Constitutive Activation of the β2 Adrenergic Receptor Alters the Orientation of Its Sixth Membrane-spanning Segment*

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The binding site of the β2 adrenergic receptor, like that of other homologous G-protein-coupled receptors, is contained within a water-accessible crevice formed among its seven membrane-spanning segments. Methanethiosulfonate ethylammonium (MTSEA), a charged, hydrophilic, lipophobic, sulfhydryl-specific reagent, had no effect on the binding of agonist or antagonist to wild-type β2 receptor expressed in HEK 293 cells. This suggested that no endogenous cysteines are accessible in the binding site crevice. In contrast, in a constitutively active β2 receptor, MTSEA significantly inhibited antagonist binding, and isoproterenol slowed the rate of reaction of MTSEA. This implies that at least one endogenous cysteine becomes accessible in the binding site crevice. In the constitutively active β2 receptor, Cys-285, in the sixth membrane-spanning segment, is responsible for the inhibitory effect of MTSEA on ligand binding to the constitutively active mutant. The acquired accessibility of Cys-285 in the constitutively active mutant may result from a rotation and/or tilting of the sixth membrane-spanning segment associated with activation of the receptor. This rearrangement could bring Cys-285 to the margin of the binding site crevice where it becomes accessible to MTSEA.

The interaction of a diverse array of signals, including neurotransmitters, peptides, hormones, light, and odorants, with G-protein-coupled receptors results in a conformational change which enhances their interaction with heterotrimeric G-proteins and thereby promotes GDP release and subsequent GTP binding and G-protein activation (1). In rhodopsin, for example, photoisomerization of retinal leads to rigid body movements of the third (M3) and sixth (M6) membrane-spanning segments relative to each other (2). Moreover, disulfide cross-linking of these membrane-spanning segments prevents the activation of transducin, the G-protein associated with rhodopsin, further supporting the relevance of their movement in the activation of rhodopsin. The generality of this movement to other G-protein-coupled receptors, however, is not known. Moreover, additional details of the structural mechanisms of receptor activation are necessary to better understand this process.

The binding sites of the β2 adrenergic receptor and of the homologous receptors for other biogenic amines are formed among their seven, mostly hydrophobic, membrane-spanning segments (1, 3) and are accessible to charged, water-soluble agonists like norepinephrine. Thus, for each of these receptors, the binding site is contained within a water-accessible crevice, the binding site crevice, extending from the extracellular surface of the receptor into the plane of the membrane. The surface of this crevice is formed by residues that can contact specific agonists and/or antagonists and by other residues that may play a structural role and affect binding indirectly.

To identify the residues that form the surface of the binding site crevice in the dopamine D2 receptor and the β2 adrenergic receptor, we have used the substituted cysteine accessibility method (4–10). Consecutive residues in the membrane-spanning segments are mutated to cysteine, one at a time, and the mutant receptors are expressed in heterologous cells. If ligand binding to a cysteine substitution mutant is near-normal, we assume that the structure of the mutant receptor, especially around the binding site, is similar to that of wild type, and thus, that the substituted cysteine lies in a similar orientation to that of the wild-type residue. In the membrane-spanning segments, the sulfhydryl of a cysteine can face either into the binding site crevice, into the interior of the protein, or into the lipid bilayer; sulfhydryls facing into the binding site crevice should react much faster with charged, hydrophilic, lipophobic, sulfhydryl-specific reagents. For such polar sulfhydryl-specific reagents, we use derivatives of methanethiosulfonate (MTS). These reagents form mixed disulfides with the cysteine sulfhydryl, covalently linking −SCH2CH2X, where X is NH3 in the case of MTS ethylammonium (MTSEA) (11). The reagents reportedly react with the ionized thiolate (RS−) more than a billion times faster than with the unionized thiol (RSH) (12), and only cysteines accessible to water are likely to ionize. We use two criteria for identifying an engineered cysteine as forming the surface of the binding site crevice: (i) the reaction with an MTS reagent alters binding irreversibly and (ii) this reaction is retarded by the presence of agonists or antagonists. To identify activation-induced structural changes in the residues forming the surface of the binding site crevice, we sought to determine the rates of reaction of MTSEA with a series of engineered cysteines in the resting and activated receptor. Agonist cannot be used to activate receptor, however, because the presence of a ligand within the binding site would interfere with access of the MTSEA to the engineered cysteines. Alternatively, the activated state of the receptor can be achieved by

membrane-spanning segment; M7, the seventh membrane-spanning segment; MTS, methanethiosulfonate; MTSEA, MTS ethylammonium; CAM, constitutively active mutant; PBS, phosphate-buffered saline; IANBD, N,N'-dimethyl-N-(iodoacetyl)-N'-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)ethylenediamine; ANOVA, analysis of variance.

