Agonist-induced conformational changes in the G-protein-coupling domain of the $\beta_2$ adrenergic receptor

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The majority of extracellular physiologic signaling molecules act by stimulating GTP-binding protein (G-protein)-coupled receptors (GPCRs). To monitor directly the formation of the active state of a prototypal GPCR, we devised a method to site specifically attach fluorescein to an endogenous cysteine (Cys-265) at the cytoplasmic end of transmembrane 6 (TM6) of the $\beta_2$ adrenergic receptor ($\beta_2$AR), adjacent to the G-protein-coupling domain. We demonstrate that this tag reports agonist-induced conformational changes in the receptor, with agonists causing a decline in the fluorescence intensity of fluorescein-$\beta_2$AR that is proportional to the biological efficacy of the agonist. We also find that agonists alter the interaction between the fluorescein at Cys-265 and fluorescence-quenching reagents localized to different molecular environments of the receptor. These observations are consistent with a rotation and/or tilting of TM6 on agonist activation. Our studies, when compared with studies of activation in rhodopsin, indicate a general mechanism for GPCR activation; however, a notable difference is the relatively slow activation in rhodopsin, indicating a general mechanism for GPCR agonist activation. Our results indicate that during agonist-induced activation, this portion of the receptor’s G-protein-coupling domain rotates and/or tilts to a more hydrophobic environment, which is closer to both the surface of the micellar compartment and TM5. These observations provide insight into the real-time agonist-induced movement of the G-protein-coupling domain of a GPCR activated by diffusible ligands.

Materials and Methods

Construction, Expression, and Purification of the $\beta_2$AR. Construction, expression, and purification of human $\beta_2$AR were performed as described (15). The mutations Glu-224→Lys, Cys-378→Ala, and Cys-406→Ala were all generated on a background in which all of the lysines in the receptor had been mutated to arginine (16). A sequence coding for the cleavage site for the tobacco etch virus protease (GIBCO/BRL) was added to the 5’ end of the receptor construct via the linker-adapter method. All mutations were confirmed by restriction enzyme analysis and sequenced. The mutant receptor demonstrated only minor alterations in the general pharmacological properties of the receptor, as assessed by the affinity of the mutant receptor for (–)isoproterenol (ISO) and (–)alprenolol (ALP) [K$_D$ for ISO = 150 ± 40 μM for the mutant receptor vs. 210 ± 21 μM for the wild-type receptor (17); $K_D$ for ALP = 4.3 ± 0.6 nM for the mutant receptor vs. 1.7 ± 0.9 nM for wild-type receptor (18)].

FluorescentLabeling of Purified $\beta_2$AR. Purified detergent-soluble receptor was diluted to 1 μM in HS buffer [20 nM Tris, pH 7.5, 500 mM NaCl/0.1% N-dodecyl maltoside (NDM)] and reacted with 1 μM FM (Molecular Probes) for 2 h on ice in the dark. The reaction was quenched with the addition of 1 mM cysteine. The receptor was bound to a 250 μl Ni-chelating structure is available (5), Cys-265 is predicted to be at the cytoplasmic end of the α-helix of transmembrane (TM) 6 (Fig. 1A and B). Mutagenesis studies have demonstrated that sequences immediately carboxyl-terminal to Cys-265 are important for G-protein coupling (6–10), and mutation of the nearby Leu-272 leads to constitutive activation (11). In addition, synthetic peptides representing sequences from the carboxyl-terminal portion of IC3 are capable of directly stimulating G proteins (12–14). Therefore, a fluorophore covalently bound to Cys-265 is well positioned to detect agonist-induced conformational changes relevant to G-protein activation. Our results indicate that during agonist-induced activation, this portion of the receptor’s G-protein-coupling domain rotates and/or tilts to a more hydrophobic environment, which is closer to both the surface of the micellar compartment and TM5. These observations provide insight into the real-time agonist-induced movement of the G-protein-coupling domain of a GPCR activated by diffusible ligands.

