Agonist-induced Conformational Changes at the Cytoplasmic Side of Transmembrane Segment 6 in the β2 Adrenergic Receptor Mapped by Site-selective Fluorescent Labeling*

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The environmentally sensitive, sulphhydryl-reactive, fluorescent probe N,N’-dimethyl-N-(iodoacetyl)-N’-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) ethylene-diamine (IANBD) was used as a molecular reporter of agonist-induced conformational changes in the β2 adrenergic receptor, a prototype hormone-activated G protein-coupled receptor, with a minimal number of endogenous cysteine residues. New cysteines were introduced in positions 2696.31, 2706.32, 2716.33, and 2726.34 at the cytoplasmic side of transmembrane segment (TM) 6. The resulting mutants were fully functional and bound both agonists and antagonist with high affinities also upon IANBD labeling. Fluorescence spectroscopy analysis of the purified and site-selectively IANBD-labeled mutants suggested that the covalently attached fluorophore was exposed to a less polar environment at all four positions upon agonist binding. Whereas evidence for only a minor change in the molecular environment was obtained for positions 2696.31 and 2706.32, the full agonist isoproterenol caused clear dose-dependent and reversible increases in fluorescence emission at positions 2716.33 and 2726.34. The data suggest that activation of G protein-coupled receptors, which are activated by “diffusible” ligands, involves a structural rearrangement corresponding to the cytoplasmic part of TM 6. The preferred conformations of the IANBD moiety attached to the inserted cysteines were predicted by employing a computational method that incorporated the complex hydrophobic/hydrophilic environment in which the cysteines reside. Based on these preferred conformations, it is suggested that the spectral changes reflect an agonist-promoted movement of the cytoplasmic part of TM 6 away from the receptor core and upwards toward the membrane bilayer.

G protein-coupled receptors (GPCRs),1 or seven-transmembrane segment receptors, comprise the largest superfamily of mammalian proteins with now more than 1000 different members (1, 2). The β2-adrenergic receptor (β2AR) was cloned more than a decade ago and has since served as a prototypic member of the receptor family (3, 4). A key issue in our understanding of GPCR function is the nature of the molecular mechanisms that couple agonist binding to receptor activation and transmission of the signal across the plasma membrane. Only recently spectroscopic techniques on purified receptor preparations have been taken into use and permitted the first direct insight into structural changes that occur during receptor activation (4). The use of EPR spectroscopy by Hubbell, Khorana, and co-workers (5–7) has showed evidence that activation of the light-sensing receptor rhodopsin involves movements of transmembrane segment (TM) 3 and 6 relative to one another. In addition, movements of TM 6 in rhodopsin have been predicted from fluorescence spectroscopy studies (8). Our application of fluorescence spectroscopy to the β2AR has supported a role of TM 3 and 6 also for activation of GPCRs activated by “diffusible” ligands. The sulphhydryl-reactive fluorescent probe, IANBD, was covalently incorporated into the purified β2AR and used as a molecular reporter of the structural changes that takes place following agonist binding to the receptor (9). Subsequent mutagenesis identified two cysteines, Cys-125.34.44 in the middle of TM 3 and Cys-285.46.47 in middle of TM 6, as responsible for the observed agonist-induced fluorescence changes (10).2

While converging on the involvement of TM 3 and 6 in receptor activation, the spectroscopic studies on rhodopsin and the β2AR also indicated some possible important differences between the two receptors. The EPR spectroscopic read-outs for rhodopsin demonstrated a rapid formation of the active metarhodopsin II state (within microseconds) following light-induced conversion of the prebound cis-retinal to all-trans-retinal, whereas the conversion of metarhodopsin back to the inactive metarhodopsin III state was slow, with a t1/2 of about 6 min (12). In contrast to the rapid activation and the slow inactivation kinetics observed for rhodopsin, the spectroscopic analyses of the β2AR indicated slow agonist-induced conformational changes (t1/2 = ~2–3 min), significantly slower than the predicted association rate of the agonist (9, 10). However, the reversal of the agonist-induced conformational change was relatively fast (t1/2 = ~30 s) (9, 13). These differences in activation

methyl-N-(iodoacetyl)-N’-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) ethylene-diamine; ISO, isoproterenol; ALP, alprenolol; DHA, dihydroalprenolol; DijM, n-dodecyl-p-n-maltoside; DOB, dobutamine; SAL, salbutamol.

2 The numbers of the residues according to the Ballesteros-Weinstein numbering scheme are indicated in superscript (see Ref. 11).
kinetics could either reflect inherent differences between the two receptors or be a consequence of differences in how the changes were detected.

To clarify this issue and to obtain further insight into the molecular mechanisms involved in GPCR activation, we wished to establish new read-outs for agonist-induced conformational changes in the β2AR. We decided to focus on the cytoplasmic end of TM 6 for two major reasons. First, the evidence for TM 6 movements in response to light activation of rhodopsin, was based on spectroscopic analysis of mutants that contained cysteine residues in this particular region of TM 6, labeled with either nitroxide spin labels or fluorescent probes (6–8). Labeling of the β2AR in this region with a molecular reporter of conformational changes would thus allow a more direct comparison between rhodopsin and the β2AR. Second, many mutagenesis-based studies have indicated the importance of the cytoplasmic region of TM 6 in receptor activation and G protein coupling (3, 14); nonetheless, its precise role is still not well understood, and conformational changes at the cytoplasmic side of TM 6 have not been described for receptors activated by diffusible ligands (4). Notably, our earlier evidence for TM 6 movements in the β2AR was based on fluorescent labeling of a naturally occurring cysteine (Cys-2856.47) situated in the hydrophobic middle part of TM 6 (10). Labeling at this position did not allow reliable predictions about the character of potential movements at the cytoplasmic side of the helix.

To explore the putative conformational changes in the predicted cytoplasmic region of TM 6 of the β2AR, four residues in this region were substituted with cysteines. The cysteines were inserted in a mutant β2AR containing a reduced number of endogenous cysteines (β2AR-Cys-min) (see Fig. 1). The spectroscopic analyses of the purified and fluorescently labeled β2AR cysteine mutants provided evidence that activation of the β2AR involves structural changes at the cytoplasmic side of TM 6. Employment of a computational method, which incorporates the complex hydrophobic/hydrophilic environment in which the inserted cysteines reside, allowed a prediction of the preferred conformations of the attached IANBD moiety. Analysis of these preferred conformations in context of a receptor model supported the possibility that TM 6 undergoes movements that are similar to those predicted to take place in rhodopsin during photo-activation.

**EXPERIMENTAL PROCEDURES**

**Mutagenesis**—The template used for site-directed mutagenesis was a cDNA named β2AR-Cys-min, which encoded the human β2AR, containing the mutations C77V, C265A, C327S, C378A, and C406A (10). In addition, the construct was tagged at the N terminus with the cleavable influenza-hemagglutinin signal sequence followed by the FLAG epitope (Sigma), and at the C terminus with six histidines (15). Mutations were all generated by polymerase chain reaction-mediated mutagenesis using Pfui polymerase according to the manufacturer’s instructions (Stratagene, La Jolla, CA). The generated polymerase chain reaction fragments were digested with the appropriate enzymes, purified by agarose gel electrophoresis, and cloned into the baculovirus expression vector pVL1392 containing β2AR-Cys-min (10) or β2AR-Cys-min-Gα (16). All mutations were confirmed by restriction enzyme analysis and DNA sequencing.

