Agonists induce conformational changes in transmembrane domains III and VI of the β_2 adrenoceptor

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Agonist binding to G protein-coupled receptors is believed to promote a conformational change that leads to the formation of the active receptor state. However, the character of this conformational change which provides the important link between agonist binding and G protein coupling is not known. Here we report evidence that agonist binding to the β_2 adrenoceptor induces a conformational change around ¹²⁵Cys in transmembrane domain (TM) III and around ²⁸⁵Cys in TM VI. A series of mutant β_2 adrenoceptors with a limited number of cysteines available for chemical derivatization were purified, site-selectively labeled with the conformationally sensitive, cysteine-reactive fluorophore IANBD and analyzed by fluorescence spectroscopy. Like the wild-type receptor, mutant receptors containing ¹²⁵Cys and/or ²⁸⁵Cys showed an agonist-induced decrease in fluorescence, while no agonist-induced response was observed in a receptor where these two cysteines were mutated. These data suggest that IANBD bound to ¹²⁵Cys and ²⁸⁵Cys are exposed to a more polar environment upon agonist binding, and indicate that movements of transmembrane segments III and VI are involved in activation of G protein-coupled receptors.

Keywords: fluorescence spectroscopy/G protein-coupled receptors/molecular modeling/signal transduction

Introduction

The majority of hormones and neurotransmitters exert their physiological response by binding to cell-surface receptors belonging to the large family of G protein-coupled receptors (GPCRs) (Probst *et al.*, 1992; Schwartz *et al.*, 1996). The chemical variety among the endogenous ligands for this superfamily of seven transmembrane segment receptors is exceptional, ranging from monoamines, amino acids and purines to lipids, peptides and glycoproteins (Probst *et al.*, 1992; Schwartz *et al.*, 1996). GPCRs also act as important sensors of exogenous stimuli, such as light and odors (Probst *et al.*, 1992; Schwartz *et al.*, 1996). Moreover, the receptors for certain chemo-

kines have recently been shown to play an essential role in the cellular entry of human immunodeficiency virus (HIV) (Choe *et al.*, 1996; Feng *et al.*, 1996). In spite of this impressive functional variability among GPCRs, it is believed that the receptors share both a common topology and a common mechanism of activation; however, the molecular processes underlying activation of this class of receptors remain unknown.

Our current knowledge about structure–function relationships in GPCRs is primarily based on the results from mutagenesis studies performed over the last decade on many members of the receptor family (reviewed in Kobilka, 1992; Savarese and Fraser, 1992; Schwartz, 1994; Strader *et al.*, 1994). These studies have led to identification of domains involved in ligand binding and G protein coupling (Kobilka, 1992; Savarese and Fraser, 1992; Schwartz, 1994; Strader *et al.*, 1994). In addition, mutagenesis techniques have provided evidence for distinct intramolecular interactions supporting an anticlockwise orientation of the seven transmembrane helices in the plasma membrane (Suryanarayana *et al.*, 1992; Zhou *et al.*, 1994; Liu *et al.*, 1995; Elling and Schwartz, 1996; Mizobe *et al.*, 1996). An increasing number of three-dimensional models of the receptors have also been developed (MaloneyHuss and Lybrand, 1992; Trumpp-Kallmeyer *et al.*, 1992; Baldwin *et al.*, 1993; Cronet *et al.*, 1993; Roper *et al.*, 1994; Ballesteros and Weinstein, 1995; Fanelli *et al.*, 1995; Scheer *et al.*, 1996). These models were initially developed based on the known tertiary structure of bacteriorhodopsin, but later models have incorporated the structural information obtained from the 9 Å electron density projection map of rhodopsin (Schertler *et al.*, 1993). Unfortunately, the models remain only qualified predictions of the actual tertiary structure as long as no accurate structural information at the atomic level is available for this family of receptor proteins.

It is generally assumed that binding of the agonist to the receptor induces a set of finely orchestrated changes in the tertiary structure of the receptor which are recognized by the associated G protein alpha subunit. However, mutagenesis studies have not been able to elucidate the conformational changes that are critical for transferring information across the plasma membrane. Current models for activation of GPCRs are based on studies in which the conformation of the receptor was inferred from activation of messenger systems and/or from computational simulations (Samama *et al.*, 1993; Luo *et al.*, 1994; Ballesteros and Weinstein, 1995; Fanelli *et al.*, 1995; Scheer *et al.*, 1996). Techniques that can directly measure conformational changes have only recently been applied to this family of receptor proteins (Gether *et al.*, 1995; Farrens *et al.*, 1996; Turcatti *et al.*, 1996). Hubbell, Khorana and co-workers have used electron paramagnetic resonance (EPR) spectroscopy to study conformational

Fig. 1. 'Helical net' representation of the human β_2 adrenoceptor. The receptor contains 13 Cys residues of which five (^{77}Cys , ^{116}Cys , ^{125}Cys , ^{285}Cys and ^{327}Cys) (large black circles) are predicted to be in the transmembrane domain. Three Cys residues are predicted to be in the cytoplasmic regions (^{265}Cys , ^{378}Cys and ^{406}Cys) (large black circles). Five Cys residues are not expected to be available for chemical derivatization (small black circles); four residues (^{106}Cys , ^{184}Cys , ^{190}Cys and ^{191}Cys) form two disulfide bridges (Fraser, 1989; Dohlman *et al.*, 1990; Noda *et al.*, 1994) and ^{341}Cys in the intracellular C-terminal tail has been shown to be palmitoylated (O'Dowd *et al.*, 1989; Mouillac *et al.*, 1992). Residues shown shaded delineate the faces of TM III and TM VI segments that are oriented towards other transmembrane domains and not towards the lipid. Note that residues ^{125}Cys (TM III) and ^{285}Cys (TM VI) are seen on the edge of the patch of shaded residues oriented towards the interior of the protein, i.e. the fluorophores would be predicted to lie at the interface between the protein and lipid environments. The specific residues shown shaded have been identified by: (i) the substituted Cys accessibility method on TM III of the D2 receptor and (ii) from mutations that significantly affect ligand binding and/or activation of neurotransmitter receptors (Javitch *et al.*, 1995; VanRhee, 1996). Residues known to be involved in agonist binding in βAR (^{113}Asp in TM III, ^{203}Ser and ^{207}Ser in TM V, ^{290}Phe and ^{293}Asn in TM VI) are indicated by white letters in dark shaded circles (Tota *et al.*, 1990; Strader *et al.*, 1991, 1994; Wieland *et al.*, 1996).

