Functionally Different Agonists Induce Distinct Conformations in the G Protein Coupling Domain of the β_2_ Adrenergic Receptor*

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G protein-coupled receptors represent the largest class of drug discovery targets. Drugs that activate G protein-coupled receptors are classified as either agonists or partial agonists. To study the mechanism whereby these different classes of activating ligands modulate receptor function, we directly monitored ligand-induced conformational changes in the G protein-coupling domain of the β_2_ adrenergic receptor. Fluorescence lifetime analysis of a reporter fluorophore covalently attached to this domain revealed that, in the absence of ligands, this domain oscillates around a single detectable conformation. Binding to an antagonist does not change this conformation but does reduce the flexibility of the domain. However, when the β_2_ adrenergic receptor is bound to a full agonist, the G protein coupling domain exists in two distinct conformations. Moreover, the conformations induced by a full agonist can be distinguished from those induced by partial agonists. These results provide new insight into the structural consequence of antagonist binding and the basis of agonism and partial agonism.

G protein-coupled receptors (GPCRs) are remarkably versatile biological sensors. They are responsible for the majority of cellular responses to hormones and neurotransmitters, as well as for the senses of sight, smell, and taste. Our current models of the mechanism of GPCR activation by diffusible agonists have been deduced from indirect measures of receptor conformation, such as G protein or second messenger activation (1–4). These indirect assays of GPCR activity provide only limited insight into the agonist-induced structural changes that define the active state of the receptor.

To elucidate the mechanism of GPCR activation by diffusible agonists, we developed a means for directly monitoring the active conformation of purified, detergent-solubilized β_2_ adrenergic receptor (β_2_ AR) by site-specific labeling of an endogenous cysteine (Cys^265) with fluorescein maleimide (FM-β_2_ AR) (5). Based on homology with rhodopsin (6), Cys^265 is located in the third intracellular loop (IC3) at the cytoplasmic end of the transmembrane domain 6 (TM6) α helix (Fig. 1A). Mutagenesis studies have shown this region of IC3 to be important for G protein coupling (7, 8). An environmentally sensitive fluorophore covalently bound to Cys^265 is therefore well positioned to detect antagonist-induced conformational changes relevant to G protein activation. The effect of agonists and partial agonists on the fluorescence intensity of FM-β_2_ AR correlates well with their biological properties (5). Binding of the full agonist isoprotrenanol induces a conformational change that decreases the fluorescence intensity of FM bound to Cys^265 by ~15% (Fig. 1B), whereas binding of partial agonists results in smaller changes in intensity, and binding of an antagonist has no effect (5).

Agonist-induced movement of FM bound to Cys^265 was characterized by examining the interaction between the fluorescein at Cys^265 and fluorescence quenching reagents localized to different molecular environments of the receptor. The results of these experiments are most consistent with either a clockwise rotation of TM6 and/or a tilting of the cytoplasmic end of TM6 toward TM5 (5). Our findings suggest that the conformational changes associated with β_2_ AR activation are similar to those in rhodopsin (9) and indicate a shared mechanism of GPCR activation.

These results provide insight into the nature of the structural changes that occur upon agonist binding. However, several mechanistic questions remain. Using conventional spectroscopy, we observe no change in the fluorescence intensity from FM-β_2_ AR upon antagonist binding. This could indicate that antagonists do not alter receptor structure or that the structural changes are not detectable by FM bound to Cys^265. Of greater interest is the structural basis of partial agonism. Partial agonists induce a smaller change in intensity of FM-β_2_ AR than do full agonists. Two models could explain this observation. If we assume that the receptor exists in two functional conformational states, inactive or active, then a partial agonist may simply induce a smaller fraction of receptors to undergo the transition to the active state than does the full agonist. Alternatively, partial agonists may induce a conformation distinct from that induced by full agonists. Conventional fluorescence spectroscopy, which represents an average intensity over a population of fluorescent molecules, does not allow us to distinguish between these two models. We therefore used fluorescence lifetime spectroscopy, which is capable of distinguishing subsets within a population of fluorescent molecules, to look for ligand-specific conformational states. Our results indicate that partial agonists and agonists induce distinct conformations. Moreover, we observe structural effects of antagonist binding that could not be detected by conventional
Fluorescence Spectroscopic Studies of the β2AR—Construction, expression, and purification of human β2AR were performed as described (10). Purified, detergent-solubilized wild-type receptor was labeled with FM at Cys265 and examined by fluorescence spectroscopy. Change in intensity of FM-bound β2AR on fluorescence intensity of FM-bound β2AR subsequent to ISO followed by the reversal by the neutral antagonist ALP. These results help elucidate the structural mechanisms that underlie ligand efficacy.