**Expression in Sf-9 Insect Cells—β2AR-Cys-min and mutant constructs were expressed in Sf-9 insect cells and high titer virus stocks generated as described using the BaculoGold transfection kit (Pharmingen, San Diego, CA) (17).**

**Membrane Preparation, Adenyllyl Cyclase Assay, and GTPase Assay—**Membranes were prepared as described from 30-ml SF-9 cell cultures in 125-ml disposable Erlenmeyer flasks, grown at a density of 3 × 10⁶ cells/ml, and infected with baculovirus encoding the different receptor constructs for 48 h before harvesting (9). Adenyllyl cyclase assays were preformed in the SF-9 cell membranes as described (18). GTPase assays were carried out on SF-9 cell membranes expressing β2AR-Gα fusion constructs as described (16). Protein was determined using the Bio-Rad DC protein assay kit.

**Binding Assays—**Saturation binding assays and competition binding assays on membrane preparations from cells expressing the different receptors were carried out as previously described using [3H]dihydroliprenol (DHA) (Amersham Pharmacia Biotech) as radioligand (19). Binding assays on solubilized and purified β2AR were also performed using [3H]DHA (Amersham Pharmacia Biotech) and radioligand (19). Binding data were analyzed by nonlinear regression analysis using Prism 2.0 from GraphPad Software (San Diego, CA).

**Purification Procedure—**The β2AR-Cys-min and mutant receptors were solubilized in 0.8% n-dodecyl-β-D-maltoside (DjBM) (Anastare) and subsequently purified by nickel chromatography using Chelating Sepharose Fast Flow Sepharose Resin (Amersham Pharmacia Biotech) followed by alprenolol affinity chromatography as previously described (17, 20). Approximately 1–2 nmol of purified protein could generally be obtained from a 500-ml culture. The specific activity of the purified receptors varied between 3 and 9 nmol/mg protein. Protein was determined using the detergent-insensitive Bio-Rad DC protein assay kit. Purified receptors were analyzed by 10% SDS-polyacrylamide gel electrophoresis and visualized by standard Coomassie staining.

**IANBD Labeling of Purified Receptor and Fluorescence Spectroscopy Analysis—**The purified receptor was labeled with IANBD ( Molecular Probes, Eugene, OR) according to described procedures (17). Briefly, 1–3 nmol receptor was bound to a 150-μl nickel column (Chelating Fast Flow Sepharose Resin, Amersham Pharmacia Biotech), equilibrated in high salt buffer (20 mM Tris-buffer, pH 7.5, with 500 mM NaCl and 0.08% DjBM), and labeling achieved by recycling 1.0 ml of 0.5 mM IANBD in high salt buffer several times over the nickel column for 20 min. Excess dye was removed by extensive washing of the column with high salt buffer. The labeling procedure resulted in incorporation of 1.1–1.5 mol of IANBD/mol of receptor, as determined by measuring absorption at 451 nm and using an extinction coefficient of 21,000 M⁻¹ cm⁻¹ for IANBD and a molecular mass of 50,000 Da for the receptor. Fluorescence spectroscopy was performed at room temperature on a SPEX Fluoromax-2 spectrophotometer connected to a PC equipped with the Datamax 2.2 software package (Jobin Yvon Inc., Edison, NJ) as described (9, 17). In all experiments the excitation and emission bandpass were set at 4.2 nm. Both emission scans and time course experiments were done with 10–20 pmol of IANBD-labeled receptor in 500 μl of buffer (20 mM Tris-buffer, pH 7.4, containing 100 mM NaCl and 0.08% DjBM) under constant stirring. In emission scan experiments the excitation wavelength was 481 nm, and emission was measured from 490 to 625 nm. During time scan experiments the excitation wavelength was 481 nm, and emission was measured at a wavelength of 530 nm. The volume of the added ligands was one percentage of total volume, and fluorescence was corrected for this dilution. The bands obtained in the fluorescence experiments had an absorbance of less than 0.01 at 481 and 525 nm in the concentrations used excluding inner filter effects.

**Collisional Quenching Experiments—**Stock solutions (1.0 μl) of the hydrophilic quencher potassium iodide containing 10 mM Na₂S₂O₃ was prepared freshly for each round of experiments. The experiments were preformed with 10–20 pmol of IANBD-labeled receptor in 400 μl of buffer (20 mM Tris-buffer, pH 7.4, containing 100 mM NaCl and 0.08% DjBM). To correct for dilution/ionic strength effects on fluorescence, measurements were performed in parallel using a 1.0 μl stock of KCl. 10 μl of quencher (potassium iodide) or control solution (potassium chloride) were added sequentially followed by thorough mixing after each addition and subsequent recording of fluorescence using the Constant wavelength Analysis program in the Datamax software package (Jobin Yvon Inc.). The excitation wavelength was 481 nm, and the emission wavelength was 530 nm. The data were plotted according to the Stern-Volmer equation, \[ F/F_0 = 1 + K_{sv}[Q], \] where \[ F/F_0 \] is the ratio of fluorescence intensity in the absence and presence of quencher (Q), and \[ K_{sv} \] is the Stern-Volmer quenching constant (21).

**Computational Methods—**TM 6 was modeled from residues Ser-2626.24–Val-2976.59 as an α-helix as described under “Results” and according to Ref. 11. The conformational memories method (22, 23) was performed with 10–20 pmol of IANBD-labeled receptor in 100 μM of Tris-buffer, pH 7.4, containing 100 mM NaCl and 0.08% DjBM. To correct for dilution/ionic strength effects on fluorescence, the simulations were performed in parallel using a 1.0 μl stock of KCl. 10 μl of quencher (potassium iodide) or control solution (potassium chloride) were added sequentially followed by thorough mixing after each addition and subsequent recording of fluorescence using the Constant Wavelength Analysis program in the Datamax software package (Jobin Yvon Inc.). The excitation wavelength was 481 nm, and the emission wavelength was 530 nm. The data were plotted according to the Stern-Volmer equation, \[ F/F_0 = 1 + K_{sv}[Q], \] where \[ F/F_0 \] is the ratio of fluorescence intensity in the absence and presence of quencher (Q), and \[ K_{sv} \] is the Stern-Volmer quenching constant (21).