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using a constitutively active mutant (CAM) $\beta_2$ adrenergic receptor as a background for further cysteine substitution. The CAM used here, L266S/K267R/H269K/L272A, has been well characterized previously (13, 14) and is intrinsically active and has a higher affinity for agonist than does the wild-type receptor. The high affinity state for agonist is typically associated with the activated receptor-G-protein complex. That agonist affinity is higher in the CAM even in the absence of G-protein suggests that the structure of the binding site of the CAM is likely to be similar to that of the agonist-activated wild-type receptor binding site. Thus, using wild-type $\beta_2$ receptor and the CAM, we can compare the resting and active forms of the receptor by determining the accessibility of residues in the binding site crevice in these two states.

To determine the accessibilities of substituted cysteines, the background receptor into which we substitute cysteines must be unaffected by our sulphydryl reagents. Although we find here that ligand binding to the wild-type $\beta_2$ adrenergic receptor was unaffected by MTSEA, surprisingly, MTSEA inhibited antagonist binding to the CAM form of the $\beta_2$ receptor. We identify Cys-285 in M6 as the sole cysteine responsible for this susceptibility of CAM. Because this Cys is protected by ligand, it is likely accessible within the binding site crevice in the CAM, but not in wild-type $\beta_2$ receptor.

EXPERIMENTAL PROCEDURES

$\beta_2$ Plasmids—The DNA sequence encoding the human $\beta_2$ adrenergic receptor, epitope-tagged at the amino terminus with the cleavable influenza-hemagglutinin signal sequence followed by the "FLAG"-epitope (IBI, New Haven, CT) and tagged with six histidines at the carboxyl terminus, was a gift from Dr. B. Kobilka (Stanford, CA) (15). This DNA was subcloned into the bicistronic expression vector pci4 (16), a gift from Dr. S. Rees (Glaxo, UK), thereby creating the vector pci4-FLAG$\beta_2$. The CAM $\beta_2$ receptor (13) (L266S/K267R/H269K/L272A) was a gift from Dr. R. J. Lefkowitz (Durham, NC). A fragment encoding the CAM mutation was subcloned into pci4-FLAG$\beta_2$ to create the vector pci4-FLAGCAM$\beta_2$.

Mutations of endogenous cysteines were generated using the Chameneon mutagenesis kit (Stratagene). Mutations were confirmed by DNA sequencing. Mutants are named as (wild-type residue) (residue number) (mutant residue), where the residues are given in the single-letter code. Plasmids containing the mutations C77V and C116V were a gift from Dr. B. Kobilka (Stanford, CA), and fragments containing these mutations were subcloned into the pci4-PCAM2H plasmid as were fragments encoding the mutations C125Y, C295S, and C327S.

Transient and Stable Transfection—HEK 293 cells were grown in Dulbecco's modified Eagle's medium/F-12 (1:1) containing 3.15 g/liter glucose in 10% bovine calf serum at 37 °C and 5% CO$_2$. Thirty-five mm dishes of 293 cells at 60–80% confluence were transfected with 2 $\mu$g of wild-type or mutant pci4-FLAG$\beta_2$H using 9 $\mu$l of LipofectAMINE (Life Technologies, Inc.) and 1 ml of Opti-MEM® (Life Technologies, Inc.). Five hours after transfection, the medium was changed. For transient transfection, the medium was changed after 24 h, and the cells were harvested after 48 h. For stable transfection, 24 h after transfection the cells were split to a 100-mm dish, and 700 $\mu$g/ml G418 (Genetech, Inc.) was added to select for a stably transfected pool of cells.

Harvesting Cells—Cells were washed with phosphate-buffered saline (PBS) (8.1 mM NaH$_2$PO$_4$, 1.5 mM KH$_2$PO$_4$, 138 mM NaCl, 2.7 mM KCl, pH 7.2), briefly treated with PBS containing 5 mM EDTA (no trypsin), and then dissociated in PBS. Cells were pelleted at 1000 × g for 5 min at 4 °C and resuspended for binding or treatment with MTS reagents.