Abbreviations: G protein, GTP-binding protein; $\beta_2$AR, $\beta_2$ adrenergic receptor; TM, transmembrane; ISO, (–)isoproterenol; ALP, (–)alprenolol; NHS, N-hydroxysuccinimide; S-DOX, S-doxyl stearate; CAT-16, 4-(N,N-dimethyl-N-hexadecyl)ammonium-2,2,6,6-tetramethylpiperidine-1-oxyl iodide; GPCR, G-protein-coupled receptor.

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receptor, on the basis of an extinction coefficient of 83,000 M

control samples without receptor was negligible. The labeling

in HS buffer for fluorescence measurements. Fluorescence in

FM. The labeled protein (FM-

Tris, pH 7.5

under the conditions described in

Fig. 1: Schematic diagram of the secondary structure of the β2AR illustrating

the FM-labeling site at Cys-265. (A) There are 13 cysteines (yellow circles) in the

β2AR, yet only Cys-265 is labeled with the relatively large polar fluorophore FM

under the conditions described in Materials and Methods. Cys-106, Cys-184,

Cys-190, and Cys-191 have been shown to be disulfide bonded (38, 39), and

Cys-341 is palmitoylated (40). Cys-378 and Cys-406 in the carboxyl terminus

form a disulfide bond during purification (data not shown). Labeling speci-

ficity was confirmed by peptide mapping and mutagenesis of potential reac-

tive cysteines (data not shown). The sites of peptide cleavage by Factor Xa

were plotted according to the Stern–Volmer equation, Fo/F = 1 + Ksv[QI], where FOF

is the ratio of fluorescence intensity in the absence and presence of QI, and Ksv is the Stern–Volmer

quenching constant. The Ksv values thus obtained were then used

with the measured fluorescence lifetimes (τo) to determine the bimolecular quenching constant, kq (Ksv = kq · τo) (19). For quenchers, a time scan was initiated after the emission scan and 100 μM (-)-ISO was added after 2 min. At 10 min, 20 μM ALP was added and the extent of reversal determined. The quenchers used did not alter the ability of ISO or ALP to compete with [3H]DHA.

Results and Discussion

The Effect of Full and Partial Agonists on the Fluorescence of FM-β2AR Correlates with the Biological Properties of the Agonists. Only Cys-265 is labeled when purified detergent-solubilized β2AR

amino-terminal after labeling the receptor with an

amine-reactive tag. The location of the FM-labeling site at

Cys-265 in both the wild-type and mutant receptors was verified by peptide mapping with protease factor Xa and cyanogen bromide (the cleavage sites are indicated in Fig. 1A).
(1 μM) is reacted with FM at a 1:1 stoichiometry. This polar fluorophore does not label TM cysteines, and the two potential accessible cysteines in the carboxyl terminus (Fig. 1) form a disulfide bond during purification (data not shown). The biological efficacy (GTP binding or G protein coupling in a reconstitution assay (data not shown)) that affect the baseline. ALP alone did not induce any changes in fluorescence, and treatment with ligands did not cause a change in the wavelength of maximum emission (data not shown). (B) Agonist and partial agonist effects on the intensity of FM-β2AR are compared with an assay of biological efficacy (GTPγS binding). FM-β2AR was treated with different agonists, and the change in fluorescence was measured at a time equal to 5 times the calculated t1/2 for each drug. All agonists were used at 100 μM to ensure saturation of the receptors and eliminate the effect of variations in agonist affinities. The ability of these ligands to stimulate GTPγS binding or G protein coupling in a reconstitution assay (data not shown) correlates with the biological efficacy of these drugs in β2AR-mediated activation of G proteins in membranes (Fig. 2B). These experiments verify that fluorescence intensity changes in FM-β2AR reflect biologically relevant ligand-induced conformational changes.

**Kinetics of Agonist-Induced Conformational Change.** Rhodopsin has long been used as a model system for direct biophysical analyses of GPCR activation because of its natural abundance, inherent stability, and spectroscopically defined activation scheme (20). The recent crystal structure of bovine rhodopsin (5) provides the first high-resolution picture of the inactive state of this highly specialized GPCR. Although the general features of this structure presumably apply across the broad family of GPCRs, the mechanism of rhodopsin activation is unique among GPCRs because of the presence of a covalent linkage between the receptor and its ligand, retinal. Thus, the dynamic processes of agonist association and dissociation common to the GPCRs for hormones, neurotransmitters, and other sensory stimuli are not part of the activation mechanism of rhodopsin. In contrast to rhodopsin, the β2AR is activated by a functionally broad spectrum of diffusible ligands.

