Sequential Binding of Agonists to the \( \beta_2 \) Adrenoceptor

KINETIC EVIDENCE FOR INTERMEDIATE CONFORMATIONAL STATES*

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The \( \beta_2 \) adrenoreceptor (\( \beta_2 \)AR) is a prototypical G protein-coupled receptor (GPCR) activated by catecholamines. Agonist activation of GPCRs leads to sequential interactions with heterotrimeric G proteins, which activate cellular signaling cascades, and with GPCR kinases and arrestins, which attenuate GPCR-mediated signaling. We used fluorescence spectroscopy to monitor catecholamine-induced conformational changes in purified \( \beta_2 \)AR. Here we show that upon catecholamine binding, \( \beta_2 \)ARs undergo transitions to two kinetically distinguishable conformational states. Using a panel of chemically related catechol derivatives, we identified the specific chemical groups on the agonist responsible for the rapid and slow conformational changes in the receptor. The conformational changes observed in our biophysical assay were correlated with biologic responses in cellular assays. Dopamine, which induces only a rapid conformational change, is efficient at activating \( G_s \) and receptor internalization. In contrast, norepinephrine and epinephrine, which induce both rapid and slow conformational changes, are efficient at activating \( G_s \) and receptor internalization. These results support a mechanistic model for GPCR activation where contacts between the receptor and structural determinants of the agonist stabilize a succession of conformational states with distinct cellular functions.

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G protein-coupled receptors (GPCRs)† represent the largest family of membrane proteins in the human genome. They are responsible for the majority of cellular responses to hormones and neurotransmitters and are the largest group of targets for drug discovery. GPCRs are remarkably versatile biological sensors, responding to a broad spectrum of chemical entities ranging in size from a single photon of light, to ions, to small organic compounds, to peptides and protein hormones (1).

Rhodopsin has long been used as a model system for studying the structure and mechanism of activation of GPCRs. It is the only GPCR for which a high-resolution structure is available (2). Light-induced conformational changes have been elucidated by a series of elegant biophysical studies (3–8). Electron paramagnetic resonance spectroscopy studies provide evidence that photoactivation of rhodopsin involves a rotation and tilting of transmembrane domain 6 (TM6) relative to TM3 (3). Light-induced conformational changes have also been observed in the cytoplasmic domain spanning TM1 and TM2 (6) and the cytoplasmic end of TM7 and helix 8 (5).

Structural and biophysical studies on other GPCRs are more limited. Fluorescence spectroscopic analysis of \( \beta_2 \)ARs labeled with environmentally sensitive fluorescent probes detects movement of both TM3 and TM6 upon agonist binding (9). More detailed analysis of conformational changes in TM6 of the \( \beta_2 \)AR provide evidence for a rigid body motion similar to that observed upon activation of rhodopsin (10, 11). Additional support for movement of TM3 and TM6 in the \( \beta_2 \)AR comes from zinc cross-linking studies (12) and chemical reactivity measurements in constitutively active \( \beta_2 \)AR mutants (13, 14). Cysteine cross-linking studies in the M3 receptor provide evidence for the movement of the cytoplasmic ends of TM5 and TM6 toward each other upon agonist activation (15).

Based on this limited set of experiments, it appears that the agonist-induced conformational changes leading to G protein activation for monoamine receptors are similar to those observed for rhodopsin. However, the process linking agonist binding to these conformational changes for rhodopsin may not be generalizable to the larger family of GPCRs for hormones and neurotransmitters because of the unique covalent interaction between rhodopsin and its agonist trans-retinal. Thus, the dynamic processes of agonist association and dissociation common to other GPCRs are not part of the activation process of rhodopsin, and the mechanism by which ligand binding leads to structural changes in GPCRs is poorly understood.

A number of kinetic models have been developed to describe the process of agonist activation. These models are based on indirect measures of receptor conformation such as ligand binding affinity and G protein activation studies. Perhaps the most widely cited model is the extended ternary complex model (16–18). A simplified version of this model (referred to as the two-state model) is commonly used as a conceptual framework to discuss experimental results. The two-state model proposes that a receptor exists primarily in two states, the inactive state (R) and the active state (R*). In the absence of ligands, the level of basal receptor activity is determined by the equilibrium between R and R*. The efficacy of ligands reflects their ability to alter the equilibrium between these two states. Full agonists stabilize R*, whereas inverse agonists stabilize R. Although often discussed in the context of two receptor states, the extended ternary complex model is compatible with multiple receptor states, and several lines of experimental evidence support the existence of multiple states (18, 19).