changes in rhodopsin accompanying photoisomerization of retinal (Farrens *et al.*, 1996). Their data provided evidence that light activation of rhodopsin may involve changes in the arrangement of hydrophobic segments C and F [transmembrane segments (TM) III and VI in the nomenclature of GPCRs] (Farrens *et al.*, 1996). In the β_2 adrenoceptor ($\beta_2\text{AR}$), we have reported the use of the environmentally sensitive and cysteine-reactive fluorescent probe IANBD to monitor agonist-induced structural changes in the receptor molecule (Gether *et al.*, 1995). We found that agonists caused a dose-dependent and reversible decrease in fluorescence from the purified IANBD-labeled $\beta_2\text{AR}$ (Gether *et al.*, 1995). This suggested that agonists promote a conformational change in the receptor that leads to an increase in the polarity of the environment around one or more IANBD-labeled cysteines (Gether *et al.*, 1995). The wild-type receptor contains eight free cysteines, and mutagenesis and peptide mapping experiments have indicated that several of these sites are accessible for chemical derivatization (U.Gether and B.K.Kobilka, unpublished observation). To identify the cysteine(s) involved in the agonist-induced change in fluorescence and thereby map agonist-induced conformational changes in the $\beta_2\text{AR}$, we generated a series of mutant receptors having limited numbers of cysteines available for fluorescent labeling. The fluorescence spectroscopy analysis of the purified and site-selectively

labeled mutants showed that IANBD-labeled ^{125}Cys and ^{285}Cys are responsible for the observed changes in fluorescence consistent with movements of TM III and VI in response to agonist binding.

Results

Cysteines in the β_2 adrenoceptor can be mutated with minimal effect on receptor function

IANBD is a sulfhydryl-reactive, environmentally sensitive fluorophore. Covalent incorporation of IANBD into the $\beta_2\text{AR}$ receptor is associated with an increase in fluorescence intensity and a 15–20 nm decrease in λ_{max} (wavelength at which maximal fluorescence occurs) relative to free IANBD in aqueous solution (Gether *et al.*, 1995). This suggests that the observed fluorescence is derived from IANBD-labeled cysteines in the hydrophobic core of the receptor. This was further supported by quenching experiments with the hydrophilic quencher, potassium iodide, demonstrating a substantially lower accessibility to IANBD in the receptor as compared with IANBD free in solution (data not shown). The $\beta_2\text{AR}$ contains 13 cysteines of which five (^{77}Cys , ^{116}Cys , ^{125}Cys , ^{285}Cys and ^{327}Cys) are predicted to be in the hydrophobic transmembrane core of the receptor (Figure 1). One cysteine, ^{265}Cys , is predicted to be in the third intracellular loop and two cysteines, ^{378}Cys and ^{406}Cys , are located close to the

Table I. Binding properties and functional coupling of the wild-type β_2 AR and mutant receptors

Receptor	$B_{\max}$ (pmol/mg protein)	K_D , [3 H]DHA (nM)	Maximum cyclase (% of basal)	EC ₅₀ , ISO, cyclase (nM)	IC ₅₀ , ISO (μ M, purified receptor)
Wild-type β_2 AR (high expression)	9.0	0.29 (4)	259 (3)	16 (3)	1.6 (3)
Wild-type β_2 AR (low expression)	~1.2	—	199 (2)	85 (2)	—
Cys(285)	2.2	0.23 (4)	347 (3)	26 (3)	1.9 (3)
Cys(116, 285)	5.7	0.33 (4)	331 (2)	21 (2)	3 (3)
Cys(116, 125)	4.1	0.46 (2)	471 (3)	17 (3)	19 (2)
Cys(116, 125, 285)	11.1	0.31 (4)	421 (2)	10 (2)	1.6 (3)
Cys(77, 116, 265, 327, 378, 406)	2.7	0.51 (2)	346 (3)	103 (3)	17 (2)

$B_{\max}$ and K_D values for [3 H]DHA were determined from saturation binding experiments by non-linear regression analysis using Inplot 4.0 (GraphPad Software, San Diego, CA). Sf-9 cells expressing high and low levels of wild-type receptor were obtained by infecting cells for 48 and 24 h, respectively. Note that the $B_{\max}$ value for the low expressing wild-type membranes was determined using a single saturating concentration of [3 H]DHA. Adenylyl cyclase activity was measured in Sf-9 cell membranes according to Materials and methods. Maximum cyclase activity and EC₅₀ values were determined from dose-response curves with isoproterenol (ISO) by non-linear regression analysis. IC₅₀ values for inhibition of [3 H]DHA binding by ISO to the purified receptor were determined from competition binding experiments by non-linear regression analysis. All data are means of the number of experiments performed in duplicate shown in parentheses.