This difference between rhodopsin and the β2AR is reflected in the rate of agonist-induced structural changes. Conformational changes induced in detergent-solubilized preparations of rhodopsin by light activation are very rapid, occurring with a t1/2 of milliseconds (21, 22). In contrast, as shown in Fig. 2, agonist activation of the β2AR is slow, despite the rapid on-rate of agonist binding (t1/2 ~ 20 sec) calculated from the agonist affinity, the off-rate estimated from the ALP reversal of the agonist effect (Fig. 2A) and the concentration of agonists used in these experiments (100 μM). Under these conditions, the on-rate of agonist is comparable to the more rapid rate of reversal of the agonist effect by the antagonist ALP (t1/2 at 25°C = 22.8 ± 3.6 sec, mean ± SEM, n = 3). We have observed the same slow rate of agonist-induced conformational change with a different fluorescent reporter on Cys-125 in TM3 and on Cys-285 in TM6 of the β2AR (Fig. 1A) (23), and Salamone et al. observed a similar rate of agonist-induced conformational changes in the δ-opioid receptor analyzed by surface plasmon resonance spectroscopy (24). Thus, agonist binding precedes the conformational change. The rate of conformational change is temperature dependent, with the rate at 37°C ~3 times that at 25°C (data not shown). The slow temperature-dependent rate of conformational change and the rapid reversal suggest that the active state is a relatively high energy state that may be reached through one or more intermediate states (Eq. 1).

$$\frac{k_1}{k_2} A + R \underset{k_4}{\overset{k_3}{\rightleftharpoons}} AR' \underset{k_4}{\overset{k_3}{\rightleftharpoons}} AR^*$$

where R is the inactive receptor, R' is the agonist-bound inactive receptor, and R* is the active receptor.

Our data would predict that k3 is slow relative to k1, k2, and k4. Moreover, the agonist-binding site in R' may not be identical to the binding site in R*. The ligand-binding site for the β2AR has been well characterized by mutagenesis studies and lies relatively deep in the TM domains (Fig. 1A) (2, 4). A possible explanation for the sequence of binding followed by conformational change is that agonist binding is a stepwise process in which some receptor-ligand interactions form rapidly, whereas other interactions cannot form until stochastic conformational fluctuations make the complete binding pocket accessible (25). In the case of

**Fig. 1.** and cyanogen bromide (which cleavage at methionines, shown in form a disulfide bond during purification (data not shown). The biological efficacy (GTP binding or G protein coupling in a reconstitution assay (data not shown)) that affect the baseline. ALP alone did not induce any changes in fluorescence, and treatment with ligands did not cause a change in the wavelength of maximum emission (data not shown). (B) Agonist and partial agonist effects on the intensity of FM-β2AR are compared with an assay of biological efficacy (GTPγS binding). FM-β2AR was treated with different agonists, and the change in fluorescence was measured at a time equal to 5 times the calculated t1/2 for each drug. All agonists were used at 100 μM to ensure saturation of the receptors and eliminate the effect of variations in agonist affinities. The ability of these ligands to stimulate GTPγS binding or G protein coupling in a reconstitution assay (data not shown) correlates with the biological efficacy of these drugs in β2AR-mediated activation of G proteins in membranes (Fig. 2B). These experiments verify that fluorescence intensity changes in FM-β2AR reflect biologically relevant ligand-induced conformational changes.