**Conformational Changes in the β2 Adrenergic Receptor**
as a dielectric gradient. Because solutions of the Poisson-Boltzmann equation are iterative, it is not possible to perform such calculations within a statistical mechanics simulation-like conformational memories. Thus, the following procedure was implemented: 1) the 15Å interfacial region was grid into 0.2Å spacing; 2) the water-interface boundary was set to a dielectric constant of 5; 4) the penetration dielectric constant was set to $\epsilon(x) = 78(1 - x^2) + 5z$, $z = 0.2n/15$, $n = 0.1,2,7,75$; 5) the Poisson Boltzmann equation for each dielectric slice was solved, and the potentials were stored on the grids numerically in data tables; 6) potentials were obtained over a coverage of at least the 60 Å region spanning the membrane; and 7) a linear interpolation of the potentials between grids was implemented. Still and co-workers (26) have developed a generalized Born surface area continuum solvation model for water and chloroform. Creation of the membrane interface was implemented as follows: 1) for each new configuration of the peptide, either a conformational change or a rigid body translation or rotation, both the chloroform and water solvation energy was computed; 2) atoms that have a $z$ coordinate less than 15 were assigned the chloroform solvation energy; 3) atoms that have a $z$ coordinate greater than 0 were assigned the water solvation energy; 4) atoms that have $15 < z < 0$ are assigned a solvation energy $e_{solv}(z(x)\text{water}) + (1 - x)\text{chloroform}$, $x = (15 + z)/15$. The starting spatial configuration of the peptide consisted of placing the $\alpha$-carbon of the IANBD-labeled cysteine mutant 0.5 Å into the interface-lipid boundary (i.e. 0 Å within the interface-lipid boundary and 0.5 Å in the chloroform environment) such that the vector between $i$ and $i + 7$ are perpendicular to the $xy$ plane. The $i + 7$-carbon residue resides at an approximate coordinate of 0, 0, -24. The different Pro-kink conformations were selected from our previous and published simulations on TM 6 of the $\beta$2AR (10). The preferred IANBD conformations defined by the computational simulations described above were analyzed in the context of a three-dimensional model of the seven-helix $\beta$2AR transmembrane domain modeled after the rhodopsin template (27, 28).

**RESULTS**

**Cysteine Substitutions at the Cytoplasmic Side of TM 6**—To achieve the possibility of site-selective, covalent incorporation of the sulphydryl-reactive fluorophore IANBD into the purified $\beta$2AR, we wanted to reduce the number of endogenous cysteines available for chemical derivatization. All of the free cysteines could not be substituted without severe decrease in receptor expression; however, the expression was preserved in a mutant where Cys-772.52, Cys-2656.27, Cys-3277.54, Cys-378, and Cys-406 were substituted with valine or serine (10). This mutant still contains three cysteines available for chemical derivatization, Cys-1163.35, Cys-1253.44, and Cys-2856.47, but additional mutation of any of these residues substantially reduced expression (data not shown and Ref. 10). Therefore, we chose this construct, which we named $\beta$2AR-Cys-min, for further studies. Importantly, $\beta$2AR-Cys-min binds agonists and antagonists with the same affinities as the wild type $\beta$2AR and couples to adenyl cyclase with the same efficiency (10).

Four consecutive residues in the cytoplasmic region of TM 6, His-2696.31, Lys-2706.32, Ala-2716.33, and Leu-2726.34, were mutated to cysteines in the transmembrane segments that were mutated to cysteines are indicated by black letters in black circles. The positions of the four mutated residues are indicated by their generic number in the $\beta$2AR followed by their number according to the Ballesteros-Weinstein nomenclature (11), which assigns the identifier 6.50 to the conserved Pro-289 (Pro-285-290) in TM 6. The cysteines in the transmembrane segments that are present in $\beta$2AR-Cys-min (Cys-1163.35, Cys-1253.44, and Cys-2856.47) are indicated by black letters in white circles. The ability to activate adenyl cyclase in SF-9 cell membrane preparations. All mutants exhibited evidence for efficient coupling to adenyl cyclase, and the observed EC$_{50}$ values for ISO were comparable to that of $\beta$2AR-Cys-min (Table I). Of the four mutants, only $\beta$2AR-Cys-min-L272C showed evidence of increased agonist-independent activity as indicated by a small increase in basal receptor activity in comparison with the wild type (Table I).

Next we wished to assess whether IANBD labeling of cysteines introduced in positions 2696.31, 2706.32, 2716.33, or 2726.34 would affect the ability of the receptor to assume the activated state and transmit the signal to the G protein. Because the GTPase activity of Gs shows less sensitivity to cysteine modifications as compared with downstream effectors such as adenyl cyclase, we subcloned all four mutant into the previously described fusion construct between the $\beta$2AR and Gs. This fusion construct allows direct and efficient assessment of G protein coupling by measurement of GTPase activity in membrane preparations (16, 29). IANBD labeling was achieved directly in situ by treating SF-9 cell membranes, expressing the $\beta$2AR-Gs fusion constructs, with a large excess of IANBD (0.5 mM) for 30 min. Following this labeling procedure, ISO was found to cause clear increases in GTPase activity in both $\beta$2AR-Cys-min-Gs and in the cysteine-substituted fusion constructs. The GTP hydrolysis was linear for at least 30 min, and the relative increases in GTPase activity were similar to those observed without IANBD labeling (Table II). Furthermore, the EC$_{50}$ values for ISO also were well preserved upon IANBD labeling. For $\beta$2AR-Cys-min-H269C, $\beta$2AR-Cys-min-A271C, and $\beta$2AR-Cys-min-L272C, the values were essentially unchanged, whereas in Cys-2706.32 a 4–5-fold increase in the EC$_{50}$ value for ISO was found after IANBD derivatization. Conceivably, this could reflect a partial steric inhibition of the receptor:G protein interaction because of covalent modification of a cysteine inserted in a region known to form part of the G protein binding site (30).

Fluorescence Spectroscopy Analysis of Site-selectively Labelled Mutant $\beta$2AR—The emission from the IANBD fluorophore is highly dependent on the polarity of the surrounding solvent. Decreasing the polarity of the solvent is accompanied by a concurrent increase in fluorescence emission and a blue shift of the $\lambda_{\text{max}}$ (wavelength at which maximal fluorescence

**Fig. 1. Snake diagram of the $\beta$2AR.** The residues at the cytoplasmic side of TM 6 that were mutated to cysteines are indicated by white letters in black circles. The positions of the four mutated residues are indicated by their generic number in the $\beta$2AR followed by their number according to the Ballesteros-Weinstein nomenclature (11), which assigns the identifier 6.50 to the conserved Pro-289 (Pro-285-290) in TM 6. The cysteines in the transmembrane segments that are present in $\beta$2AR-Cys-min (Cys-1163.35, Cys-1253.44, and Cys-2856.47) are indicated by black letters in white circles.
Conformational Changes in the β2 Adrenergic Receptor

Table I

| Receptor Mutant | K<sub>d</sub> M | K<sub>i</sub> M | IC<sub>50</sub> M | EC<sub>50</sub> M | Affinity for Basal | Affinity for ISO |
|----------------|--------------|--------------|--------------|-------------|-----------------|-----------------|
| β2AR-Cys-min   | 4.9 ± 0.2    | 0.35 ± 0.03  | 0.25 ± 0.04  | 1.0 c ± 0.2  | 0.003 ± 0.002   | 0.003 ± 0.002   |
| A271C          | 5.4 ± 0.2    | 0.66 ± 0.02  | 1.00 ± 0.00  | 1.00 ± 0.00  | 0.003 ± 0.002   | 0.003 ± 0.002   |
| L272C          | 3.5 ± 0.2    | 0.46 ± 0.06  | 0.46 ± 0.06  | 0.46 ± 0.06  | 0.003 ± 0.002   | 0.003 ± 0.002   |

The data are means ± S.E. for three experiments. The K<sub>d</sub> values for [3H]DHA binding to the purified and IANBD-labeled receptors were determined from competition binding experiments using the nonradioactive, structurally identical compound, alprenolol, as competitor. The K<sub>i</sub> values were calculated from the equation K<sub>i</sub> = IC<sub>50</sub>/L<sub>max</sub> - L<sub>max</sub>/K<sub>d</sub> where L<sub>max</sub> is the concentration of [3H]DHA. Data are the means of 3–5 with S.E. intervals calculated from the percentage of basal [3H]DHA activity (means ± S.E., n = 3–5). The IC<sub>50</sub> values were subsequently converted to EC<sub>50</sub> values (means ± S.E., n = 3–5 with S.E. intervals shown in parentheses and percentages of maximum cyclase (means ± S.E., n = 3–5).
around Cys-2696.31 and Cys-2706.32 is a likely interpretation. Moreover, because IANBD derivatization of Cys-2696.31 and Cys-2706.32 in membranes still permit receptor activation and G protein coupling (see previous section), it is also reasonable to assume that the lack of response is not due to inability of the IANBD-labeled receptor constructs to adopt the activated state.