C-terminus of the β_2 AR (Figure 1). There are five cysteines in the receptor which are not expected to be available for chemical derivatization. In the extracellular loops, four cysteines (¹⁰⁶Cys, ¹⁸⁴Cys, ¹⁹⁰Cys and ¹⁹¹Cys) form two disulfide bridges (Figure 1) (Vauquelin *et al.*, 1979; Fraser, 1989; Dohlman *et al.*, 1990; Noda *et al.*, 1994; Mejean-Galzi *et al.*, 1995) and in the intracellular C-terminal tail, ³⁴¹Cys has been shown to be palmitoylated (O'Dowd *et al.*, 1989; Mouillac *et al.*, 1992).

To identify the cysteine(s) that are responsible for the agonist-induced change in fluorescence from the IANBD labeled β_2 AR, we mutated cysteines in the receptor and generated a series of mutant receptors with one, two or three cysteines in the TM regions of the receptor (Table I and Figure 1). Cysteine residues ⁷⁷Cys, ¹¹⁶Cys and ¹²⁵Cys were all changed to valines, and ³²⁷Cys was changed to serine based on previous experience with single substitution of these residues in the β_2 AR (Fraser, 1989; Dohlman *et al.*, 1990). ²⁸⁵Cys is highly conserved among G protein-coupled receptors and was mutated to serine, since this is the most frequently observed substitution in this position (Probst *et al.*, 1992). The combined cysteine substitutions in the β_2 AR had little effect on the general pharmacological properties of the receptor (Table I). Antagonist binding was unchanged in all mutants as assessed by saturation binding experiments with [3 H]dihydro-alprenolol ([3 H]DHA) (Table I). However, mutation of several cysteine residues led to a reduction in receptor expression (Table I). Notably, a mutant receptor with all free cysteines substituted was expressed so poorly that purification in sufficient quantities for fluorescence spectroscopy analysis was impossible (data not shown). The reduced expression of cysteine mutants may be due to inefficient post-translational processing of these proteins so that a larger fraction of the mutant receptor protein is incorrectly folded. However, this should not affect our spectroscopy data since the last step of the purification procedure involves a ligand affinity chromatography which separates functional from non-functional receptors.

The mutants were also analyzed for their ability to activate adenylyl cyclase. As shown in Table I, all mutants demonstrated efficient coupling to adenylyl cyclase with 3- to 4-fold increases in basal activity in response to

stimulation with the agonist, isoproterenol (Table I). The observed EC₅₀ values were found to be highly dependent on receptor expression. Two different expression levels of the wild-type receptor, 1.2 pmol/mg protein and 9.0 pmol/mg protein, resulted in a 5-fold difference in the EC₅₀ value (85 nM at the low expression level and 16 nM at the high expression level; Table I). Taking the different levels of expression into account, the EC₅₀ values for the mutants were all comparable with the EC₅₀ of the wild-type (Table I). Overall these data show that cysteine residues ⁷⁷Cys, ¹¹⁶Cys, ¹²⁵Cys, ²⁶⁵Cys, ²⁸⁵Cys, ³²⁷Cys, ³⁷⁸Cys and ⁴⁰⁶Cys are not essential for antagonist binding, nor are they required for the functional coupling of the β_2 AR.

Following purification, the mutant receptors were analyzed for their ability to bind the agonist, isoproterenol (Table I). We found that the IC₅₀ values for inhibition of [3 H]DHA binding to the Cys(285) and Cys(116, 125, 285) mutants were identical to the wild-type receptor but that the IC₅₀ values for the mutants, Cys(116, 285), Cys(116, 125) and Cys(77, 116, 265, 327, 378, 406), were ~10-fold higher than that for the wild-type (Table I). Nevertheless, the efficient coupling of these mutants to adenylyl cyclase demonstrates that they are able to undergo agonist-induced conformational changes similar to the wild-type receptor (Table I). We should also mention that the binding and functional coupling properties of the β_2 AR expressed in insect cells are comparable with those of the receptor expressed in mammalian cells (Benovic *et al.*, 1984; Bouvier *et al.*, 1989).

Fluorescence spectroscopy analysis of site-selectively labeled mutant β_2 adrenoceptors

The mutant receptors described in Table I were all purified and fluorescently labeled with the IANBD fluorophore. As expected, the IANBD-labeled mutants all demonstrated emission maxima around 525 nm (data not shown). In addition, the fluorescence intensity of the labeled mutants was reduced relative to the wild-type receptor consistent with the reduced number of derivatizable cysteines (data not shown). We have previously demonstrated that agonist stimulation of the purified IANBD-labeled wild-type receptor causes a reversible decrease in fluorescence

Fig. 2. Time course experiments with purified, IANBD-labeled wild-type and mutant β_2 adrenoceptors. Emission from IANBD-labeled wild-type β_2 adrenoceptor (**A**), mutant Cys(285) (**B**), mutant Cys(116, 125) (**C**), mutant Cys(116, 285) (**D**), mutant Cys(116, 125, 285) (**E**) and mutant Cys(77, 116, 265, 327, 378, 406) (**F**) measured over time in response to stimulation with the full agonist isoproterenol (10^{-3} M). The responses were reversed by either the antagonist (-)alprenolol (10^{-4} M) or by (-)propranolol (10^{-5} M) both having a more than 100-fold higher affinity for the receptors than isoproterenol (data not shown). By themselves, alprenolol and propranolol caused a relative increase in baseline fluorescence over time but with a slower time-course than the reversal of the agonist response (data not shown and Gether *et al.*, 1995). The ligand concentrations used were chosen to ensure saturation of the receptors eliminating any influence from different agonist affinities. Excitation was set at 481 nm and emission was measured at 525 nm. Fluorescence in the individual traces was normalized to the fluorescence observed immediately before addition of ligand. The experiments shown are representative of at least three identical experiments. (**G**) Bar diagram of changes in fluorescence in response to the full agonist isoproterenol for the wild-type receptor and indicated mutants. Data are given as percent change in fluorescence (mean $\pm$ SE, $n = 3-6$). The percent change was calculated as the change in fluorescence relative to the extrapolated baseline at $t = 15$ min after addition of ligand.