**Fig. 2.** Effect of agonists and partial agonists on fluorescence intensity of FM-β2AR. (A) The change in intensity of FM-β2AR in response to the addition of the full agonist ISO and the strong partial agonist epinephrine was reversed by the neutral antagonist ALP. In most experiments, we use the ALP reversal to quantitate the magnitude of the agonist-induced change. We found the ALP reversal to be the most consistent measure for comparison of agonist-induced conformational changes, because ALP reversal occurs over a shorter period relative to agonist responses and therefore is less subject to nonspecific effects on fluorescence intensity (e.g., photobleaching, receptor denaturation) that affect the baseline. ALP alone did not induce any changes in fluorescence, and treatment with ligands did not cause a change in the wavelength of maximum emission (data not shown). (B) Agonist and partial agonist effects on the intensity of FM-β2AR are compared with an assay of biological efficacy (GTPγS binding). FM-β2AR was treated with different agonists, and the change in fluorescence was measured at a time equal to 5 times the calculated t1/2 for each drug. All agonists were used at 100 μM to ensure saturation of the receptors and eliminate the effect of variations in agonist affinities. The ability of these ligands to stimulate GTPγS binding in a β2AR-Gus fusion protein was determined, as previously described (41). All data represent experiments performed in triplicate.
the β2AR, the energy to stabilize this conformational change comes from the energy of binding, which is calculated to be 6–10 Kcal/mol [on the basis of the binding affinity of ISO to detergent-solubilized β2AR (18) or to β2AR in membranes (26)]. In contrast, the energy released by the photoisomerization of retinal is ~33 Kcal/mol (27). Thus, the difference in the rate of conformational change between rhodopsin and the β2AR can be attributed to the need for the ligand to diffuse into the binding pocket and the smaller energy associated with agonist binding.

It should be emphasized that our studies are done in the absence of G protein. Precoupling of the β2AR to its cognate G protein in cell membranes may accelerate agonist-induced conformational changes.

Agonist-Induced Movement of FM Bound to Cys-265 Relative to Lys-224. To distinguish between the movement of Cys-265 toward either TM3 or TM5, we generated a modified β2AR that permits site-specific attachment of an amine-reactive spin-labeled quencher at the cytoplasmic border of TM5 (Fig. 1C). To position the quencher at the base of TM5, we began with a template β2AR in which all of the lysines have been replaced by arginine (16) and changed Glu-224 to lysine. We purified this mutant and studied the interaction between FM at Cys-265 and oxyl-NHS at Lys-224. Although the baseline quenching of FM on Cys-265 with oxyl-NHS bound to Lys-224 was less that 10%, we observed that the effect of ISO on the FM fluorescence intensity (as reflected in the magnitude of the ALP reversal) was enhanced by more than 50% with the quencher bound to Lys-224 (Fig. 3B). Because the effect of this quencher is distance dependent, the increase in the extent of quenching reflects an agonist-induced conformational change that brings these regions of TM6 and TM5 closer together.

Agonist-Induced Movement of FM Bound to Cys-265 Relative to a Lipophilic Quencher in the Detergent Micelle. Because of the location of the fluorophore close to the predicted protein-lipid interface (Fig. 1B) of TM6, we next studied the interaction between the fluorophore and nitroxide spin-labeled fatty acids that partition into the detergent micelle to observe relative motion between the Cys-265 and the micelle (Fig. 4A). Because of their ability to quench the excited state of a variety of fluorophores in a distance-dependent manner, these spin-labeled fatty acid derivatives have been used extensively to study the distribution, location, and dynamics of fluorescently tagged proteins and lipids (30). We examined fatty acid derivatives with spin labels at two different locations along the carbon chain (Fig. 4A) and observed the best quenching of fluorescein by 4-(N,N-dimethyl-N-hexadecyl)ammonium-2,2,6,6-tetramethylpiperidine-1-oxyl iodide (CAT-16), which has a charged spin label on the head group of the fatty acid (Fig. 4B). The magnitude of the change in fluorescence intensity of FM-β2AR in response to the agonist ISO is dramatically increased in the presence of CAT-16 compared with the control fatty acid stearate (Fig. 4C). This effect was not observed with 5-doxyl stearate (5-DOX) (Fig. 4C). For example, 100 μM 5-DOX quenched baseline fluorescence by 12% (Fig. 4B) but had no significant effect on the magnitude of the agonist-induced change in fluorescence (Fig. 4C). In contrast, 50 μM CAT-16 produced a similar (~12%) soluble quencher KI, as expected on the basis of the predicted position of the fluorescein bound to Cys-265 (Fig. 1C). To determine the effect of agonist on KI quenching, we measured the fluorescence lifetimes of FM-β2AR in the presence ISO and ALP, which permitted us to calculate the bimolecular quenching constant, Φq, by using the average value of the lifetime of FM-β2AR in the presence of either ISO (Φq = 0.45 ± 0.01 × 10−9 M−1 s−1) or ALP (Φq = 0.51 ± 0.01 × 10−9 M−1 s−1) (28). There was no difference between the extent of KI quenching in the ligand-free or ALP-bound receptor (data not shown). However, the lower Φq in the ISO-bound state clearly shows that the fluorescein label on the β2AR is less accessible to the water-soluble quenching reagent KI in the presence of the agonist ISO (29). As a result, the fractional change in fluorescence intensity induced by ISO in the presence of 250 mM KI is smaller than in the presence of 250 mM KCl (Fig. 3B). Thus, ISO induces a conformational change that enhances the intrareceptor quenching of FM bound to Cys-265 but reduces access of Cys-265 to exogenous aqueous quencher KI. The burial of Cys-265 away from the aqueous milieu could be accomplished by a movement of TM6 toward the plasma membrane (Fig. 1B) and/or a movement of TM6 that would bring Cys-265 closer to either TM3 or TM5 (Fig. 1C).
Lipid concentration was kept constant at 100 nM with stearic acid. All values are mean ± SEM, n = 5.