Agonist stimulation of both β2AR-Cys-min-A271C and β2AR-Cys-min-L272C lead to clear increases in fluorescence emission relative to the extrapolated base line with the largest change observed for β2AR-Cys-min-L272C (Fig. 2B). Both responses could be completely reversed by the agonist ALP (Fig. 2A). In addition, the agonist-induced fluorescence changes for both mutant β2AR-Cys-min-A271C and β2AR-Cys-min-L272C could be blocked by addition of ALP before addition of ISO (Fig. 2B). Please note that the affinity of ALP is ~50–100-fold higher than the affinity for ISO, and therefore, 10^(-4) M ALP should easily displace a 3- or 10-fold higher concentration of ISO. It should also be noted that the ISO-induced increases in fluorescence intensity observed for positions Cys-2716.33 and Cys-2726.34 were not accompanied by detectable changes in λmax (data not shown). In summary, the data suggest that binding of the agonist ISO causes a conformational change in the receptor that exposes IANBD bound to Cys-2716.33 and Cys-2726.34 to a more hydrophobic or less hydrophilic environment.

The kinetics of the agonist-induced changes in fluorescence in IANBD-labeled β2AR-Cys-min, β2AR-Cys-min-A271C, and β2AR-Cys-min-L272C were analyzed by nonlinear regression analysis using a single exponential function to determine τ1/2 values for the observed changes. To carry out this analysis, raw traces were transformed into the kinetic curves (shown in Fig. 3) by subtracting all data points from an extrapolated base line with a slope defined from the fluorescence trace after the fluorescence intensity has stabilized upon ISO stimulation (last 300 s before ALP is added). This was chosen instead of a simple extrapolation of the base line prior to ISO addition to eliminate potential nonspecific effects of ISO on the base-line drift. In β2AR-Cys-min-A271C and β2AR-Cys-min-L272C, a normalized β2AR-Cys-min trace was also subtracted to exclude the influence of the background decrease on the kinetics of the ISO induced increase in fluorescence. If the agonist-induced increase in fluorescence observed in β2AR-Cys-min-A271C and β2AR-Cys-min-L272C represent new read-outs of the same series of conformational changes that are also reported by IANBD bound to Cys-1253.44 and Cys-2856.47, it would be expected that the changes would occur with similar kinetics. As shown in Fig. 3, the agonist-induced changes in fluorescence in β2AR-Cys-min, β2AR-Cys-min-A271C, and β2AR-Cys-min-L272C did display similar kinetics with τ1/2 values of 197 ± 24 s (mean ± S.E., n = 3), 155 ± 6 s (mean ± S.E., n = 4), and 181 ± 11 s (mean ± S.E., n = 4), respectively (Fig. 3). The τ1/2 value for the response observed in the wild type β2AR was 135 ± 7 s, (mean ± S.E., n = 2) (data not shown). Although not identical, these values are within a similar range, in particular in comparison with the microsecond time scale reported for conformational changes in rhodopsin. It is, therefore, reasonable to assume that the fluorescence changes represent different read-outs of the same sequence of structural changes (Fig. 3).

Collisional Quenching Experiments with Iodide—To further elaborate the character of the ISO-induced change in the molecular environment surrounding Cys-2716.33 and Cys-2726.34, a series of collisional quenching experiments were carried out on IANBD-labeled β2AR-Cys-min, β2AR-Cys-min-A271C, and β2AR-Cys-min-L272C. Collisional quenching requires a bimolecular interaction between the quencher and the fluorophore and, accordingly, such experiments can determine the accessibility of the fluorophore to the surrounding solvent (21). The aqueous quencher iodide (I⁻) is a strong quencher of the fluorescence from IANBD as illustrated by the linear Stern-Volmer plot in Fig. 4, in which F/F0 is plotted against the potassium iodide concentration. The slope of the line represents the Stern-Volmer constant (KSV), which was 5.9 M⁻¹ for the free probe. The iodide quenching of IANBD-labeled β2AR-Cys-min was substantially smaller but still apparent with a linear Stern-Volmer plot and a KSV value of 3.1 M⁻¹ (Fig. 4A). This is consistent with the localization of the major labeling sites, Cys-1253.44 and Cys-2856.47, in a hydrophilic environment that is only partially accessible to the aqueous solvent. Interestingly, the KSV values for both β2AR-Cys-min-A271C and β2AR-Cys-min-L272C were markedly higher (4.6 and 4.5 M⁻¹), suggesting that IANBD bound to Cys-2716.33 and Cys-2726.34 are more exposed to the solvent than Cys-1253.44 and Cys-2856.47 (Fig. 4, B and C). These data provide additional direct support for the prediction that Cys-2716.33 and Cys-2726.34 are situated close to the cytoplasmic membrane-water interface (Fig. 1).
The quenching experiments were also carried out in the presence of ISO. In β2AR-Cys-min, iodide quenching was identical in presence of ISO, indicating that the change in environment surrounding Cys-1253.44 and Cys-2856.47 observed in the time course experiments is not accompanied by a measurable change in iodide accessibility (Fig. 4A). However, a minor decrease in iodide accessibility was observed for both β2AR-Cys-min-A271C and β2AR-Cys-min-L272C (Fig. 4B and C). Importantly, this observation is consistent with the time course experiments shown in Fig. 2, which indicated a movement of IANBD-labeled Cys-2716.33 and Cys-2726.34 to a more hydrophobic and expectably a less solvent-accessible environment.

Dose-response Analysis and Investigation of Correlation between Agonist Efficacy and Fluorescence Response at IANBD-labeled β2AR-Cys-min-A271C and β2AR-Cys-min-L272C—The ISO-mediated response at both β2AR-Cys-min-A271C and β2AR-Cys-min-L272C displayed a clear dose dependence (Fig. 5). The maximum response at β2AR-Cys-min-L272C was twice as big as the response at β2AR-Cys-min-A271C. Fitting the data points to a single-site hyperbolic function showed EC50 values of 32 μM for β2AR-Cys-min-A271C and 3.9 μM for β2AR-Cys-min-L272C. These values should be compared with the observed EC50 value of 29 μM for the IANBD-mediated response caused by labeling of Cys-1253.44 and Cys-2856.47 (9). It must be emphasized that these EC50 values are determined on purified protein in absence of G protein and thus cannot be compared with the EC50 values determined in functional assays (adenylyl cyclase or GTPase activity). As outlined under “Discussion” it can be expected that the G protein can affect the kinetic parameters of agonist-promoted structural changes substantially.