(Gether *et al.*, 1995). A typical experiment with wild-type receptor is shown in Figure 2A. Analysis of the mutants revealed that agonist-induced changes in fluorescence are observed only in receptors in which ^{285}Cys or ^{125}Cys are present (Figure 2). A mutant lacking only these two cysteines (Cys77, 116, 265, 327, 378, 406) showed no response to agonist binding (Figure 2F).

Both ^{285}Cys -NBD (^{285}Cys labeled with IANBD) and ^{125}Cys -NBD appeared to contribute to the agonist-induced change in fluorescence (Figure 2G). A decrease in fluores-

cence of similar amplitude was observed in IANBD-labeled Cys(116, 125) and in IANBD-labeled Cys(116, 285). The change in fluorescence in IANBD-labeled Cys(116, 285) was similar to that in IANBD-labeled Cys(285), confirming the lack of contribution of ^{116}Cys -NBD to the change in fluorescence. Moreover, the change in fluorescence from IANBD-labeled Cys(116, 125, 285) was nearly twice that of IANBD-labeled Cys(116, 125) or IANBD-labeled Cys(116, 285), further confirming that the molecular environment around both ^{285}Cys -NBD and

^{125}Cys -NBD is altered following agonist binding. In conclusion, the data suggest that agonist binding to the $\beta_2\text{AR}$ promotes a conformational change in the receptor that exposes NBD attached to ^{125}Cys in TM III and to ^{285}Cys in TM VI to a more polar environment.

Molecular modeling

To define the possible spatial orientation of NBD bound to ^{285}Cys and ^{125}Cys , we performed a series of computational simulations of TM III and VI. The MonteCarlo simulations demonstrated that the conformational space available for Cys-NBD in TM III and VI is significantly restricted, even though each Cys-NBD residue has eight rotatable side-chain angles (Figure 3). The restrictions are imposed by the intrinsic rigidity within the NBD moiety, and by additional steric hindrance from the helix backbone (e.g. the χ_1 angle of Cys-NBD is restricted to the *trans* configuration on both ^{125}Cys and ^{285}Cys). This considerable restriction, evident from preliminary explorations of the molecular models, was further tested by starting all MonteCarlo simulations from different χ angles; the final angles returned the conformations to the previously observed preferred geometries. The preferred conformations for ^{125}Cys -NBD and ^{285}Cys -NBD are shown in Figure 3A in the context of a three-dimensional model of the $\beta_2\text{AR}$. This preferred conformation is extracted from the results of the conformational memories simulation (see Materials and methods) depicted in Figure 3B by 100 representative conformations of Cys-NBD at each site. The space spanned by these conformations provides an indication of the conformational freedom of the NBD molecules. The conformational space available for the fluorophore is clearly confined to a limited number of preferred orientations adopted by the 100 representative structures, illustrating the conformational rigidity mentioned above. This conformational restraint for NBD attached to cysteines suggests that changes in emission from the fluorophore most likely reflects changes in the orientation of the TM helix to which it is attached, rather than movement of the NBD relative to the transmembrane helix.

Residues in TM III and VI known to be involved in agonist binding in the βAR are shown in white in Figure 3. Other residues known to face toward the protein interior are shown in red to illustrate their orientations towards the protein interior. These residues are at the positions identified from mutagenesis and/or by the substituted cysteine accessibility method to affect ligand binding or activation of neurotransmitter receptors (Javitch *et al.*, 1995; Van Rhee, 1996). Within this reference framework, the preferred conformations of ^{125}Cys -NBD and ^{285}Cys -NBD are seen to be oriented mostly towards the lipid milieu (or the hydrophobic tails of the detergent micelle in purified receptor). This conclusion is in agreement with the experimentally determined emission maxima which indicate a highly apolar environment for the bound fluorophore (Gether *et al.*, 1995).

Chemical modification of the wild-type cysteine residues could potentially perturb the structure of the receptor and hence alter its activation mechanism. However, MonteCarlo simulations indicated that the conformational space available to residues facing the protein interior was not significantly altered by incorporation of NBD on

to ^{125}Cys and ^{285}Cys (data not shown). Moreover, the observation that NBD bound to ^{125}Cys and ^{285}Cys is oriented predominantly towards the lipid suggests that these probes, when bound to the receptor, are unlikely to significantly alter the overall structure of the receptor. This is in agreement with data showing similar binding affinities for wild-type and IANBD-labeled receptor (data not shown and Gether *et al.*, 1995).

Discussion

The purpose of the present study was to obtain insight into the activation mechanism of G protein-coupled receptors by mapping agonist-induced conformational changes in the purified β_2 adrenoceptor ($\beta_2\text{AR}$) using fluorescence spectroscopy analysis. A series of mutant receptors with a limited number of cysteines available for chemical derivatization were generated and site-selectively labeled with the conformationally sensitive and sulfhydryl-reactive IANBD fluorophore. Fluorescence spectroscopy analysis of the purified receptor mutants revealed that agonist binding leads to a conformational change that alters the molecular environment around NBD bound to ^{125}Cys in TM III and ^{285}Cys in TM VI. Importantly, computational simulations demonstrated a significant conformational restraint for the NBD bound to these two cysteines as illustrated in Figure 3. This suggests that the change in molecular environment around the bound NBD reflects movement of the transmembrane helix to which it is attached, rather than movement of the NBD relative to the transmembrane helix.