Comparison of effects of quenchers localized to the micelle on the agonist-induced fluorescence change of FM-β2AR by increasing concentrations of CAT-16 or 5-DOX, as well as the putative location of these quenching groups in the micelle. The quenching group on CAT-16 is localized on the polar surface of the micelle. The quenching group on 5-DOX is located within the hydrophobic core of the micelle. (B) Stern–Volmer plots depicting the extent of quenching of FM-β2AR by increasing concentrations of CAT-16 or 5-DOX. Quenchers were added to labeled receptor, and fluorescence was measured and plotted as in Fig. 3 and Materials and Methods. The total lipid concentration was kept constant at 100 μM with stearic acid. The quenching constant $K_q$ was 2.4 ± 0.1 M$^{-1}$ in the presence of CAT-16 and 1.4 ± 0.2 M$^{-1}$ in the presence of 5-DOX. (C) Differences in real-time movement of the G-protein-coupling domain of this receptor. By site-specific labeling with a single fluorophore on the cytoplasmic end of TM6, we obtained evidence for movement of TM3 and TM6 on agonist activation (23). Recent fluorescence spectroscopic analysis of mutant β2ARs labeled on the cytoplasmic side of TM6 suggests that a rigid body motion occurs in this region during agonist-induced activation (34), whereas additional support for movement of TM3 and TM6 in the β2AR comes from zinc crosslinking studies (35) and chemical reactivity measurements and fluorescence spectroscopy (29), as well as by UV absorbance spectroscopy (32) and zinc crosslinking of histidines (33).

Earlier biophysical studies with β2AR labeled at multiple TM cysteines, we obtained evidence for movement of TM3 and TM6 on agonist activation (23). Recent fluorescence spectroscopic analysis of mutant β2ARs labeled on the cytoplasmic side of TM6 during rhodopsin activation was provided by chemical reactivity measurements and fluorescence spectroscopy (29), as well as by UV absorbance spectroscopy (32) and zinc crosslinking of histidines (33).

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Electron paramagnetic resonance spectroscopic studies provide evidence that photoactivation of rhodopsin involves a rotation and tilting of TM6 relative to TM3 (31). Further support for motion of TM6 during rhodopsin activation was provided by chemical reactivity measurements and fluorescence spectroscopy (29), as well as by UV absorbance spectroscopy (32) and zinc crosslinking of histidines (33).

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interface between TM5 and TM6, rendering it less accessible to the aqueous quencher KI. Also, these conformational changes would bring FM on Cys-265 closer to both a nitroxide spin quencher covalently bound to Lys-224 and to a nitroxide quencher in the micelle surrounding the TM domains. Our data are not consistent with a large piston-like movement of TM6 into the membrane, although they do not exclude a small screw-like movement of the fluorophore toward the membrane that might accompany a clockwise rotation of TM6. Thus, our findings suggest that the conformational changes associated with β2AR activation are similar to those in rhodopsin and indicate a shared mechanism of GPCR activation.

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