We have previously shown that the agonist-induced changes in fluorescence measured on IANBD-labeled wild type β2AR correlates directly with the intrinsic efficacy of the agonist (9). We inferred from this correlation that IANBD attached to the receptor were reporting conformational changes associated with receptor activation (9). A similar experiment was carried out on IANBD-labeled β2AR-Cys-min-A271C and β2AR-Cys-min-L272C. In addition to the full agonist ISO, we tested the partial agonists dobutamine (DOB) and salbutamol (SAL). Before measuring the effect of these compounds on fluorescence, we wanted to ensure that they bound with high affinity to the receptor and were able to act as strong partial agonists. Competition binding experiments demonstrated that both dobutamine and salbutamol bind with high affinity to the purified and IANBD-labeled β2AR-Cys-min-A271C and β2AR-Cys-min-
It should be noted that the absence of response to dobutamine with the S.E. intervals in parentheses calculated from pIC50 (2.1–2.3) and 1.4 (1.2–1.5) min-L272C, respectively. The observed increases in emission at both efficiencies of dobutamine, salbutamol, and ISO, and the observed increases in emission at both 2AR-Cys-min-A271C and 2AR-Cys-min-L272C (DOB < SAL < ISO) (Fig. 6). It should be noted that the absence of response to dobutamine at 2AR-Cys-min-A271C does not necessarily reflect that dobutamine is not causing a conformational change at Cys-271 6.3 (means ± S.E., n = 3), 155 ± 6 s (mean ± S.E., n = 4), and 181 ± 11 s (mean ± S.E., n = 4) for 2AR-Cys-min, 2AR-Cys-min-A271C, and 2AR-Cys-min-L272C, respectively.

Conformational Changes in the β2 Adrenergic Receptor

**Fig. 3.** The kinetics of the isoproterenol induced changes in fluorescence. Representative kinetic curves for IANBD-labeled β2AR-Cys-min (upper panel), β2AR-Cys-min-A271C (middle panel), and Cys-min-L272C (lower panel) are shown. The kinetic curves for ISO stimulation were obtained from representative raw traces by subtracting all data points from an extrapolated base line with a slope defined from the fluorescence trace after the fluorescence intensity has stabilized upon ISO stimulation (last 300 s before ALP is added). The resulting curves were analyzed by nonlinear regression analysis using a single exponential function to determine t1/2 values for the fluorescent changes (means ± S.E., n = 4). The t1/2 values were 179 ± 24 s (mean ± S.E., n = 3), 155 ± 6 s (mean ± S.E., n = 4), and 181 ± 11 s (mean ± S.E., n = 4) for 2AR-Cys-min, 2AR-Cys-min-A271C, and 2AR-Cys-min-L272C, respectively.

**Fig. 4.** Stern-Volmer plots of iodide quenching in β2AR-Cys-min, β2AR-Cys-min-A271C, and β2AR-Cys-min-L272C. A, quenching of β2AR-Cys-min with the aqueous quencher potassium iodide in presence (open circles) and absence (closed triangles) of 300 μM ISO. B, quenching of β2AR-Cys-min-A271C with the aqueous quencher potassium iodide in presence (open circles) and absence (closed triangles) of 300 μM ISO. C, quenching of β2AR-Cys-min-L272C with the aqueous quencher potassium iodide in presence (open circles) and absence (closed triangles) of 300 μM ISO. The quenching experiments were carried out and data plotted as described under “Experimental Procedures.” In absence of ISO the Stern-Volmer quenching constants (KSV) were 3.13 ± 0.07 M−1 (n = 2), 4.6 ± 0.3 M−1 (n = 3) and 4.5 ± 0.12 M−1 (n = 3) (means ± S.E.) for β2AR-Cys-min, β2AR-Cys-min-A271C, and β2AR-Cys-min-L272C, respectively. In presence of 300 μM ISO the Stern-Volmer quenching constants (KSV) were 3.09 ± 0.05 M−1 (n = 2), 4.0 ± 0.2 M−1 (n = 3), and 4.1 ± 0.23 M−1 (n = 3) (means ± S.E.), respectively. In all experiments the excitation was 480 nm, and emission was recorded at 530 nm. The β2AR-Cys-min Stern-Volmer plot (A) is shown in B and C as a dashed line for comparison.

change in baseline fluorescence was observed in response to the inverse agonist, ICI 118,551 (data not shown).

**Molecular Modeling—**TM 6 was modeled as a Pro-kinked α-helix from Ser-2624.24 to Val-2975.59 (Fig. 7, A and B). A proline kink motif around the highly conserved Pro-2885.50 has been directly indicated from the electron density maps of rhodopsin (27) and supported by application of the substituted cysteine accessibility method to the dopamine D2 receptor (32). Moreover, this kink is apparent in the very recently published high resolution structure of rhodopsin (33). The spin labeling studies in rhodopsin have identified the N terminus of TM 6 at position 6.23–6.24 (7), whereas substituted cysteine accessibility method studies identified the C terminus of TM 6 at position 6.59 (32) (Fig. 7B). These limits are also very close to those observed in the high resolution structure of rhodopsin showing the N terminus at position 6.27 and the C terminus at position 6.59 (33). The sequence identity among these GPCRs suggests that similar helical segments should be expected for the β2AR. However, because of the presence of four positively charged

L272C. The K_{1} values for dobutamine and salbutamol were 2.2 (2.1–2.3) and 1.4 (1.2–1.5) μM, respectively, at A271C and 3.1 (2.0–5.0) and 0.27 (0.25–0.30) μM, respectively, at L272C. This should be compared with 1.3 (0.7–2.4) and 0.7 (0.3–1.3) μM, respectively, in β2AR-Cys-min (all data are means of n = 3 with the S.E. intervals in parentheses calculated from pIC_{50} ± S.E.). As shown in Fig. 6A, dobutamine and salbutamol also acted as strong partial agonist as determined from adenylyl cyclase experiments on SF-9 cell membranes expressing β2AR-Cys-min-A271C and β2AR-Cys-min-L272C. The observed efficacies correspond well to those reported previously for the wild type β2AR (9). The apparent higher efficacy at β2AR-Cys-min-L272C is most likely a reflection of the weak constitutive receptor activity observed in this mutant (Table I) (31). In the fluorescence experiments we observed a strong correlation between these efficacies of dobutamine, salbutamol, and ISO and the observed increases in emission at both 2AR-Cys-min-A271C and 2AR-Cys-min-L272C (DOB < SAL < ISO) (Fig. 6). It should be noted that the absence of response to dobutamine at 2AR-Cys-min-A271C does not necessarily reflect that dobutamine is not causing a conformational change at Cys-271 6.3 but only that the expected increase in fluorescence either counterbalances the decrease in fluorescence because of labeling of Cys-1253.44 and Cys-2856.47 or is below our detection limit. No
residues (Lys-2636.25, Lys-2676.29, Lys-2706.32, and Lys-2736.35), the hydrophobicity plot, on which most common prediction methods for transmembrane segments rely, is not predicting that the segment between Ser-2626.24 and Lys-2736.35 is helical in the β2AR. (Fig. 7C). The simplest interpretation would be that TM 6 differs substantially from rhodopsin and that the TM 6 helix does not commence before Lys-2736.35. We find it likely that this is not the correct interpretation and, thus, that the hydrophobicity plot is misleading. The same hydrophobicity criterion that considers the membrane as a hydrophobic medium predicts that in a helical transmembrane segment, polar residues would be expected to face the protein interior while lipid-facing residues are expected to be apolar. This is not the case in the β2AR where the four lysines are predicted to face the lipids if they are a part of the TM 6 helix (Fig. 7B). Interestingly, it is known from structures of membrane proteins that Arg and Lys residues tend to concentrate at the cytoplasmic boundary of the helices, where they face the lipids and their positive charge interact with the phospholipid headgroups (11, 34). This Arg/Lys cytoplasmic motif could explain why the hydrophobicity plot of TM 6 in the β2AR is not reflecting the actual secondary structure of the segment from Ser-2626.24 to Lys-2736.35.