It is important to note that, based on the experimental data and the computational simulations, it is highly unlikely that the agonist-induced changes in fluorescence reflect quenching due to interaction between NBD and the ligand. Labeling of the receptor with IANBD does not alter agonist or antagonist binding properties (Gether *et al.*, 1995), as would be expected if the bound NBD was positioned within the ligand-binding pocket. The results from mutagenesis studies have also provided substantial evidence that amino acids involved in forming the ligand-binding pocket are on a different side of the transmembrane α helix, and 1–2 α -helical turns closer to the membrane surface relative to ^{125}Cys and ^{285}Cys . This is illustrated in the receptor models in Figure 3 and by the side view of TM III and VI in Figure 4C, showing that the fluorophore is well separated from the residues forming interactions with the agonist. Moreover, we have obtained convincing evidence that the change in fluorescence represents a conformational change which is important for receptor activation. Thus, the magnitudes of the fluorescence changes were found to correlate with the intrinsic biological efficacy of the ligand, as demonstrated by comparing the effect of a series of partial and full agonists on adenylyl cyclase activity with their effect on the magnitude of the fluorescence changes (Gether *et al.*, 1995). Interestingly, analysis of a constitutively activated $\beta_2\text{AR}$ showed that constitutive activation is associated with greater changes in fluorescence, especially in response to partial agonists (Gether *et al.*, 1997). This is in agreement with biological data showing higher biological efficacy of partial agonists on the constitutively activated mutant $\beta_2\text{AR}$.

Fig. 3. Orientation of $^{125}\text{Cys-NBD}$ and $^{285}\text{Cys-NBD}$ in the context of a model of the transmembrane domains of the β_2 adrenoceptor, seen from the extracellular side. **(A)** In the preferred conformation, $^{125}\text{Cys-NBD}$ and $^{285}\text{Cys-NBD}$ lie at the protein-lipid interface, oriented predominantly towards the lipid. This orientation is substantiated by comparison with the residues that are known to be oriented towards the interior of the protein (shown in red and identified in Figure 1), and by residues in TM III and VI known to be involved in agonist binding in βAR (^{113}Asp , ^{290}Phe and ^{293}Asn , shown in white) (Tota *et al.*, 1990; Strader *et al.*, 1991, 1994; Wieland *et al.*, 1996). The seven transmembrane helical domains are shown schematically by helical ribbons (yellow) following the electron density footprint of rhodopsin (Schertler *et al.*, 1993). Note the highly conserved Pro residue in TM VI and the kink that it induces in this helix. **(B)** Superposition of 100 representative conformations of $^{125}\text{Cys-NBD}$ and $^{285}\text{Cys-NBD}$ obtained from the conformational memories calculations, shown in the same structural context as in (A). The close clustering of these conformations that identify the conformational space available to the Cys-bound fluorophores indicates that the preferred conformation of the fluorophores is restricted to a well defined region.

The most likely interpretation of our results is therefore that agonist binding promotes conformational changes involving movements of helix III and VI. As shown in Figure 3, ^{285}Cys is predicted to be at the helix VI–VII

interface in a boundary zone between the lipid bilayer (or the hydrophobic tails of the detergent micelle) and the more polar interior of the protein. Thus, an agonist-induced movement of helix VI could lead to movement

Fig. 4. The relationship of $^{285}\text{Cys-NBD}$ to the Pro-kink of TM VI. (A) The fluorophore is positioned within the region of TM VI (yellow ribbon) that contains the kink induced by ^{288}Pro , which spans from ^{284}Leu to ^{288}Pro . Note the position of the preferred conformation of $^{285}\text{Cys-NBD}$ at the protein-lipid interface, defined by the residues that are known to be oriented inwards towards the protein (shown in red and identified in Figure 1), and by residues in TM VI known to be involved in agonist binding in βAR (^{290}Phe and ^{293}Asn , shown in white) (Tota *et al.*, 1990; Strader *et al.*, 1994; Wieland *et al.*, 1996). The location of residues involved in G protein coupling is illustrated by the position of ^{285}Leu shown in red at the cytoplasmic end of TM VI. (B) Superposition of 100 representative conformations of $^{285}\text{Cys-NBD}$ and of the corresponding helix backbones (identified by their helix axes, in blue), obtained from the exploration of conformational space with the conformational memories method. Note that the kink induced by ^{288}Pro provides a flexible hinge connecting the binding site residues (in white, at the top of the figure) with the putative G protein-coupling domain at the cytoplasmic end of the helix. Varying degrees of kinking indicated by the helix axis (blue) have been implicated in the activation mechanism (Zhang and Weinstein, 1993). Thus, the positioning of $^{285}\text{Cys-NBD}$ at the Pro-kink of TM VI makes it particularly sensitive to agonist-induced changes in the orientation of this TM helix. (C) Side view of the seven TM model including 100 representative conformations of $^{285}\text{Cys-NBD}$ and $^{125}\text{Cys-NBD}$, highlighting TM VI and III. Note that the NBD moieties are well within the space representing the membrane environment, at the lipid-protein interface, but not in contact with the βAR -binding site residues (shown in white). The flexible hinge provided by the Pro-kink is illustrated by the wide margin span of the 100 representative TM VI helix axes (in blue).

