker affinity for the β<sub>2</sub>AR than does isoproterenol.

**DISCUSSION**

*Structurally similar, but functionally diverse ligands.* Ligand binding affinity consists of the sum of the energies of interaction between different structural components of the ligand and the amino acids within the binding site of the receptor. The energetic costs associated with ligand-induced conformational changes and ligand desolvation also contribute to binding affinity. Ligands for the β<sub>2</sub>AR share remarkable structural homology (Figure 1B). Common features include a primary or secondary amine, a chiral β-hydroxyl, and an aromatic ring. For agonists and partial agonists, the aromatic ring and the amine are separated by two carbons, while for antagonists and inverse agonists they are separated by three carbons and an oxygen. Interactions between the agonist isoproterenol and the β<sub>2</sub>AR have been mapped in considerable detail by a series of mutagenesis experiments from several labs (Fig. 1A) (5-7). Evidence suggests that the amine of agonists, partial agonists and antagonists all share an interaction with Asp113<sup>3,32</sup> (22); therefore, structural differences in the aromatic components of these ligands are the primary determinants of efficacy.

The structural differences between isoproterenol and salbutamol are relatively subtle (Figure 1B), and one might expect them to occupy a similar space within the β<sub>2</sub>AR binding pocket, and activate the β<sub>2</sub>AR by a similar mechanism. We used catechol as a probe to explore the differences between binding and activation by catecholamines and salbutamol. Our results show that the binding site for catechol is the same as the binding site for the catechol ring in catecholamines. Catechol cannot induce a detectable conformational change in TMR-β<sub>2</sub>AR occupied by a catecholamine agonist (Fig. 3A and C), and catechol competes with isoproterenol in binding to the β<sub>2</sub>AR (Figure 4B). Moreover, we observe that catechol is a weak partial agonist (Fig. 5A, C). In contrast, binding experiments (Fig. 4C) demonstrate that there is no competition between catechol and salbutamol for binding to the β<sub>2</sub>AR, and fluorescence experiments demonstrate that catechol can bind and induce a conformational change in TMR-β<sub>2</sub>AR occupied by salbutamol (Fig. 3B and C).

Based on these observations, we conclude that the aromatic ring of salbutamol occupies a binding space in the β<sub>2</sub>AR that does not overlap with the binding space occupied by the aromatic ring of catechol or of catecholamines. Thus, a difference in the meta position of the aromatic ring (-OH for isoproterenol, -CH<sub>2</sub>OH for salbutamol, Figure 1B) has a dramatic effect on its location in the binding site and on the mechanism of activation. A catechol moiety optimizes the interactions with the serine residues in TM5, through the formation of a complex network of hydrogen bonds. However, the apparently minor substitution of the meta -OH for a -CH<sub>2</sub>OH group destabilizes this network, such that the aromatic ring of salbutamol no longer occupies this space in the β<sub>2</sub>AR binding pocket. Based on the study of the structure of the binding site of β<sub>2</sub>AR, we propose that the aromatic ring of salbutamol may interact with aromatic residues in the second extracellular loop and the carboxyl terminal end of TM6 (Fig. 6B). In this position, the chiral β-hydroxyl would not be expected to interact with Asn293<sup>6,55</sup>. This is consistent with previous studies showing that the binding affinities of other non-catechol partial agonists are not affected by mutation of Asn293<sup>6,55</sup> to Ala (6).

*Differences in the mechanism of activation of salbutamol and catecholamines.* It has been suggested that interactions between the aromatic ring of catecholamine agonists and Phe290<sup>6,52</sup> in TM6 play an important role in some of the
conformational changes associated with receptor activation by a rotamer toggle switch mechanism (26). Monte Carlo simulations suggest that rotameric positions of Phe290<sup>6.52</sup> and Trp286<sup>6.48</sup> are coupled, and modulate the bend angle of TM6 around the highly conserved proline-kink at Pro288<sup>6.50</sup>, leading to the movement of the cytoplasmic end of TM6 (26). It is likely that the rapid change in fluorescence observed upon binding of catechol and catecholamine agonists to TMR-β<sub>2</sub>AR represents the conformational changes associated with this movement of TM6 relative to TM5.

Based on our experimental results and the model in Fig. 6B, salbutamol does not interact with this aromatic cluster and therefore does not directly activate the receptor by this rotamer toggle switch mechanism. One may hypothesize that the inability of salbutamol to fully activate the receptor may be due to its failure to directly engage the toggle switch. In this case, the combination of catechol and salbutamol might be expected to induce a more active conformation; however, we found that catechol partially inhibits activation by salbutamol (Figure 5B). These results are most consistent with salbutamol inducing an active conformation distinct from the conformation induced by catecholamine agonists. They suggest that this active conformation does not involve the same movement of TM6 around Pro288<sup>6.50</sup> that occurs upon activation of the β<sub>2</sub>AR by catecholamines. These studies provide mechanistic insight into previous fluorescence lifetime experiments demonstrating that salbutamol and isoproterenol induce distinct conformational changes in the cytoplasmic end of TM6 of the β<sub>2</sub>AR (3,9,27).