We have used fluorescence lifetime spectroscopy to characterize the diversity of conformational states of the \( \beta_2 \)AR (20). We found that the conformational states induced by full and
partial agonists are distinguishable. Moreover, we found that a single catecholamine agonist induces or stabilizes at least two conformational states that are distinguishable from the unliganded state. Based on these studies, we proposed the existence of an intermediate state between the inactive (unliganded state) and the fully activated state. We now show that it is possible to observe and characterize an intermediate state by kinetic analysis. We use fluorescence spectroscopy to monitor agonist-induced conformational changes in purified β2AR over time. Our results support a mechanistic model for GPCR activation, where contacts between the receptor and structural determinants of the agonist stabilize a succession of conformational states with distinct cellular functions.

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 and 1 mM EDTA; Buffer C; Buffer A with 300 μM alprenolol (Sigma) and 1 mM CaCl2; Buffer D; Buffer A with 1 mM CaCl2; Buffer E; Buffer A with 0.01% cholestoldemethane succinate.

Receptor Purification and Labeling—β2AR was expressed in SF9 cells and solubilized using methods described previously (21). CaCl2 was added to a final concentration of 1 mM, and the detergent-solubilized β2AR was purified by M1-FLAG affinity chromatography (Sigma). The receptor was eluted from the M1-FLAG resin in Buffer B. The concentration of functional, purified receptor was determined using a saturation concentration (10 nM) of [3H]dihydroprenolol as described previously (21). FLAG-purified receptor was diluted to a concentration of 1 μM and labeled with tetramethylrhodamine-5-maleimide (Molecular Probes) at a final concentration of 1 μM for 1 h on ice. Labeled receptor was then purified by alprenolol-Sepharose chromatography as described previously (21). 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. Two liters of SF9 cells typically yield 500 μl of a 5 μM solution of tetramethylrhodamine-labeled β2AR (TMR-β2AR).

Fluorescence Spectroscopy—Experiments were performed on a SPEX FluoroMax-3 spectrofluorometer with a photon counting mode using an excitation and emission bandpass of 3.2 nm. Approximately 25 pmol of TMR-β2AR was desalted into 500 μl of Buffer E immediately before spectroscopy. For time course experiments, excitation was at 541 nm, and emission was monitored at 571 nm. Unless otherwise indicated, all experiments were performed at 30 °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 in the concentrations used, excluding any inner filter effect in the fluorescence experiments. Spectra were corrected for fluorescence of the compounds tested and labeled with tetramethylrhodamine-5-maleimide (Molecular Probes) at a final concentration of 1 μM for 1 h on ice.

cAMP Accumulation—The production of cAMP was determined by adenyl cyclase activation FlashPlate assay (PerkinElmer Life Sciences) as described previously (22).

Agonist-induced Internalization—HEK293 cells expressing FLAG-β2AR were cultured in poly-lysine-coated 12-well plates for 24 h. The cells were stimulated with different drugs for 10 min before fixing without permeabilization with 4% paraformaldehyde in phosphate-buffered saline (with Ca2+ and Mg2+). After blocking with 2.5% goat serum in phosphate-buffered saline (with Ca2+ and Mg2+), the cells were stained with Alexa-488 (Molecular Probes)-conjugated M1 antibody (clone 1A6) at a concentration of 1 μg/ml for 30 min. The unbound antibody was removed by washing four times with phosphate-buffered saline (with Ca2+ and Mg2+). The cells were harvested with 1% SDS in phosphate-buffered saline, and the intensity of Alexa-488 emission was measured on a FluorMax-3 spectrofluorometer. The excitation was at 485 nm, and the emission was from 495 to 580 nm with an integration time of 0.3 s/nm. The intensity traces from 495 to 580 nm was normalized after subtracting the background from cells without M1 antibody.

Statistical Analysis—Curve fitting and statistical analysis were performed using Prism (GraphPad Software, Inc.).

RESULTS AND DISCUSSION

Monitoring Agonist-induced Conformational Changes in the β2AR with a Fluorescent Probe at the Cytoplasmic End of TM6—To study agonist-induced conformational changes in the β2AR, we monitor fluorescence intensity of purified β2AR labeled at Cys-265 with tetramethylrhodamine maleimide (TMR-β2AR) as a function of time. We have shown previously that it is possible to monitor agonist-induced conformational changes in β2ARs labeled at Cys-265 with either fluorescein maleimide (10, 20) or tetramethylrhodamine maleimide (23). An environmentally sensitive fluorophore covalently bound to Cys-265 is well positioned to detect agonist-induced conformational changes relevant to G protein activation. Based on homology with rhodopsin (2), Cys-265 is located in the third intracellular loop (IC3) at the cytoplasmic end of the transmembrane 6 (TM6) (Fig. 1). Mutagenesis studies have shown this region of IC3 to be important for G protein coupling (24, 25). Moreover, TM6, along with TM3 and TM5, contain amino acids that form the agonist binding site. The sites of interaction between catecholamines and the β2AR have been extensively characterized (26–28) and are summarized in Fig. 1. The amine nitrogen interacts with Asp-293 in TM6 (27), and the aromatic ring interacts with Phe-290 in TM6 (26). Also shown is the relative position of Cys-265, the labeling site for tetramethylrhodamine.