of NBD bound to ^{285}Cys from the non-polar environment of the lipid bilayer (or detergent micelle) to the more polar environment of the interior of the protein, explaining the observed changes in fluorescence. Notably, ^{285}Cys is situated one α -helical turn below ^{288}Pro , which is highly conserved among GPCRs and provides a flexible hinge in TM VI. Figure 4 shows how varying degrees of kinking of TM VI around the proline hinge affect the position of the fluorophore. Consequently, the movement of ^{285}Cys -NBD to a more polar environment in the protein interior would be directly facilitated by this flexible hinge connecting the binding site residues (in white, at the top of Figure 4A, B and C) with the putative G protein-coupling domain at the cytoplasmic end of the helix. Involvement of the proline in receptor activation has been suggested from modeling studies (Ballesteros and Weinstein, 1995). Like ^{285}Cys -NBD, ^{125}Cys -NBD is predominantly exposed to the lipid bilayer (or detergent micelle) (Figure 3). Our data indicate that the extent of lipid exposure is changed in response to agonist binding. This suggests that the agonist induces a movement of TM III, causing the fluorophore to be exposed to a more polar face of TM IV and/or the more polar interior of the receptor (Figure 3). It is however important to emphasize that the amplitude of the fluorescent change is only a rough indicator of the magnitude of conformational change. For example, we cannot assume that there is a linear correlation between change in fluorescence and magnitude of movements. Therefore, the movement of TM III may not be of the same magnitude as movement of TM VI.

The present data with the $\beta 2\text{AR}$ demonstrate striking parallels to recent studies in rhodopsin where EPR spectroscopy was used to delineate structural changes associated with activation (Farahbakhsh *et al.*, 1995; Altenbach *et al.*, 1996; Farrens *et al.*, 1996). The studies suggested that rhodopsin activation entails a rigid-body movement of TM VI relative to TM III. Specifically, Farrens *et al.* (1996) propose an anticlockwise rotation (viewed from the extracellular side as in Figure 3) of the TM VI segment and a translation away from the TM III segment at the cytoplasmic side. This rotation of TM VI proposed for rhodopsin activation, if applied to the $\beta 2\text{AR}$ model shown in Figure 3, would rotate ^{285}Cys -NBD in an anticlockwise direction and thus reorient the fluorophore towards the interior of the protein in full agreement with our experimental observations. Rearrangement of TM III and VI as a key element in receptor activation has also been supported by the observation that binding of Zn^{2+} to a bis-His metal ion-binding site constructed between TM III and VI in rhodopsin can block transducin activation (Sheik *et al.*, 1996).

The binding of catecholamine agonists to the $\beta 2\text{AR}$ also involves important interactions in both TM III and TM VI (Figure 4C). In TM III the highly conserved aspartic acid (^{113}Asp) is believed to form an ionic interaction with the positively charged nitrogen of epinephrine (Strader *et al.*, 1991, 1994). In TM VI the agonist is believed to form hydrophobic interactions with ^{290}Phe (Tota *et al.*, 1990), and evidence suggests the presence of a hydrogen bond between the β -OH-group of epinephrine and ^{293}Asn (Wieland *et al.*, 1996). Critical agonist interactions in TM VI have also been described for other G protein-coupled receptors (Wess *et al.*, 1991; Choudary *et al.*, 1995) and,

in rhodopsin, retinal has been shown to interact with the highly conserved ^{265}Trp (^{286}Trp in $\beta 2\text{AR}$) (Nakayama and Khorana, 1991). An essential role of TM VI in receptor activation is further indicated by several studies demonstrating that discrete mutations at the bottom of TM VI can constitutively activate many GPCRs including the $\beta 2\text{AR}$ (Allen *et al.*, 1991; Lefkowitz *et al.*, 1993; Samama *et al.*, 1993). These mutations may disrupt constraining intramolecular interactions, thereby leading to agonist-independent movements of TM VI. Importantly, we have observed a substantially enhanced fluorescence change following agonist stimulation of an IANBD-labeled, constitutively active mutant of the βAR as compared with the wild-type receptor (Gether *et al.*, 1995).

In light of the suggested movements of TM III as a part of receptor activation (Farrens *et al.*, 1996; Sheik *et al.*, 1996; this study), it is noteworthy that protonation of the aspartic acid in the highly conserved DRY-motif at the cytoplasmic border of TM III has been proposed to be essential for receptor activation (Arnis *et al.*, 1994; Scheer *et al.*, 1997). Thus, molecular dynamics simulations in the adrenergic α_{1B} receptor comparing the protonated state of the aspartic acid with the deprotonated state indicated that protonation caused significant movements of TM III due to a shift of the arginine in the DRY-motif out of a 'polar pocket' formed by residues in TM I, II and VII (Scheer *et al.*, 1996).

In conclusion, the present data represent the first mapping of agonist-induced conformational changes in a hormone-activated G protein-coupled receptor. Computational simulation demonstrated that the changes are consistent with significant movements of TM III and VI in agreement with recent spin-labeling studies in rhodopsin. This strongly suggests that our observations can be generalized to other members of the superfamily of G protein-coupled receptors. Nevertheless, the fact that TM III and VI may undergo significant conformational changes during receptor activation does not exclude the possibility that other domains also move in response to agonist binding. For example, movements of TM V and VII have been indicated from computational simulations comparing the agonist bound and unliganded form of the 5-HT_{2A} receptor (Zhang and Weinstein, 1993). Hence, additional experiments based on biophysical techniques are required in the future to clarify further the changes involved in activation of G protein-coupled receptors.