$[^3]$H]CGP-12177 Binding—Whole cells from a 35-mm dish were resuspended in 400 $\mu$l of buffer A. Aliquots (50 $\mu$l) of cell suspension were incubated with freshly prepared MTSEA (Toronto Research Biochemicals). The means ± S.E. are shown for triplicate determinations from a representative experiment; the experiment was performed three times with similar results. The fraction of initial binding, $Y$, was fit to $e^{\pm k}$, where $k$ is the second-order rate constant, $c$ is the concentration of MTSEA, and $t$ is the time (120 s). B, cells expressing the CAM $\beta_2$ receptor were incubated for 20 min without or with 1 $\mu$M or 10 $\mu$M isoproterenol, and then 25 mM MTSEA was added for 2 min. The cells were washed as described previously (9), and $[^3]$H]CGP-12177 binding was performed as described under “Experimental Procedures.” The data are expressed as a fraction of the binding measured in the absence of MTSEA treatment.

$[^3]$H]CGP-12177 binding was defined as total binding less nonspecific binding in the presence of 1 $\mu$M alprenolol (Research Biochemicals).

Reactions with MTS Reagents—Whole cells from a 35-mm dish were resuspended in 400 $\mu$l of buffer A. Aliquots (50 $\mu$l) of cell suspension were incubated with freshly prepared MTSEA (Toronto Research Biochemicals) at the stated concentrations at room temperature for 2 min. Cell suspensions were then diluted 20-fold, and 300-$\mu$l aliquots were used to assay for $[^3]$H]CGP-12177 (400–800 pM) binding in triplicate as described above. The fractional inhibition was calculated as 1−(specific binding after MTS reagent)/specific binding without reagent).

We used the SPSS for Windows (SPSS, Inc.) statistical software to analyze the effects of MTS reagents by one-way ANOVA according to Student-Neuman-Keuls post hoc test ($p < 0.05$).

RESULTS AND DISCUSSION

MTSEA, at concentrations as high as 100 mM for 2 min, had no significant effect on $[^3]$H]CGP-12177 binding to wild-type $\beta_2$ receptor (Fig. 1A). This finding is consistent with previous observations that even the less polar N-ethylmaleimide had no effect on antagonist binding to the $\beta_2$ receptor (18). MTSEA also had no effect on the affinity of isoproterenol in competition with $[^3]$H]CGP-12177 binding (data not shown). Thus, it is likely that none of the 13 endogenous cysteine residues is accessible for reaction in the binding site crevice of wild-type $\beta_2$ receptor.

In contrast, MTSEA did inhibit $[^3]$H]CGP-12177 binding in the CAM $\beta_2$ receptor up to 46 ± 8% (mean ± S.E., $n = 6$) at the highest concentration of MTSEA tested (Fig. 1A). Because the CAM mutation does not add cysteines, an endogenous cysteine...
likely became accessible for reaction with MTSEA in the CAM. Isoproterenol retarded the reaction of MTSEA with the CAM (Fig. 1B), consistent with access to the endogenous cysteine being through the binding site crevice.

The \( \beta_2 \) receptor contains five cysteines in the putative membrane-spanning segments. These include Cys-77 in M2, Cys-116 and Cys-125 in M3, Cys-285 in M6, and Cys-327 in M7. To determine which cysteine was responsible for the MTSEA inhibition in the CAM, we created a series of mutations in the CAM background in which we substituted the endogenous cysteines, one at a time or in combination. Mutation of Cys-285 to serine decreased the inhibitory effect of MTSEA on binding to the CAM receptor to a level indistinguishable from that observed in wild-type receptor (Fig. 2). In contrast, substitution of Cys-77, Cys-116, Cys-125, or Cys-327 had no significant effect on the inhibition of binding caused by MTSEA (Fig. 2).

There are two ways in which the mutation of Cys-285 to serine could abolish the inhibitory effect of MTSEA on binding to the CAM receptor. If MTSEA reacts with Cys-285 to inhibit binding, then mutation of this residue to serine would abolish the inhibition. Another possibility, however, is that mutation of Cys-285 to serine might act as a second site revertant and eliminate the CAM phenotype. In this case, Cys-285 need not be the reactive cysteine. We investigated the phenotype of the CAM/C285S mutant by determining the affinity of isoproterenol for the CAM/C285S receptor to a level indistinguishable from that observed in wild-type receptor (Fig. 2). Based on our findings in the D2 receptor, however, a rotation of less than 40 degrees and/or a tilting of M6 might be sufficient to make Cys-285 accessible in the binding site crevice (Fig. 4).