**Catechol and inverse agonism.** The mechanism of inverse agonism is poorly understood. As shown in Fig. 5C, we can detect inverse agonism of ICI-118,551 in a functional assay by its inhibition of basal GTPγS binding; yet our conformational reporter on Cys265 is not sensitive to this conformational change. Nevertheless, we can conclude that ICI-118,551 does not restrict movement of TM6, as it is still possible to detect a conformational response (Fig. 3C) and functional response (Fig. 5C) to catechol in the presence of a saturating concentration of this inverse agonist.

**Using ligand fragments to dissect the process of ligand binding and activation.** Evidence from several studies suggests that agonists activate GPCRs through a series of conformational intermediates (3,9,27-30). These studies form the basis for our current hypothesis regarding the mechanism of activation (9). The unliganded receptor is maintained in a relatively inactive state by a series of intramolecular interactions that stabilize a specific arrangement of the TM segments. Ligands activate the receptor by disrupting these stabilizing interactions and/or by stabilizing new, more active arrangements of the TM segments. The fully active conformation occurs when all of the stabilizing intramolecular interactions have been broken, and the new ones have been formed. The unliganded state of the receptor is conformationally dynamic and at any one moment in time the amino acids that interact with the ligand are not arranged to form a complete binding site for the ligand (as envisioned in a lock and key model of ligand binding). As such, all of the contacts between receptor and agonist do not form at the same time, but sequentially through a series of conformational intermediates. As one component of the ligand engages the receptor, some of the stabilizing intramolecular interactions are broken and there is an increased probability that additional contacts will form as the receptor explores its conformational space. As each contact is formed, the receptor assumes a more active state.

Catechol and dopamine can be considered fragments of catecholamine agonists, and can therefore provide some insight into the functional properties of intermediate conformational states. Catechol disrupts and/or stabilizes a specific interaction between TMs 5 and TM6, and even this relatively small conformational change is associated with an increase in activity towards Gs (Fig. 5C). Catechol has a remarkably high affinity (K<sub>D</sub> = 160 μM, based on a conformational assay) considering its size (f.w. 110). This is consistent with an agonist fragment, where a high proportion of the catechol atoms are involved in binding interactions with the receptor. Moreover, the relatively high binding affinity suggests that energetic cost of the conformational changes required for optimal interactions between the β<sub>2</sub>AR and catechol are small. The observation that sensitive biophysical (Fig. 2C) and functional assays (Fig. 5C) can detect binding and activation
by a small agonist fragment such as catechol suggests that fragment-based screening strategies may be applicable to drug discovery for GPCRs (31).

The binding of the catechol ring of dopamine results in the same structural change that occurs upon binding of catechol alone, but the interaction between its amine and Asp113^{3.32} also stabilizes a specific arrangement of TM3 relative to TM5 and TM6. This additional conformational change imparts a much greater activity towards Gs (Fig. 5A). It is interesting to note that binding affinity for dopamine (K_{i} = 350 µM) is similar to that for catechol. This is surprising considering that the interaction between the primary amine and Asp113^{3.32} makes the strongest contribution to the binding energy. Part of the binding energy associated with the interaction between dopamine and Asp113^{3.32} might be offset by the energetic cost of the conformational change needed for the binding interaction to occur. Thus, in the inactive state, TM5 and TM6 are positioned such that little energy is needed to accommodate the binding of the catechol ring. In contrast, the movement of TM3 relative to TM5 and TM6 required for binding of dopamine may involve breaking of intramolecular interactions thereby consuming part of the energy provided by the ionic interaction.

The process of binding and activation by a full agonist such as isoproterenol and epinephrine involves the same interactions that form with catechol and dopamine as well as additional interactions between the receptor and the β-OH and the alkyl substituent on the amine. These interactions make significant contributions to binding affinity and to the active state of the receptor.

**Conclusion.** We have used catechol as a molecular probe to investigate the location of the binding site for the partial agonist salbutamol, and the mechanism by which activation of the receptor by this partial agonist differs from the mechanism of activation by full agonist catecholamines. In spite of the structural similarity of salbutamol and catecholamine agonists, the aromatic ring of salbutamol occupies a different space in the β_{2}AR, and thereby induces a functionally different active state. The results provide further evidence for structural plasticity of GPCRs, and the possibility of achieving more than one active state through different interactions between receptors and ligands.

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**REFERENCES**

FIGURE LEGENDS

Figure 1. A. Binding site for norepinephrine in the \( \beta_2 \)AR. Amino acids in the \( \beta_2 \)AR involved in ligand binding have been identified using a combination of site-directed mutagenesis and modified ligands \( (6,7,22) \). The catecholamine nitrogen interacts with Asp113 in TM3 \( (22) \). Hydroxyls on the catechol ring interact with serines 203 \( (7) \), 204 and 207 \( (19) \) in TM6. The chiral \( \beta \)-hydroxyl interacts with Asn293 in TM6 \( (6) \) and the aromatic ring interacts with Phe290 in TM6 \( (22) \). Also shown is the relative position of Cys265, the labeling site for tetramethylrhodamine maleimide. B. The structures of agonist and antagonists used or discussed in the manuscript.