**EXPERIMENTAL PROCEDURES**

**Fluorescence Spectroscopic Studies of the β2AR**

**Construction and expression**.

The receptor was expressed in Escherichia coli to yield a soluble protein that could be purified by affinity chromatography. The purified receptor was then solubilized in dodecyl-β-D-maltoside, a detergent that allows the receptor to maintain its native conformation. The solubilized receptor was then labeled with fluorescein maleimide to generate FM-bound β2AR. The labeling reaction was monitored by fluorescence spectroscopy, which allowed detection of lifetimes ranging from several nanoseconds to a few picoseconds. Samples were placed in a 10-mm path length cuvette. The excitation was provided by the frequency-doubled output of a cavity-dumped pyridine-2 dye laser tuned at 370 nm synchronously pumped by a mode-locked argon ion laser. Sample emission was filtered through Corning 3–72 and 4–96 filters. For the reference signal, 4-(dimethylamino)4′-cyanostilbene in methanol (463-ps fluorescence lifetime) was observed through the same filter combination. The governing equations for the time-resolved intensity decay data were assumed to be a sum of discrete exponentials as in Equation 2,

\[ I(t) = I_0 \sum \alpha_i e^{\alpha_i \tau_i} \]  

(Eq. 2)

where \( I(t) \) is the intensity decay, \( \alpha \) is the amplitude (pre-exponential factor), and \( \tau \) is the fluorescence lifetime of the \( i \)-th discrete component or a sum of Gaussian distribution functions as in Equation 3,

\[ I(t) = I_0 \sum \alpha_i(\tau_i)e^{\alpha_i \tau_i} \]  

(Eq. 3)

and Equation 4,

\[ \alpha_i(\tau) = \frac{1}{\sigma \sqrt{2\pi}} e^{-\frac{1}{2}(\frac{\tau - \tau_i}{\sigma})^2} \]  

(Eq. 4)

where \( \tau \) is the center value of the lifetime distribution, and \( \sigma \) is the standard deviation of the Gaussian, which is related to the full width at half maximum by 2.354 \( \sigma \). In the frequency domain, the measured quantities at each frequency \( \omega \) are the phase shift (\( \phi \)) and demodulation factor (\( m \)) of the emitted light versus the reference light. Fractional intensity, amplitude, and lifetime parameters were recovered by a non-linear least-squares procedure using the software developed at the Center for Fluorescence Spectroscopy. The measured data were compared with calculated values (\( \phi, m \)) and the goodness of fit was characterized by Equation 5,

\[ \chi^2 = \frac{1}{v} \sum_{\alpha} \left( \frac{\theta - \phi}{\delta \theta} \right)^2 + \frac{1}{v} \sum_{\alpha} \left( \frac{m - m_\alpha}{\delta m} \right)^2 \]  

(Eq. 5)

where \( v \) is the number of degrees of freedom, and \( \delta \theta \) and \( \delta m \) are the uncertainties in the measured phase and modulation values, respectively. The sum extends over all frequencies (\( \omega \)).

**RESULTS AND DISCUSSION**

**Using Fluorescence Lifetime Spectroscopy to Study Ligand-induced Conformational Changes in the β2AR**

The receptor was purified and labeled at Cys265 with fluorescein maleimide to generate FM-bound β2AR as described previously (5). We examined ligand-dependent changes in fluorescence lifetime of FM-bound β2AR in an effort to identify the existence of agonist-specific conformational states. Fluorescence lifetime analysis can detect discrete conformational states in a population of molecules, whereas fluorescence intensity measurements reflect the weighted average of one or more discrete states. Based on the changes in steady-state fluorescence intensity we observed, we predicted that ligand-induced conformational changes in the receptor would alter the fluorescence lifetime of the fluorophore. Fluorescence lifetime, \( \tau \), refers to the average time that a fluorophore that has absorbed a photon remains in the excited state before returning to the ground state. The lifetime of fluorescein (nanoseconds) is much faster than the predicted off-rate of the agonists we examined (\( \mu \)-ms) and much shorter than the half-life of conformational states of bacteriorhodopsin (\( \mu \)-s) (12) or rhodopsin (\( \mu \)-s) (13, 14) or of ion channels (\( \mu \)-s) (15). Therefore, lifetime analysis of fluorescein bound to Cys265 is well suited to capture even short-lived, agonist-induced conformational states.