Materials and methods

Nomenclature

The cysteine mutants constructed in the present study were named according to the cysteines still present in the receptor and available for chemical derivatization. Thus, $\text{Cys}(285)$ describes a construct where ^{285}Cys is present but where ^{77}Cys , ^{116}Cys , ^{125}Cys , ^{265}Cys , ^{327}Cys , ^{378}Cys and ^{406}Cys have been mutated. ^{106}Cys , ^{184}Cys , ^{190}Cys and ^{191}Cys , which form two-disulfide bridges, and ^{341}Cys , which is palmitoylated, were excluded from the nomenclature, since they are not available for chemical derivatization (Fraser, 1989; Dohlman *et al.*, 1990; Noda *et al.*, 1994).

Mutagenesis

The cDNA encoding the human β_2 adrenoceptor, epitope-tagged at the N-terminus with the cleavable influenza hemagglutinin signal sequence followed by the 'FLAG'-epitope (IBI, New Haven, CT) and tagged at the C-terminus with six histidines (SF-h $\beta 2$ -6H), was used as a template for the mutagenesis (Guan *et al.*, 1992). To facilitate the cloning

procedures, an *NheI* site at bp 426 (as counted from the initiator codon) and a *SacI* site at bp 1083 were introduced in SF-h β_2 -6H by mutagenesis before constructing the cysteine mutants. The *NheI* and *SacI* sites and cysteine mutations C77V, C116V, C125V, C265A, C285S, C327S, C378A and C406A were all generated by PCR-mediated mutagenesis using *Pfu* polymerase according to the manufacturer's instructions (Stratagene, La Jolla, CA). The generated PCR fragments were digested with the appropriate enzymes, purified by agarose gel electrophoresis and cloned into the baculovirus expression vector pVL1392 containing SF-h β_2 -6H (Guan *et al.*, 1992). The constructs, Cys(285), Cys(116, 125, 285), Cys(116, 125), Cys(116, 285) and Cys(77, 116, 265, 327, 378, 406), were obtained by combining selected restriction enzyme fragments. All mutations were confirmed by restriction enzyme analysis and sequenced.

Expression of receptors in Sf-9 insect cells

SF-h β_2 -6H and mutant constructs in the baculovirus expression vector pVL1392 were co-transfected with linearized BaculoGold DNA into Sf-9 insect cells using the BaculoGold transfection kit (Pharmingen, San Diego, CA). The resulting viruses were harvested after 4–5 days and amplified once before plaque purification. The plaque-purified viruses were amplified several times to obtain 500 ml of a high titer virus stock ($\sim 1 \times 10^9$ p.f.u.). Sf-9 insect cells were grown in suspension culture in SF 900 II medium (Gibco, Grand Island, NY) containing 5% fetal calf serum (Gemini, Calabasas, CA) and 0.1 mg/ml gentamicin (Gibco). For purification, the cells were grown in 1000–1200 ml cultures. Cells were infected with a 1:30–40 dilution of a high titer virus stock at a density of $3.5\text{--}5.5 \times 10^6$ cells/ml and harvested after 48 h by centrifugation for 10 min at 5000 g. The resulting cell pellets were kept at -70°C until used for purification.

PCR analysis of virus stocks

The virus stocks were routinely checked for expression of the correct mutant by PCR analysis. Briefly, virus was isolated by centrifugation of 5–10 ml virus stock for 20 min at 40 000 g. The pelleted virus was resuspended in 400 μl TE-buffer (10 mM Tris–HCl plus 0.1 mM EDTA) and incubated for 30 min at 37°C with 10 $\mu\text{g}/\text{ml}$ RNase followed by incubation for 30 min at 37°C in the presence of 10 $\mu\text{g}/\text{ml}$ proteinase K and 0.5% SDS. The viral DNA was isolated from the lysate by phenol–chloroform extraction and ethanol precipitation. The entire coding sequence of the receptor was amplified by PCR using *Taq* polymerase, and the resulting fragments analyzed by restriction enzyme analysis and agarose gel electrophoresis.

Membrane preparation and adenylyl cyclase assay

Membranes were prepared as described (Gether *et al.*, 1995) from Sf-9 cells (30 ml cultures in 125 ml disposable Erlenmeyer flasks at a density of 3×10^6 cells/ml) that were infected with baculovirus encoding the different receptor constructs for 24 or 48 h. Protein was determined using the Bio-Rad DC protein assay kit (Bio-Rad, Hercules, CA). Adenylyl cyclase in membranes was measured as described (Suryanarayana *et al.*, 1991).

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]dihydro-alprenolol (Amersham, Arlington Heights, IL) as radioligand (Suryanarayana *et al.*, 1991). Binding assays on solubilized and purified β_2 receptor were also performed as described using [^3H]dihydro-alprenolol as radioligand (Gether *et al.*, 1995). Binding data were analyzed by non-linear regression analysis using Inplot 4.0 from GraphPad Software, San Diego, CA.