Fig. 1. Binding site for norepinephrine in the β2AR. Amino acids in the β2AR involved in ligand binding have been identified using a combination of site-directed mutagenesis and modified ligands (26–28). The catecholamine nitrogen interacts with Asp-113 in TM3 (26). Hydroxyls on the catechol ring interact with serines 203 (28), 204, and 207 (28) in TM6. The chiral β-hydroxyl interacts with Asp-293 in TM6 (27), and the aromatic ring interacts with Phe-290 in TM6 (26). Also shown is the relative position of Cys-265, the labeling site for tetramethylrhodamine.
Fig. 2. Norepinephrine (Norepi) induces a biphasic conformational change in TMR-β2AR. As shown in A, agonist-induced conformational changes in response to norepinephrine and dopamine were examined by monitoring changes in the fluorescence intensity of TMR-β2AR as a function of time. The curves represent the average of three experiments. The response to norepinephrine was best fit with a two-site exponential association function (p < 0.0001), whereas there was no significant difference between a one-site and a two-site fit for the response to dopamine. The rapid and slow components of the biphasic response to norepinephrine are shown as dotted lines. B, a comparison of the conformational response to (+) and (−) enantiomers of norepinephrine. The chiral β-hydroxyl (red) determines the direction of the slow component of the biphasic response. The tracings are representative of three independent experiments performed on the same preparation of TMR-β2AR. All drugs were added to achieve a final concentration of 100 μM.

Fig. 3. Conformational changes in TMR-β2AR in response to a panel of catecholamine-related ligands reveal the structural features of catecholamine ligands responsible for the rapid and slow components of the biphasic conformational change. A, changes in fluorescence intensity in response to the natural catecholamines dopamine, norepinephrine, and epinephrine and the synthetic βAR agonist isoproterenol. A biphasic conformational response is observed in compounds that have a chiral β-hydroxyl. The magnitude and rate of the slow component of the biphasic response are influenced by the alkyl substituent of the amine on the ligand. As shown in B, changes in fluorescence intensity in response to dopamine, catechol, resorcinol, and tyramine demonstrate the role of the catechol hydroxyls in the rapid component of the biphasic conformational response to catecholamines. The tracings are representative of at least three independent experiments performed on the same preparation of TMR-β2AR. All drugs are added to achieve a final concentration of 100 μM.

TABLE I

| Drugs      | Fast component, t½ (sec) | Slow component, t½ (sec) | Number |
|------------|--------------------------|--------------------------|--------|
| Isoproterenol | 2.6 ± 0.2                | 147 ± 10                 | 19     |
| Epinephrine     | 4.5 ± 0.6                | 164 ± 20                 | 10     |
| Norepinephrine  | 2.8 ± 0.4                | 70.2 ± 6.1               | 10     |
| Dopamine       | 4.2 ± 0.3                |                          | 10     |

* n = number of experiments.

the amine nitrogen (present in both dopamine and norepinephrine) are rapid, whereas conformational changes associated with interactions between the receptor and the β-hydroxyl (present in norepinephrine) are ~10 times slower. This is somewhat surprising considering that the β-hydroxyl contributes ~2 kCal/mol of binding energy (as determined by equilibrium binding affinity). The relatively slow rate of formation of this interaction between the receptor and the β-hydroxyl suggests that the conformational transition required to accommodate this interaction involves traversing a relatively large energy barrier. Comparing the effects of (−) and (+) enantiomers of norepinephrine on TMR-β2AR further demonstrates the importance of the chiral β-hydroxyl in the slow phase of the conformational response to norepinephrine. As shown in Fig. 2B, a rapid phase of comparable magnitude is observed for both isomers; however, during the slow phase for (−) norepinephrine, there is a relative decrease in fluorescence. Thus, it appears that the β-hydroxyl of (−) norepinephrine binds to the β2AR but stabilizes a different conformational state. It is interesting that the rate of the slow component for (+) norepinephrine is comparable with that for (−) norepinephrine. However, we do not know whether the β-hydroxyl of (+) norepinephrine binds to the same amino acid side chain in the β2AR as the β-hydroxyl of (−) norepinephrine.