**Antagonist Binding Narrows the Distribution of Fluorescence Lifestyles**

Data from fluorescence lifetime experiments on FM-bound β2AR bound to different drugs at equilibrium were analyzed in two ways. Traditionally, fluorescence decays are fit to single and multiple discrete exponential functions, and the best
Agonist-specific Conformations of the β2 Adrenoceptor

The observed fluorescence decay was resolved into one or more exponential components, with each component, \(i\), being described by \(\tau_i\) and \(a_i\), where \(a_i\) represents the fractional contribution of \(\tau_i\) to the overall decay. The best fit to single or multiple components was determined by \(\chi^2\) analysis. If different agonists induce a single active state, then the fluorescence lifetime associated with that state \((\tau_{ISO})\) should be the same for different drugs, and only the fractional contributions \((a_{ISO}/a_x)\) should differ. However, if there are agonist-specific conformational states we should observe unique, agonist-specific lifetimes (e.g., \(\tau_{ISO}, \tau_{SAL}, \text{and } \tau_{DOB}\)).

<table>
<thead>
<tr>
<th>Drug</th>
<th>(\tau_1 (\text{ns}))</th>
<th>(\tau_2 (\text{ns}))</th>
<th>(a_2)</th>
<th>(\chi^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No Drug</td>
<td>4.22 ± 0.02</td>
<td></td>
<td>2.9 ± 0.4</td>
<td></td>
</tr>
<tr>
<td>ALP</td>
<td>4.21 ± 0.01</td>
<td></td>
<td>3.3 ± 0.8</td>
<td></td>
</tr>
<tr>
<td>ISO</td>
<td>4.30 ± 0.01</td>
<td>0.77 ± 0.05</td>
<td>0.19 ± 0.03</td>
<td>3.3 ± 1.0</td>
</tr>
<tr>
<td>SAL</td>
<td>4.35 ± 0.02</td>
<td>1.45 ± 0.16</td>
<td>0.08 ± 0.01</td>
<td>2.0 ± 0.2</td>
</tr>
<tr>
<td>DOB</td>
<td>4.36 ± 0.01</td>
<td>1.68 ± 0.3</td>
<td>0.07 ± 0.01</td>
<td>1.8 ± 0.4</td>
</tr>
</tbody>
</table>

**FIG. 2.** Effect of drugs on fluorescence lifetime distributions of FM-β2AR. Fluorescence lifetimes were determined by phase modulation, and lifetime distributions of FM-β2AR were calculated in the absence of ligand (black trace), with the neutral antagonist ALP (red trace), or in the presence of the full agonist ISO (green trace). The mean lifetime and the full width at half maximum for the distributions are as follows: No Drug, \(\tau = 4.21 ± 0.01 \text{ ns}; \text{FWHM} = 1.1 ± 0.1; \chi^2 = 2.8\); ALP, \(\tau = 4.21 ± 0.01 \text{ ns}; \text{FWHM} = 0.7 ± 0.2; \chi^2 = 2.9\); ISO, \(\tau_{LONG} = 4.36 ± 0.08 \text{ ns}; \text{FWHM}_{LONG} = 0.5 ± 1.1; \text{FWHM}_{SHORT} = 0.76 ± 0.33 \text{ ns}; \chi^2 = 3.2\).

fit is determined by \(\chi^2\) analysis (Table I). This discrete component analysis assumes that the receptor exists in one or a few rigid protein conformations and does not accurately reflect the dynamic nature of proteins. Proteins that are functionally in a single conformational state actually undergo small conformational fluctuations around a minimum energy state (16), and these small structural perturbations can lead to small changes in the environment around an attached fluorophore. These perturbations are thought to reflect local unfolding reactions within the three-dimensional structure of proteins (17). Such flexibility in protein structure can be modeled using fluorescence lifetime distributions (18), wherein the width of the distributions reflects the conformational flexibility of the protein (Fig. 2). The mobility of fluorescein relative to the receptor is minimal, as determined by its high measured anisotropy \((r = 0.30 ± 0.02; n = 3)\) and therefore would be expected to contribute little to the width of the lifetime distribution. Thus, the width of the distribution can be attributed to conformational flexibility in the receptor itself.

Lifetime analysis of unliganded FM-β2AR reveals a single, flexible state. This is indicated by both the single, broad Gaussian distribution of lifetimes centered around 4.2 ns (Fig. 2, black trace) and the discrete component analysis, where the fluorescence decay rate of FM-β2AR in the absence of any drug is best fit by a single exponential function (Table I). Binding of the neutral antagonist ALP to FM-β2AR does not significantly change the fluorescent lifetime (Table I) but does narrow the distribution of lifetimes (Fig. 2, red trace), suggesting that ALP stabilizes the receptor and reduces conformational fluctuations. This interpretation is consistent with the results of experiments demonstrating that the β2AR is more resistant to protease digestion when bound to ALP (19).