To further explore this, a sequence alignment of 56 GPCRs homologous to the β2AR was constructed, and the Arg and Lys residues within TM 6 of any aligned GPCR were identified. The Arg/Lys cytoplasmic motif predicts that Arg/Lys in any homologous receptor that belongs to the TM 6 α-helix at the cytoplasmic interface should concentrate on one face of the α-helix facing the phospholipid headgroups. Whenever an Arg or Lys residue was found in any of the 56 neurotransmitter GPCRs aligned (data not shown), the position is highlighted by a thicker circle in Fig. 7B. Interestingly, we observed that the arginines and lysines are clustering in a pattern consistent with the presence of an α-helix from Ser-2626.24 to Lys-2736.35. In contrast, all positions prior to Ser-2626.24 and Lys-2736.35 are polar residues in TM 6 reside in an α-helical region. Furthermore, we predict that the residues are situated in a mixed hydrophobic-hydrophilic environment at the lipid-water interface consistent with the spin labeling data in rhodopsin, indicating the presence of a hydrophobic/hydrophilic boundary around residues 6.33–6.37 (7).

Computational Simulations—Based on the TM 6 model described above we attempted to define the preferred conformations of the IANBD moiety attached to the inserted cysteines by carrying out a series of computer simulations. In our previous study (10), the preferred conformations of the IANBD moiety attached to Cys-1256.44 and Cys-2856.47 were also predicted from computational simulations. Because both Cys-1256.44 and Cys-2856.47 reside in a highly hydrophobic environment, these simulations was carried out in vacuum. However, our TM 6 model predicts that Cys-2696.31, Cys-2706.32, Cys-2716.33, and Cys-2726.34 reside in a mixed hydrophobic-hydrophilic environment. Reliable predictions about the preferred IANBD conformations would obviously require that this complex environment is taken into consideration. Therefore, a computational method in which this mixed environment is approximated as a
A dielectric gradient was developed (see “Experimental Procedures”). This method is based on the data of White and Wimley (reviewed in Ref. 25), who showed evidence that a membrane consists of a 30-Å relatively constant, low dielectric region, sandwiched between two complex regions of variable dielectric behavior, both of which are 15 Å (reviewed in Ref. 25). The 30-Å-thick core region corresponds to the hydrophobic lipid chains while the surrounding 15 Å thick region corresponds to the phospholipid headgroup domain (Fig. 7A). Thus, the transmembrane helical domains reside in a complex environment consisting of three distinct phases: a hydrophobic core of the membrane defined by the phospholipid chains, a mixed hydrophobic-hydrophilic region comprised by the phospholipid headgroups, and the aqueous cytoplasm (25). A similar complex environment can be envisioned for detergent micelles in which the experiments in the present study were carried out, because they would be expected to follow the same polarity pattern, i.e. a hydrophobic core, a mixed hydrophobic-hydrophilic region comprised by the polar headgroups of the detergent, and the aqueous exterior. Notably, the spectral responses to agonists in IANBD-labeled β2AR-Cys-min-A271C were similar following reconstitution into phospholipid vesicles as compared with detergent micelles (data not shown). For the purpose of defining the approximate conformational space for the IANBD moiety, we therefore allowed the assumption that the mixed hydrophobic-hydrophilic region is at least roughly the same in detergent micelles as in phospholipid membranes.

The conformational memories simulations were performed on the four cysteine mutants IANBD derivatized peptides of TM 6 (see “Experimental Procedures”). The technique of conformational memories, which utilizes energy-based Monte Carlo simulations, was applied to explore the preferred conformations of the IANBD-labeled cysteines. The most likely conformations of the IANBD-moieties attached to the substituted cysteine residues are those more populated in the Monte Carlo simulation, analyzed in terms of their dihedral angles, which can be classified in terms of rotamers. Rotamer configurations populated more than 5% during the course of the simulation were selected as possible conformations of each IANBD-labeled cysteine residue. An important result of the new computational method was the observation of multiple IANBD rotamer configurations populated in all four IANBD-labeled constructs. This result contrasts the results of our previous simulations on IANBD attached to Cys-2856.47, which demonstrated a restricted conformational space for IANBD (10). Hence, the mixed dielectric medium that corresponds to the mixed hydrophobic-hydrophilic region allows apparently for considerably more conformational freedom of IANBD than the same moiety attached to Cys-2856.47, which resides in a hydrophobic environment. The composite of the populated conformations defines the set of conformations available to each IANBD-derivatized cysteine residue. Figs. 8 and 9A show the most populated conformations for each of the four IANBD-labeled cysteine residues at the bottom of TM 6 in the structural context provided by a model of the TM domain of the receptor. TM 6 was oriented based on the predictions and experimental data identifying which residues are oriented toward the protein interior or facing the lipid membrane as described above and shown in Fig. 7B.
DISCUSSION

The data from the present study provide structural evidence that agonist binding to the β2AR leads to a conformational rearrangement at the cytoplasmic side of TM 6. Cysteines were introduced in four positions at the cytoplasmic side of TM 6 and site-selectively labeled with the conformationally sensitive fluorophore IANBD. The pharmacological properties of the cysteine mutants were largely unchanged, supporting that the overall structure of the receptor is preserved and, thus, that the new fluorescence signal detected in the IANBD-labeled mutants can be attributed to labeling of the inserted cysteines rather than to altered labeling of the endogenous cysteines present in the background, β2AR-Cys-min. The spectroscopic analysis showed that binding of the full agonist ISO caused clear increases in the fluorescence quantum yields in positions Cys-2716.33 and Cys-2726.34. The increases were reversible and dose-dependent. Moreover, the magnitude of the responses correlated with the efficacy of the used agonist, suggesting that the observed changes are relevant for receptor activation (Fig. 6). The increase in quantum yield is consistent with a decrease in the polarity of the environment surrounding the IANBD-labeled cysteines as compared with Cys-2696.31 and Cys-2706.32, but only that the change in the polarity of the environment must be significantly larger.

To understand the fluorescence changes in a structural context, we generated a molecular model of TM 6. Importantly, this model corresponds well to the newly published high resolution structure of rhodopsin (33). The TM 6 model proposed that the cysteine-substituted residues (Cys-2696.31, Cys-2706.32, Cys-2716.33, and Cys-2726.34) reside in an α-helical region whose environment corresponds to a mixed hydrophobic-hydrophilic region (Fig. 7). In agreement with this, collisional quenching experiments on β2AR-Cys-min-A271C and β2AR-Cys-min-L272C revealed a higher accessibility to the hydrophilic quencher iodide consistent with the localization of both Cys-2716.33 and Cys-2726.34 in a more water-accessible environment (Fig. 4). Obviously, to interpret our data correctly, it is highly important to predict the conformations of the bound cysteine IANBD moiety as precisely as possible in this mixed hydrophobic-hydrophilic region. In this context, it should be taken into consideration, for example, that cysteine substitutions and subsequent IANBD labeling in this part of the receptor could produce local structural changes that are not affecting the overall conformation of the receptor. Therefore, to obtain the most optimal framework for our data interpretation, a new computational method was developed. Notably, current computational simulation methods do not incorporate the complexity of the mixed hydrophobic-hydrophilic region. Only recently, a bi-phasic lipid-water solvent continuum model was developed (24). In this study, this solvent model was extended by developing a computational method in which the mixed hydrophobic-hydrophilic regions are approximated as a dielectric gradient. The results of the simulations, illustrated by the most preferred conformations for each of the four IANBD-derivatized cysteine residues, are shown in context of a molecular model in Fig. 8 (viewed from the extracellular side) and Fig. 9A (viewed perpendicular to the plane of the membrane).