Figure 2. Ligand-induced changes in the fluorescence intensity of TMR-\( \beta_2 \)AR. Conformational changes in response to (A) norepinephrine \( (100 \ \mu \text{M}) \), (B) salbutamol \( (100 \ \mu \text{M}) \), and (C) catechol \( (100 \ \mu \text{M}) \) were examined by monitoring changes in the fluorescence intensity of TMR-\( \beta_2 \)AR as a function of time. The response to norepinephrine was best fit with a two-site exponential association function. The rapid and slow components of the biphasic response to norepinephrine...
are shown as dotted lines in panel A. The tracings are representative of three independent experiments. Both drugs were added to achieve a final concentration of 100 µM.

**Figure 3.** Catechol induces conformational changes in TMR-β2AR in the presence of a saturating concentration of salbutamol. Ligand-induced changes in the intensity of TMR-β2AR were monitored as a function of time. A. Intensity changes in response to 100 µM norepinephrine followed by 100 µM catechol. B. Intensity changes in response to 100 µM salbutamol followed by 100 µM catechol. The tracings in A and B are representative of three independent experiments performed on the same preparation of TMR-β2AR. C. The maximal change in intensity of TMR-β2AR following the addition of 100 µM catechol to receptor that had been preincubated with saturating concentrations of the indicated ligands (100 µM for agonists, 10 µM for antagonists). The data represent the average +/- s.e. of three determinations.

**Figure 4.** Catechol competes with isoproterenol, but not with salbutamol or alprenolol for binding to the β2AR. Competition binding studies were performed with 1 nM [³H]DHA as described in Experimental Procedures. A. Competition of isoproterenol, salbutamol and catechol for [³H]DHA binding sites on purified, reconstituted β2AR. B. Competition of isoproterenol for [³H]DHA binding sites on purified, reconstituted β2AR in the absence and presence of the indicated concentration of catechol. C. Competition of salbutamol for [³H]DHA binding sites on purified, reconstituted β2AR in the absence and presence of the indicated concentration of catechol. Binding experiments represent the average of three independent experiments performed in triplicate.

**Figure 5.** Ligand-stimulated [³⁵S]GTPγS binding to purified Tet-Gαs reconstituted with purified β2AR. Reconstitution and [³⁵S]GTPγS binding were performed as described in Materials and Methods. A. Maximal [³⁵S]GTPγS binding stimulated by isoproterenol (10µM), dopamine (1 mM) and catechol (1mM). B. Dose-response experiments for isoproterenol and salbutamol in the absence or presence of 1 mM catechol. C. The effect of the inverse agonist ICI-118,551 on basal [³⁵S]GTPγS binding in the presence and absence of catechol. Bars indicate significant differences (p < 0.05) as determined by a Student’s t test. The data for each panel represent the average +/- s.e. of three determinations, and are representative of two independent experiments.

**Figure 6.** Molecular model of the agonist binding pocket of the β2AR. The model was generated using the crystal structure of rhodopsin as a template. TM segments involved in agonist binding are colored as follows: TM3, red; TM5, green; TM6, blue. A. The proposed binding site for isoproterenol. B. Salbutamol docked into the upper (extracellular) region of the binding pocket. The aromatic ring of salbutamol interacts with Tyr174, Phe193 and Tyr199.

5.38
Figure 1

A

Norepinephrine

Asp113
TM3
Phe290
TM5
Asn293
Cys265
Ser203, 204, 207

B

Catechol
Dopamine
Norepinephrine
Epinephrine
Isoproterenol
Salbutamol
ICI118,551
Figure 2

A. Norepinephrine

B. Salbutamol

C. Catechol

% Change in fluorescence vs. Time (sec)

Rapid phase

Slow phase
Figure 3

- **A**: Graph showing the response to Catechol (% change in fluorescence) with time (sec) for Norepinephrine.
- **B**: Graph showing the response to Catechol (% change in fluorescence) with time (sec) for Salbutamol.
- **C**: Bar graph showing the response to Catechol (% change in fluorescence) for different compounds: Isoproterenol, Epinephrine, Norepinephrine, Salbutamol, No ligand, Timolol, Alprenolol, ICI-118,551.
Figure 4

A. [3H]DHA binding (% of maximal) vs Drugs (log M)
- Isoproterenol
- Salbutamol
- Catechol

B. [3H]DHA binding (% of maximal) vs Isoproterenol (log M)
- ISO
- ISO + 1 mM CAT
- ISO + 10 mM CAT

C. [3H]DHA binding (% of maximal) vs Salbutamol (log M)
- SAL
- SAL + 1 mM CAT
- SAL + 10 mM CAT
Figure 5

A

[35S]GTP\_S binding

basal CAT DOP ISO

B

[35S]GTP\_S binding

Drug (log M)

ISO ISO+CAT SAL SAL+CAT

C

[35S]GTP\_S binding

basal CAT ICI ICI +CAT

*
Figure 6