Purification procedures

The wild-type and mutant receptors were purified using a two-step purification procedure (Gether *et al.*, 1995, 1997; Kobilka, 1995). One or two pellets of cells from 1000 ml infected cultures were lysed in a 10 mM Tris–HCl buffer, pH 7.5, containing 1 mM EDTA, 10 $\mu\text{g}/\text{ml}$ leupeptin (Boehringer, Mannheim, Germany), 10 $\mu\text{g}/\text{ml}$ benzamidine (Sigma) and 0.2 mM phenylmethylsulfonyl fluoride (Sigma). Following centrifugation (30 000 g for 20 min), the lysed cells were resuspended and subsequently solubilized under constant stirring for 2 h at 4°C in a 20 mM Tris–HCl buffer, pH 7.5, containing 1.0% *n*-dodecyl- β -D-maltoside (D β M), 500 mM NaCl, 10 $\mu\text{g}/\text{ml}$ leupeptin (Boehringer, Mannheim, Germany), 10 $\mu\text{g}/\text{ml}$ benzamidine (Sigma), 0.2 mM phenylmethylsulfonyl fluoride (Sigma) and 10^{-6} M alprenolol (Sigma). The solubilized receptor was purified by nickel-column chromatography using chelating Sepharose (Pharmacia, Uppsala, Sweden) as described

(Gether *et al.*, 1995; Kobilka, 1995). The eluate from the Ni-column was further purified by alprenolol-affinity chromatography as described (Gether *et al.*, 1995; Kobilka, 1995). Approximately 5 nmol of purified protein could generally be obtained from a 1000 ml culture. The specific activity of the purified receptors varied between 4 and 12 nmol/mg protein. Protein was determined using the detergent-insensitive Bio-Rad DC protein assay kit (Bio-Rad, Hercules, CA). Purified receptors were analyzed by classical 10% SDS–PAGE. The protein was visualized by standard Coomassie staining. The results obtained with wild-type purified by the two-step purification procedure described here were indistinguishable from results obtained using our previously described three-step purification procedure (Gether *et al.*, 1995; Kobilka, 1995).

Fluorescence labeling

Purified receptors (1–1.5 nmol) were labeled according to previously described methods with 15- to 20-fold molar excess of IANBD [*N,N'*-dimethyl-*N*-(iodoacetyl)-*N'*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) ethylene-diamine] (Molecular Probes, Eugene, OR) in a total volume of 100 μl buffer (20 mM Tris-buffer, pH 7.4, containing 100 mM NaCl and 0.08% D β M). The reaction was allowed to proceed for 1 h at room temperature in the dark and was quenched by addition of 1 mM cysteine. Cysteine-reacted dye was removed by desalting on a Sephadex G50 gel filtration column (0.5 $\times$ 9 cm) followed by concentrating the resulting sample to 100 μl using a Centricon-30 concentrator (Amicon, Beverly, MA). Alternatively, purified receptor was bound to a 150 μl nickel column (chelating Sepharose) and IANBD labeling achieved by recycling 1.0 ml of 0.5 mM IANBD in buffer (Tris–HCl, pH 7.5, containing 500 mM NaCl and 0.08% D β M) over the nickel column for 20 min. Excess dye was removed by extensive washing of the column with approximately 50 column volumes of buffer. Labeled receptor was eluted with 200 mM imidazole in buffer. Both labeling procedures resulted, depending on the mutant, in incorporation of 0.5–1.5 mol IANBD per mol receptor, as determined by measuring absorption at 481 nm and using an extinction coefficient of 21 000 M/cm for IANBD and a mol. wt of 50 000 Da for the receptor. Identical results were obtained with both labeling procedures. Derivatization of the receptor with IANBD did not perturb receptor pharmacology and labeling could be selectively blocked by sulfhydryl-reactive agents (Gether *et al.*, 1995).

Fluorescence spectroscopy

Fluorescence spectroscopy was performed at room temperature on a SPEX Fluoromax spectrofluorometer with photon counting mode using an excitation emission bandpass of 4.2 nm as described previously (Gether *et al.*, 1995). Briefly, both emission scans and time course experiments were done with 50 pmol IANBD-labeled receptor in 500 μl buffer (20 mM Tris-buffer, pH 7.4, containing 100 mM NaCl and 0.08% D β M) under constant stirring. In emission scan experiments the excitation wavelength was 481 nm and emission measured from 490 to 625 nm with an integration time at 0.3 s/nm. During time scan experiments the excitation wavelength was 481 nm and emission was measured at a wavelength of 525 nm. The volume of the added ligands was 1% of total volume and fluorescence was corrected for this dilution. The compounds tested in the fluorescence experiments had an absorbance of less than 0.01 at 481 and 523 nm in the concentrations used excluding inner filter effects.

Molecular modeling

The TM segments III (residues 107–136) and VI (residues 227–197) containing the Cys–NBD labels were modeled as α -helices, except for the Pro-kink in TM VI, as described (Ballesteros and Weinstein, 1995), and subjected to MonteCarlo simulations. The simulations explored a region of the TM domains composed of 11 residues centered on the Cys–IANBD. The novel technique of conformational memories (Guarnieri and Wilson, 1995, 1996) was used to perform a search of the conformational space by variation of torsional angles. The variation of backbone dihedral angles ϕ and ψ were restrained to 20° from their initial values, except for the Pro-kink region (residues ^{284}Leu to ^{288}Pro), where ϕ and ψ angles were restrained within 50° of their initial values due to the known flexibility of Pro-kinks relative to α -helical domains (Ballesteros and Weinstein, 1995). Side chain dihedral angles were rotated freely. More than 100 rounds of independent random simulations were performed for each TM segment. In each round, repeated runs of MonteCarlo simulated annealing (Kirkpatrick, 1983) were performed from a starting temperature of $T_1 = 2070$ K, with a cooling schedule of $T_{n+1} = 0.9 \cdot T_n$, and 10 000 steps per temperature to reach 310 K. This first stage identifies the regions of the torsional angle space that are populated at 310 K for each dihedral angle, i.e. the conformational

memories. In the second stage the conformational memories are used in a biased MonteCarlo procedure to sample only from these populated regions. This step yields the ensemble of structures accessible at 310 K. The conformational space of each NBD-Cys was analyzed in the context of a model of the β 2AR constructed using previously described approaches (Ballesteros and Weinstein, 1995), that follows the electron density footprint of rhodopsin (Scherler *et al.*, 1993).

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