To further characterize the structural components responsible for the rapid and slow conformational changes, we examined the response to a panel of ligands that are structurally related to catecholamines (Fig. 3). Like norepinephrine, isoproterenol and epinephrine both have chiral β-hydroxyls and both induce a biphasic change in the intensity of TMR-β2AR (Fig.
The magnitude of the slow component (isoproterenol/epinephrine/norepinephrine) correlates with the presence of an alkyl substituent on the amine of the agonist. Moreover, the rate of the slow component for norepinephrine, which lacks an amine substituent, is significantly faster ($t_{1/2} < 70$ s) than that of the slow components for epinephrine ($t_{1/2} = 164 \pm 20$ s) and isoproterenol ($t_{1/2} = 147 \pm 10$ s) (Table I). Thus, like receptor interactions with the chiral $\beta$-hydroxyl, conformational changes involving interactions between the amine substituent and the $\beta_2$AR are relatively slow.

The Catechol Ring Is Required for the Rapid Component of the Biphasic Conformational Change—To understand the structural basis of the rapid phase of TMR-$\beta_2$AR response to catecholamines, we compared responses to dopamine, tyramine, catechol, and resorcinol (Fig. 3B). Tyramine, which differs from dopamine in lacking the meta-hydroxyl on the catechol ring, does not induce a detectable change in the fluorescence of TMR-$\beta_2$AR. Thus, the catechol structure is essential for the rapid conformational response. In fact, catechol alone induces a small, rapid response in TMR-$\beta_2$AR, whereas no detectable response is observed with resorcinol. The difference in the magnitude of the fluorescence response to dopamine and catechol can be attributed to the interaction of the amine nitrogen of dopamine and Asp-113 in TM3 of the $\beta_2$AR (Fig. 1). Therefore, this interaction must occur on a similar rapid time scale ($t_{1/2} < 5$ s).

Sequential Binding Model—These fluorescence experiments provide evidence for a sequential binding model (Fig. 4) in which the process of binding of a small organic agonist occurs by kinetically distinguishable steps through discrete intermediate conformational states. This model proposes that the unliganded receptor exists in a dynamic and relatively flexible state $R$ (see Fig. 4) that can undergo transitions to an undetermined number of states. The flexibility of the unliganded state is based on fluorescence lifetime studies (20) and the observation that $R$ is more susceptible to thermal denaturation (29) and proteolysis (30). Moreover, $R$ may undergo spontaneous transitions to a state capable of activating the $G$ protein, explaining the high basal activity observed for some GPCRs (31). We propose that in the $R$ state, the specific amino acid side chains involved in agonist binding (Fig. 1) are not arranged to simultaneously coordinate the ligand. These interactions between the receptor and the agonist are formed sequentially, such that each interaction increases the probability of the subsequent interaction. The process results in a series of intermediate states ($R^1$ and $R^2$ and $R^3$). In our model, we hypothesize that $R^1$ results from interactions between the catechol ring of the agonist and TMs 5 and 6 of the receptor. $R^2$ occurs when the amine nitrogen interacts with TM3. Our speculation that binding of the catechol ring precedes the binding of the amine is based on the

![Sequential Binding Model](sequential_binding_model.png)
observation that catechol alone induces a rapid conformational response in TMR-β2AR (Fig. 3B). In contrast, tyramine, which lacks an intact catechol ring but has the amine group, produces no conformational change that can be detected by our reporter on TM6. Nevertheless, the magnitude of the response for dopamine is greater than that for catechol, suggesting that the interaction between the amine and Asp-113 facilitates or stabilizes the interaction of the catechol ring with TM5 and TM6. Nevertheless, the magnitude of the response for dopamine is greater than that for catechol, suggesting that the interaction between the amine and Asp-113 facilitates or stabilizes the interaction of the catechol ring with TM5 and TM6.

The Rapid and Slow Conformational States May Have Different Functional Properties—The relatively large difference in the rates of the rapid and slow conformational responses suggest that the different receptor states may have distinct functional properties. Agonist binding promotes interactions between the β2AR and Gs, thereby activating signaling cascades. Agonists also promote interactions between the β2AR and Gβγ subunits, which block access to the receptor and protein hormones where there are a larger number of sites for interaction between receptor and agonist. A better understanding of this conformational heterogeneity will facilitate the design of more effective and selective pharmaceuticals.

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Fig. 5. Cellular responses to catecholamines. A, cAMP accumulation in HEK293 cells expressing β2AR in response to isoproterenol (Iso), epinephrine (Epi), norepinephrine (NEpi), dopamine (Dop), tyramine (Tyr), and catechol (Cat). Cells were treated with ligands at the indicated concentrations for 10 min at 37 °C. B, ligand-induced internalization of β2AR in HEK293 cells following a 10-min exposure at 37 °C to the indicated ligands.
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