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)\(^a\) 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 \( \beta_2 \) adrenoreceptor (\( \beta_2 \)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) (see Fig. 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 (6) and that the process of activation occurs through at least two kinetically distinguishable steps (6). 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 examined differences in the mechanism of activation of the \( \beta_2 \)AR by catecholamine agonists and the non-catechol partial agonist salbutamol. We used 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 demonstrated that salbutamol binds to and activates the \( \beta_2 \)AR in a manner different from catecholamine agonists. Moreover, they provided 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% dodecyl maltoside (Anatrace); Buffer B, Buffer A containing 0.02% cholesterol hemisuccinate (Steraloids, Inc.), 200 \( \mu \)g/ml FLAG peptide, and 1 mM EDTA; Buffer C, Buffer A with 300 \( \mu \)M alpranolol (Sigma) and 1 mM CaCl\(_2\); Buffer D, Buffer A with 0.02% cholesterol hemisuccinate (Steraloids, Inc.); Buffer F, 20 mM Hepes buffer, pH 7.5, 100 mM NaCl and 1% octylglucoside.

Receptor Purification and Labeling—\( \beta_2 \)AR was expressed in Sf9 cells and solubilized using methods described previously (10) with modifica-

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Mechanistic Differences between Agonists and Partial Agonists

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 Cys-265 (Fig. 1A) with tetramethylrhodamine maleimide (TMR-β2AR), as described previously (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. Fig. 2A shows the biphase 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, whereas interactions between the receptor and the chiral β-hydroxyl are required for the slow phase (9). Biphase conformational changes are also observed upon binding to epinephrine and isoproterenol (9).

Fig. 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 Asp-1133.32. 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 was added to TMR-β2AR, we observed only a slow phase, which is comparable with 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-$\beta_2$AR (9).

Catechol Can Bind to $\beta_2$AR 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-$\beta_2$AR (Fig. 2C) (9). The fluorescence response to catechol is saturable with an apparent $K_D$ of 160 $\mu$M (data not shown). The location of the binding site for catechol in the $\beta_2$AR 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-$\beta_2$AR occupied by catecholamine agonists (Fig. 3, A and C). In contrast, catechol produces a response in TMR-$\beta_2$AR bound to a saturating concentration of salbutamol (Fig. 3B) that is comparable with the response observed in unliganded TMR-$\beta_2$AR (Fig. 3C). Therefore, the binding sites for catechol and salbutamol do not overlap.

Antagonists are believed to share a common interaction with agonists and partial agonists at Asp-113 (10). Unlike agonists and partial agonists, $\beta$AR antagonists cause little or no change in the fluorescence intensity of TMR-$\beta_2$AR. However, as with partial agonists, catechol induces an increase in fluorescence in $\beta_2$AR bound to the antagonist alprenolol as well as timolol and ICI-118,551 (Fig. 3C). Therefore, the
Mechanistic Differences between Agonists and Partial Agonists

Molecular Modeling of the Salbutamol Binding Site—Fig. 6A shows isoproterenol in the ligand binding site (root mean square of salbutamol) A

A

B

C

Fig. 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 panels 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.

To confirm the results of the biophysical studies, we performed more conventional equilibrium competition binding assays (Fig. 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 (Fig. 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 (Fig. 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 (Fig. 4C).

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 Tct-Ga, (11) and monitored the effect of ligands on [35S]GTPγS binding. We have previously shown that Tct-Ga, couples more efficiently to β2AR than does wild type Ga, when expressed in insect cells (11). Fig. 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 nonspecific effect on Tct-Ga, as no response was observed in the absence of receptor (data not shown). Fig. 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 Fig. 3C, catechol can induce a conformational change in β2AR occupied by a saturating concentration of the inverse agonist ICI-118,551. Fig. 5C shows that this conformational change is associated with an enhanced coupling to Tct-Ga,.
which may explain why salbutamol has a weaker affinity for the \( \beta_2 \)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 \( \beta_2 \)AR share remarkable structural homology (Fig. 1B). Common features include a primary or secondary amine, a chiral \( \beta \)-hydroxyl, and an aromatic ring. For agonists and partial agonists, the aromatic ring and the amine are separated by two carbons, whereas for antagonists and inverse agonists, they are separated by three carbons and an oxygen. Interactions between the agonist isoproterenol and the \( \beta_2 \)AR have been mapped in considerable detail by a series of mutagenesis experiments from several laboratories (Fig. 1A) (5–7). Evidence suggests that the amines of agonists, partial agonists, and antagonists all share an interaction with Asp-1133.32 (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 (Fig. 1B), and one might expect them to occupy a similar space within the \( \beta_2 \)AR binding pocket...
and activate the β2AR 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-β2AR occupied by a catecholamine agonist (Fig. 3, A and C), and catechol competes with isoproterenol in binding to the β2AR (Fig. 4B). Moreover, we observe that catechol is a weak partial agonist (Fig. 5, A and C). In contrast, binding experiments (Fig. 4C) demonstrate that there is no competition between catechol and salbutamol for binding to the β2AR, and fluorescence experiments demonstrate that catechol can bind and induce a conformational change in TMR-β2AR occupied by salbutamol (Fig. 3, B and C).

Based on these observations, we conclude that the aromatic ring of salbutamol occupies a binding space in the β2AR 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, –CH2OH for salbutamol, Fig. 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 –CH2OH group destabilizes this network such that the aromatic ring of salbutamol no longer occupies this space in the β2AR binding pocket. Based on the study of the structure of the binding site of β2AR, 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 Asn-2935,55. This is consistent with previous studies showing that the binding affinities of other non-catechol partial agonists are not affected by mutation of Asn-2935,55 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 Phe2906.52 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 Phe-2906.52 and Trp-2886.48 are coupled and modulate the bend angle of TM6 around the highly conserved proline kink at Pro-2886.50, 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-β2AR 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 (Fig. 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 Pro-2886.50 that occurs upon activation of the β2AR 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 β2AR (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 Cys-265 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 TM5 and TM6, and even this relatively small conformational change is associated with an increase in activity toward Gs (Fig. 5C). Catechol has a remarkably high affinity (Kᵢ = 160 μM, based on a conformational assay) considering its size (110 Da). This is consistent with an agonist fragment, in which a high proportion of the catechol atoms is involved in binding interactions with the receptor. Moreover, the relatively high binding affinity suggests that the energetic cost of the conformational changes required for optimal interactions between the β2AR 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 (24).

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 Asp-1135,32 also stabilizes a specific arrangement of TM3 relative to TM5 and TM6. This additional conformational change imparts a much greater activity toward Gs (Fig. 5A). It is interesting to note that binding affinity for dopamine (Kᵢ = 350 μM) is similar to that for catechol. This is surprising considering that the interaction between the primary amine and Asp-1135,32 makes the strongest contribution to the binding energy. Part of the binding energy associated with the interaction between dopa-
mine and Asp-113 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 the 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 $\beta$-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. Despite the structural similarity of salbutamol and catecholamine agonists, the aromatic ring of salbutamol occupies a different space in the $\beta_2$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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Probing the $\beta_2$ Adrenoceptor Binding Site with Catechol Reveals Differences in Binding and Activation by Agonists and Partial Agonists
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