A simple rotation of TM 6 was sufficient to explain our previous data based on IANBD labeling of Cys-2856.47 (10). However, the observation in the present study that IANBD at all four inserted cysteines residues likely moves into a more hydrophilic environment upon agonist binding suggests that the helical movement is more complex. Of particular interest, EPR spectroscopy analysis of spin-labeled rhodopsin mutants has provided strong evidence that light activation of rhodopsin involves a rigid body movement of the cytoplasmic part of TM 6 away from TM 3 (6). The present data could support a similar movement of TM 6 in β2AR in response to agonist binding. As seen from the side (Fig. 9A), TM 6 is predicted to form a kinked α-helix because of the presence of a highly conserved proline (Pro-2876.50) (11). The projection maps of rhodopsin, which are believed to represent the inactive state of the receptor, indicates that the cytoplasmic part of TM 6 below the proline kink is almost perpendicular to the plane of the membrane, whereas the part above the proline kink is tilted −25° (27). A rigid body movement of the cytoplasmic part of TM 6 away from TM 3, and thus the receptor core will result in large changes in the axial positioning of all four IANBD-labeled substituted cysteines. In the inactive conformation the IANBD moieties would be predicted to reside in the polar headgroup region (Fig. 9A). However, if the cytoplasmic part of TM 6 is moved away from the receptor core all four IANBD-labeled residues are brought upwards and outwards, allowing them to penetrate further into the more hydrophobic region of the membrane/detergent micelles and away from the more hydrophilic polar headgroups as well as from the predicted more hydrophilic interior of the receptor protein (illustrated by the hypothetical active structure in Fig. 9B where the cytoplasmic part of TM 6 with the IANBD moieties attached is tilted arbitrarily away from the...
Conformational Changes in the β2 Adrenergic Receptor

Cys-2716.33 and Cys-2726.34 transfer from the protein interior phatic chains in both the inactive and active states, whereas respond to the inactive conformation (Fig. 9 the cytoplasmic portion of TM 6 moving away from the receptor core (10). Whereas the more kinked conformation would correspond to the inactive conformation (A), the active state may be represented by a less kinked conformation, resulting in the cytoplasmic portion of TM 6 moving away from the receptor core (B).

The movement can explain the observed shift for all four IANBD-labeled cysteines toward a less polar environment upon receptor activation. Because residues Cys-2696.31 and Cys-2706.32 are facing the hydrophobic aliphatic chains in both the inactive and active states, whereas Cys-2716.33 and Cys-2726.34 transfer from the protein interior to being exposed to the hydrophobic aliphatic chains upon activation, the latter residues could be expected to display a larger change in fluorescence emission upon agonist binding, in agreement with the experimental observations. Notably, the movement of the cytoplasmic part of TM 6 is shown to occur around the conserved proline kink but could as well involve a rigid body movement of the entire helix. However, our previous simulation of the TM 6 helix indicated the possibility that the kink in the TM 6 helix induced by Pro-2876.50 could behave as a flexible hinge, which can mediate the movement of the cytoplasmic side of TM 6 in relation to the extracellular region (10). Whereas the more kinked conformation would correspond to the inactive conformation (A), the active state may be represented by a less kinked conformation, resulting in the cytoplasmic portion of TM 6 moving away from the receptor core (B).

FIG. 9. Proposed conformations of the inactive and active states of the β2AR. A, inactive conformation of the receptor characterized by a highly kink TM 6 helix (blue) with the cytoplasmic end in close proximity to TM 3 and the helix bundle. An illustrative set of the preferred conformations of the IANBD moiety covalently attached to the four substituted cysteines at the cytoplasmic side of TM 6 is shown. IANBD attached to Cys-2696.31 (blue) and Cys-2706.32 (red) are facing the hydrophobic milieu, whereas IANBD attached to Cys-2716.33 (yellow) and Cys-2726.34 (purple) are facing the protein interior. B, a hypothetical active conformation of the receptor in which the cytoplasmic side of TM 6 is moved away arbitrarily from the helix bundle and upward toward the hydrophobic region, marked by straight lines. This putative rearrangement of TM 6 moves all four IANBD-labeled residues upwards and outwards allowing them to penetrate further into the more hydrophobic region of the membrane/detergent headgroups and away from the more hydrophilic interior of the receptor protein. The movement can explain the observed shift for all four IANBD-labeled cysteines toward a less polar environment upon receptor activation. Because residues Cys-2696.31 and Cys-2706.32 are facing the hydrophobic aliphatic chains in both the inactive and active states, whereas Cys-2716.33 and Cys-2726.34 transfer from the protein interior to being exposed to the hydrophobic aliphatic chains upon activation, the latter residues could be expected to display a larger change in fluorescence emission upon agonist binding, in agreement with the experimental observations. Notably, the movement of the cytoplasmic part of TM 6 is shown to occur around the conserved proline kink but could as well involve a rigid body movement of the entire helix. However, our previous simulation of the TM 6 helix indicated the possibility that the kink in the TM 6 helix induced by Pro-2876.50 could behave as a flexible hinge, which can mediate the movement of the cytoplasmic side of TM 6 in relation to the extracellular region (10). Whereas the more kinked conformation would correspond to the inactive conformation (A), the active state may be represented by a less kinked conformation, resulting in the cytoplasmic portion of TM 6 moving away from the receptor core (B).

The biophysical analyses of conformational changes in rhodopsin and in β2AR raise new interesting questions about molecular modes of agonist-induced receptor activation. Most significantly, the present data substantiate differences between the kinetics of rhodopsin and β2AR activation. In rhodopsin, formation of the activated metarhodopsin II state occurs essentially within microseconds. Interestingly, metarhodopsin II subsequently undergoes a slow (t1/2 = ~6 min) transition to the inactive metarhodopsin III (12). The rapid activation kinetics for rhodopsin is likely directly facilitated by the fact that its ligand, cis-retinal, is covalently bound to the receptor as an inverse agonist and upon absorption of a photon isomerizes to an agonist (trans-retinal) within the binding pocket (reviewed in Ref. 39). Thus, ligand binding is not part of the activation process. This specialized mechanism of activation may be necessary to facilitate the very rapid response of rhodopsin to light. In contrast to the rapid activation and the
slow inactivation kinetics observed for rhodopsin, the agonist-induced conformational changes in the β2AR are slow ($t_{1/2} = \sim 2–3$ min) (9, 13). It cannot be excluded, of course, that this difference between rhodopsin and the β2AR is caused by a difference in the methodological approach. However, because the measurements were performed under similar conditions they may reflect differences between rhodopsin and a receptor activated by a diffusible ligand. In the present study we find that the agonist-induced spectral changes observed following IANBD labeling of cysteines introduced at the cytoplasmic side of TM 6 occur with kinetics similar to those observed following labeling of the endogenous cysteines (Cys-125,44 and Cys-285,47). This consistency for the different read-outs and the clear correlation between change in fluorescence and biological efficacy (9) support the contention that the slow kinetics is an intrinsic property of the receptor, at least when conformational changes are being probed using the IANBD fluorophore.