**Agonists and Partial Agonists Induce Distinct Conformations**—Unexpectedly, binding of the full agonist ISO promotes conformational heterogeneity. In the presence of saturating concentrations of ISO, FM-β2AR has two distinguishable fluorescence lifetimes (see Fig. 2, green trace, and Table I) representing at least two distinct conformational states. The long lifetime component is only slightly longer than the lifetime observed in the absence of drugs; however, the distribution is narrower than that observed in the presence of the antagonist ALP (Fig. 2, green and red traces). In contrast, the distribution of the short lifetime component observed in the presence of ISO...
is relatively broad, suggesting that there is considerable flexibility around Cys265 in this agonist-induced conformation.

We next examined the effect of the partial agonists salbutamol (SAL) and dobutamine (DOB) on the fluorescence lifetime of FM-$\beta_2$AR. Similar to ISO, we observed two lifetimes when the receptor was bound to saturating concentrations of SAL and DOB (see Table I and Fig. 3). The long lifetime component found in the presence of these two partial agonists is indistinguishable from that observed in the ISO-bound receptor; however, the short lifetime component found in both the SAL- and DOB-bound receptor is statistically different from that for the ISO-bound receptor. We observe a strong correlation between a reduction in fluorescence intensity and drug efficacy (5), and shortening of the average fluorescence lifetime is associated with a reduction in fluorescence intensity. Therefore, the short lifetime, found only in the presence of agonists, likely represents the G protein-activating conformation of FM-$\beta_2$AR.

The different short lifetimes for the full agonist (ISO) and the partial agonists (SAL and DOB) indicate different molecular environments around the fluorophore and therefore represent different, agonist-specific active states. The narrowing and rightward shift of the long lifetime component following binding of both agonists and partial agonists indicate that this lifetime also reflects an agonist-bound state but most likely represents a more abundant intermediate state that would not be expected to alter greatly the intensity of FM bound to Cys265. It is possible that the number of conformations that we observe in these experiments represent only a few of the possible conformations that can be stabilized by drugs. Moreover, whereas the overlapping short lifetime distributions of SAL and DOB (see Fig. 3B and Table I) suggest that they induce similar conformations, it is possible that a conformationally sensitive probe positioned elsewhere on the receptor could distinguish between DOB- and SAL-bound receptor states.

Models of GPCR Activation—According to the prevailing two-state model of GPCR activation, receptors exist in an equilibrium between a resting (R) state and an active (R*) state that stimulates the G protein (20–22). Agonists preferentially enrich the R* state, whereas inverse agonists select for the R state of the receptor. Neutral antagonists possess an equal affinity for both states and function simply as competitors. In this simple system, functional differences between drugs can be explained by their relative affinity for the single active R* state (Fig. 4A). Alternatively, differences in efficacy between drugs can be because of ligand-specific receptor states (23–25). Based on our lifetime experiments, we propose a model with multiple, agonist-specific receptor states, wherein activation occurs through a sequence of conformational changes. Upon agonist binding, the receptor undergoes a conformational change to an intermediate state (I) that is associated with a narrowing and rightward shift in the long lifetime distribution. The less abundant active state, represented by the short lifetime, is different for the full agonist ISO (R*) and the partial agonists DOB and SAL (R'). The relatively slow, temperature-dependent rate of change of fluorescence intensity following agonist binding and the rapid rate of reversal by antagonist (see Ref. 5 and Fig. 1B) suggest that transitions from the intermediate state to the active state are relatively rare high energy events. It is likely that in vivo the active conformation is further stabilized by interactions between the receptor and its cognate G protein $G_{i}$. Thus, one might expect the proportion of receptor in the active state to be greater when the receptor is coupled with $G_{i}$.

Conclusions—Our results have implications for drug discovery and efforts to obtain high resolution crystal structures of GPCRs. The conformational flexibility observed in the ligand-free receptor (Fig. 2) may make it particularly challenging to obtain crystals in the absence of a bound ligand. Of greater concern, the existence of two conformational states in the presence of saturating concentrations of full and partial agonists (Fig. 3) will impact efforts to obtain a high resolution structure of the active, agonist-bound receptor. If such an agonist-bound structure is obtained, it will likely represent the more energetically stable of the two conformations and may not be the maximally active conformation. Thus, the use of such a structure for rational drug design efforts may have significant limitations. In support of this contention, recent structural analyses of the intermediate conformational states of the proton pump bacteriorhodopsin have revealed discrepancies attributed to energetic inhibition of conformational states within the three-dimensional crystal (12, 26). Finally, our results indicate that GPCRs are relatively plastic. The number of conformations that we observed in these experiments may represent only a few of a larger spectrum of possible conformations that could be stabilized by drugs. Thus, it may be possible to identify even more potent agonists or agonists that can alter G protein coupling specificity.

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