It is not clear whether the CAM represents the active form of the receptor or a form of the receptor that can more readily assume the active conformation. The change in MTSEA accessibility in the CAM may thus reflect the increased frequency with which the receptor attains the active state. The rate of reaction of Cys-285 in the CAM with MTSEA was 0.18 ± 0.02 M\(^{-1}\) s\(^{-1}\) (mean ± S.E., \( n = 3 \)). In contrast, the rate of reaction of the endogenous Cys-118 in the binding site crevice of the dopamine D2 receptor was approximately 40 M\(^{-1}\) s\(^{-1}\) (7). Thus, while Cys-285 is much more reactive in the CAM than in the wild-type receptor, it is not likely to be freely accessible in the binding site crevice but rather intermittently accessible at the margin of the crevice.

A possible mechanism for the movement of Cys-285 might involve the highly conserved Pro-288 in M6 of the \( \beta_2 \) receptor. Mobility about proline kinks (9, 20–22) has been implicated in the mechanism of activation of a number of membrane proteins (23, 24). Interestingly, highly conserved Pro residues are found in M2, M5, M6, and M7 of G-protein-coupled receptors. A possible mechanism of receptor activation utilizing proline kink flexibility in M6 of a G-protein-coupled receptor has been demonstrated (25, 26). Flexibility about the proline kink in M6 might allow rotation and/or tilting of Cys-285 toward the binding site crevice in the CAM.

Other findings are consistent with such a conformational change being part of the normal mechanism of receptor activation. Fluorescence spectroscopy has been used to monitor structural changes in the \( \beta_2 \) receptor in response to agonist binding (27). Reaction of the cysteine-reactive, fluorescent probe \( N,N' \)-dimethyl-N-(iodoacetyl)-N'-(7-nitrobenz-2-oxa-1,3-diazol-4-yi)-

\[^2^\] An alternative, less likely explanation is that the endogenous cysteine reacted with MTSEA in the wild-type receptor without exerting an effect on binding, but that, because of a conformational change in the CAM, the covalently attached ethylammonium group inhibited antagonist binding. Nonetheless, either explanation requires a motion of the Cys relative to the binding site crevice.

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\[^4^\] The rate of reaction of a sulphydryl with MTSEA will be influenced by its water accessibility, by steric and electrostatic factors, and by ionization of the sulfhydryl. Moreover, if the position of the sulphydryl is not static, an increase in the portion of time the sulphydryl is in the accessible state will be reflected as an increase in the rate of reaction.

\[^5^\] U. Gether, S. Lin, P. Ghanoumi, J. A. Ballesteros, H. Weinstein, and B. K. Kolbka, submitted for publication.
Cys-285 toward the water-accessible surface of the binding site crevice where it could react with MTSEA, thus accounting for our observations in the CAM β2 receptor. Moreover, flexibility around the proline kink, as discussed above, could facilitate the observed rotation and tilting in the cytoplasmic half of the membrane-spanning segment (20) without requiring movement of the entire segment.

The systematic application of the substituted cysteine accessibility method with both the wild-type receptor and CAM/C285S mutant as backgrounds should allow us to detect other conformational changes which accompany β2 receptor activation.

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FIG. 4. An illustration of the rotation and/or tilting of the sixth membrane-spanning segment associated with the activation of the β2 receptor. The indicated rearrangement brings Cys-285 to the margin of the binding site crevice and allows it to react with MTSEA to inhibit ligand binding. The arrangement of the membrane-spanning segments is based on the projection structure of rhodopsin (19). The accessible surface of the sixth membrane-spanning segment determined in the homologous dopamine D2 receptor is shaded.

ethylenediamine (IANBD) with purified β2 receptor did not affect ligand binding, consistent with the absence of endogenous cysteines in the binding site crevice. Addition of agonist elicited a dose-dependent decrease in fluorescence in IANBD-labeled β2 receptor, consistent with movement of the probe toward a more polar environment. Cys-125 and Cys-285 are responsible for this agonist-induced fluorescence decrease. In decreased polarity could come about by movement of the probe closer to the binding site crevice and/or to another membrane spanning segment. Our results suggest that Cys-285 moves closer to the binding site crevice in the CAM than in the wild-type receptor. Thus, the conformational change observed in the CAM is consistent with the rearrangement induced by agonist in wild-type receptor. In contrast, Cys-125 was not accessible to reaction with MTSEA.

A rotation and/or tilting of M6 that would make Cys-285 accessible (Fig. 4) is also consistent with the rotational-translational motion predicted by spin labeling studies in rhodopsin (2). These studies indicated that the distance between a particular pair of cysteines at the cytoplasmic ends of M3 and M6 decreased with activation, while the distances between other pairs increased. To explain these findings, the authors proposed that M6 tilts away from M3 and rotates in an anticlockwise direction (when viewed from the extracellular face). As illustrated in Fig. 4, the proposed rotation would reposition...