It is important to emphasize that our experiments have been carried out in the absence of G protein and that it is very likely that the G protein can affect the kinetics of the transition between the inactive AR state and the active AR* state. Clearly, the slow kinetics of the agonist-induced conformational change in the absence of G protein would predict a high activation energy barrier for this transition (13). It is conceivable, however, that the G protein is able to stabilize the agonist-receptor complex and accordingly lower the activation energy barrier substantially and cause receptor activation to occur significantly faster. This may provide an explanation for the apparent discrepancy between the slow kinetics of agonist-induced conformational changes observed for the purified β2AR with the rapid responses to agonist stimulation of GPCRs in cells, such as for example activation of ion channels. Unfortunately, it has not yet been possible to test the kinetic importance of the G protein because of technical difficulties. Credible spectroscopic analysis of the fluorescently labeled receptor reconstituted with Gα requires essentially 100% coupling efficiency between the receptor and Gα and thus a huge excess of Gα. At the current stage, we have not been able to establish these experimental conditions because of instability of the Gα protein.

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REFERENCES
1. Kolakowski, L. F. (1994) Receptors Channels 2, 1–7
2. Ji, T. H., Grossmann, M., and Ji, I. (1998) J. Biol. Chem. 273, 17299–17302
3. Strader, C. D., Fong, T. M., Tota, M. R., Underwood, D., and Dixon, R. A. F. (1994) Annu. Rev. Biochem. 63, 63101–63132
4. Gether, U. (2000) Endocr. Rev. 21, 90–113
5. Farabakhsh, Z. T., Ridge, K. D., Khorana, H. G., and Hubbell, W. L. (1995) Biochemistry 34, 8812–8819
6. Farrens, D. L., Altenbach, C., Yang, K., Hubbell, W. L., and Khorana, H. G. (1996) Science 274, 768–770
7. Altenbach, C., Yang, K., Farrens, D. L., Farabakhsh, Z. T., Khorana, H. G., and Hubbell, W. L. (1996) Biochemistry 35, 12470–12478
8. Dunham, T. D., and Farrens, D. L. (1999) J. Biol. Chem. 274, 1683–1690
9. Gether, U., Lin, S., and Koblika, B. K. (1995) J. Biol. Chem. 270, 28268–28275
10. Gether, U., Lin, S., Hanauni, P., Ballesteros, J. A., Weinstein, H., and Koblika, B. K. (1997) EMBO J. 16, 6737–6747
11. Ballesteros, J. A., and Weinstein, H. (1996) Methods Neurosci. 25, 366–428
12. Farabakhsh, Z. T., Hideg, K., and Hubbell, W. L. (1993) Science 262, 1416–1419
13. Gether, U., Ballesteros, J. A., Seifert, R., Sanders-Bush, E., Weinstein, H., and Koblika, B. K. (1997) J. Biol. Chem. 272, 2587–2590
14. Weiss, J. (1998) Pharmacol. Ther. 80, 231–234
15. Guan, X., Peroutka, S. J., and Koblika, B. K. (1992) Mol. Pharmacol. 41, 695–698
16. Seifert, R., Lee, T. W., Lam, V. T., and Koblika, B. K. (1998) Eur. J. Biochem. 259, 369–382
17. Jensen, A. D., and Gether, U. (2000) Methods Mol. Biol. 126, 345–361
18. Suryanarayana, S., Daunt, D. A., Van Zastrow, M., and Koblika, B. K. (1991) J. Biol. Chem. 266, 15488–15492
19. Rasmussen, S. G., Jensen, A. D., Laiakapis, G., Ghanouni, P., Javitch, J. A., and Gether, U. (1999) Mol. Pharmacol. 56, 175–184
20. Koblika, B. K. (1995) Annu. Rev. Biochem. 64, 269–282
21. Lakowicz, J. R. (1999) Principles of Fluorescence Spectroscopy, 2nd Ed., pp. 238–318, Kluwer Academic/Plenum Publishers, New York
22. Guarnieri, F., and Weinstein, H. (1996) J. Am. Chem. Soc. 118, 5580–5589
23. Guarnieri, F., and Wilson, S. R. (1996) J. Comput. Chem. 16, 648–653
24. Ballesteros, J., Kianovic, S., Guarnieri, F., Davies, P., Fromme, B. J., Konvicka, K., ChL, Millar, R. P., Davidson, J. S., Weinstein, H., and Sealfon, S. C. (1998) J. Biol. Chem. 273, 10445–10453
25. White, S. H., and Wimley, W. C. (1999) Annu. Rev. Biophys. Biomol. Struct. 28, 319–365
26. Mohamadi, F., Richards, N. G. J., Guide, W. C., Liskamp, R., Lipton, M., Caulfield, C., Chang, G., Hendrickson, T., and Still, W. C. (1990) J. Comput. Chem. 11, 440–467
27. Unger, V. M., Hargrave, P. A., Baldwin, J. M., and Schertler, G. F. (1997) Nature 389, 203–206
28. Baldwin, J. M., Schertler, G. F., and Unger, V. M. (1997) J. Mol. Biol. 272, 144–164
29. Seifert, R., Wenzel-Seifert, K., Lee, T. W., Gether, U., Sanders-Bush, E., and Koblika, B. K. (1998) J. Biol. Chem. 273, 5109–5116
30. Weiss, J. (1997) FASEB J. 11, 346–354
31. Samama, P., Cotech, S., Costa, T., and Lefkovitz, R. J. (1993) J. Biol. Chem. 268, 4625–4630
32. Javitch, J. A., Ballesteros, J. A., Weinstein, H., and Chen, J. (1998) Biochemistry 37, 998–1006
33. Palkowska, K., Kumasaka, T., Hori, T., Behnke, C. A., Motoshima, H., Fox, B. A., Le Trong, l., Teller, D. C., Okada, T., Stenkamp, R. E., Yamamoto, M., and Miyano, M. (2000) Science 289, 739–745
34. Ballesteros, J. A., and Weinstein, H. (1992) Biophys. J. 62, 107–109
35. Javitch, J. A., Fu, D., Liakakis, G., and Chen, J. (1997) J. Biol. Chem. 272, 18546–18550
36. Sheikh, S. P., Vilardaga, J. P., Baranski, T. J., Lichtarge, O., Iiri, T., Meng, E. C., Nissenson, R. A., and Bourne, H. R. (1999) J. Biol. Chem. 274, 17033–17041
37. Liu, J., Blin, N., Conklin, B. R., and Weiss, J. (1996) J. Biol. Chem. 271, 6172–6178
38. Sakmar, T. P. (1998) Prog. Nucleic Acids Res. Mol. Biol. 59, 1–34
39. DeBlasi, A., Reilly, K., and Metz, H. J. (1989) Trends Pharmacol. Sci. 10, 227–229