Structural Instability of a Constitutively Active G Protein-coupled Receptor

AGONIST-INDEPENDENT ACTIVATION DUE TO CONFORMATIONAL FLEXIBILITY

(Received for publication, October 9, 1996, and in revised form, November 18, 1996)

Ulrik Gether‡, Juan A. Ballestros§, Roland Seifert‡, Elaine Sanders-Bush†, Harel Weinstein§, and Brian K. Kobilka‡,

From the ‡Howard Hughes Medical Institute and the Division of Cardiovascular Medicine, Stanford University Medical School, Stanford, California 94305 and the §Department of Physiology and Biophysics, Mount Sinai School of Medicine, New York, New York 10029

Mutations in several domains can lead to agonist-independent, constitutive activation of G protein-coupled receptors. However, the nature of the structural and molecular changes that constitutively turn on a G protein-coupled receptor remains unknown. Here we show evidence that a constitutively activated mutant of the β2 adrenergic receptor (CAM) is characterized by structural instability and an exaggerated conformational response to ligand binding. The structural instability of CAM could be demonstrated by a 4-fold increase in the rate of denaturation of purified receptor at 37°C as compared with the wild type receptor. Spectroscopic analysis of purified CAM labeled with the conformationally sensitive and cysteine-reactive fluorophore, N,N'-dimethyl-N-(iodoacetyl)-N'-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)ethyleneediamine, further indicated that both agonist and antagonist elicit more profound structural changes in CAM than in the wild type protein. We propose that the mutation that confers constitutive activity to the β2 adrenergic receptor removes some stabilizing conformational constraints, allowing CAM to more readily undergo transitions between the inactive and the active states and making the receptor more susceptible to denaturation.

In classical receptor theory, binding of agonist has been considered essential for receptor activation and transmission of the biological signal across the plasma membrane. However, expression of many members of the seven-transmembrane, G protein-coupled receptor family in transfected cell lines has revealed that activation of intracellular messenger systems can occur in the absence of agonists (1–6). Moreover, it has been found that mutations in several different regions of G protein-coupled receptors can dramatically enhance agonist-independent receptor activity and in some cases confer oncogenic properties to the receptor (1–3, 7–14). Nevertheless, the molecular and structural changes in the receptor that are responsible for constitutive, agonist-independent activation are poorly understood. According to the prevailing two-state model for activation of G protein-coupled receptors, constitutive activation has been explained as a disturbance of the normal equilibrium between the inactive (R) state and the active (R*) state leading to a higher proportion of receptor molecules in the active R* state (3, 15). However, the conformational state of a constitutively active receptor protein has so far only been deduced from its effects on intracellular second messenger systems; therefore, this hypothesis has not been substantiated by any direct structural analysis.

Recently, we have described the use of fluorescence spectroscopy to directly analyze ligand-induced conformational changes in the purified wild type β2 adrenergic receptor (16). The approach is based on the sensitivity of many fluorescent molecules to the polarity of their molecular environment (17). In this study, we use the same techniques to directly study conformational changes associated with constitutive activation. Our results reveal novel characteristics of a constitutively active receptor that provide insight into the mechanism of altered signaling behavior of this mutant β2 receptor.

EXPERIMENTAL PROCEDURES

Expression of the Constitutively Activated β2 Receptor in Insect Cells—The cDNA encoding CAM was generously provided by Dr. R. J. Lefkowitz (Duke University, Durham, NC) and cloned into the baculovirus expression vector pVL1392 (Invitrogen) as described for the wild type receptor (16). The resulting construct had the cleavable influenza-hemagglutinin signal-sequence followed by the FLAG epitope (IBI) at the amino terminal and a tail of six histidines at the carboxyl terminus. Baculovirus containing the tagged CAM sequence was generated using the BaculoGold kit (Pharmingen) and plaque-purified. SF-9 cells were grown as described (16).

Assessment of Receptor Expression in the Presence and the Absence of Ligand—Suspension cultures of SF-9 cells at a density of 3 × 10^6 cells/ml were infected in the presence or the absence of ligands plus 2 mM ascorbate. After 48 h 1.0-ml samples of the cultures were collected, and ligands were added to controls before centrifugation (10 min at 10,000 × g). The pellets were frozen at −70°C, thawed, and resuspended in 1.0 ml of lysis buffer (10 mM Tris-HCl, pH 7.4, with 1 mM EDTA, 10 μg/ml leupeptin, 10 mg/ml benzamidine, and 0.2 mM phenylmethylsulfonyl fluoride). The lysed cells were centrifuged (10 min at 10,000 × g), and the membrane pellet was washed three times in 1.0 ml of binding buffer (75 mM Tris-HCl, pH 7.4, with 12.5 mM MgCl2, and 1 mM EDTA) plus 10 μg/ml leupeptin and 10 μg/ml benzamidine. For binding assay 50 μl of membrane suspension was incubated in binding buffer in a total volume of 0.5 ml with 10 nM [3H]dihydroalprenolol (Amersham Corp.) for 2 h as described (19).

Purification Procedure—For purification, SF-9 cells were grown in 600-ml cultures, infected with a 1:10 dilution of a high titer virus stock at a density of 3 × 10^6 cells/ml and harvested after 48 h. To avoid a complete loss of the unstable CAM protein it was necessary to exclude the M1 anti-FLAG antibody column from our previously described purification procedure allowing purification in 1 day instead of over 2 days (16, 20). Briefly, lysed cell membranes were solubilized in 1.0% (v/v) Nonidet P-40.
Instability of Constitutively Active $\beta_2$ Adrenoreceptor

A novel adrenoreceptor, constitutively active $\beta_2$ adrenoreceptor (CAM), was expressed in Sf-9 insect cells and its properties were investigated. CAM is characterized by an enhanced basal activity of adenyl cyclase and GTPase activity compared to the wild type receptor (WT). The expression of CAM is significantly higher than WT, with a 1.8 pmol/mg protein level. CAM also shows a longer half-life (12.3 min) compared to WT (4.9 min). This increased stability of CAM may be due to biochemical stabilization of an inherently unstable protein.

RESULTS AND DISCUSSION

Leftkowitz and co-workers previously showed that a discrete change in the carboxyl-terminal part of the third intracellular loop leads to constitutive activation of the $\beta_2$ adrenoreceptor (CAM) (3). We expressed an epitope-tagged version of this mutant in Sf-9 insect cells to obtain large quantities of receptor needed for purification and subsequent structural characterization. The functional properties of CAM in insect cell membranes were similar to those observed for CAM in membranes from transfected mammalian cells (3). As expected we observed increased agonist affinity, an elevated basal level of adenyl cyclase and GTPase activity, and a higher maximal agonist-stimulated adenyl cyclase and GTPase activity for CAM than for the $\beta_2$ WT receptor when expressed at a similar level (data not shown). Together this confirms that CAM not only possesses constitutive activity but also is “superactive” compared with the wild type receptor.

The expression of functional CAM in Sf-9 insect cells was considerably lower than for the wild type (3.4 ± 1.3 pmol/mg protein versus 8.7 ± 1.8 pmol/mg protein, mean ± S.E., n = 4) in agreement with earlier studies in mammalian cells (3). However, incubation of the cells with either an agonist or an inverse agonist (also referred to as negative antagonist) during the 48-h infection markedly increased the expression of CAM (Fig. 1). A similar increase was also observed for neutral antagonists (data not shown). This surprising lack of correlation between the increase in expression and the pharmacological properties of the added ligands strongly argues that the lowered expression of CAM in the insect cells cannot be explained by receptor down-regulation as a consequence of constitutive activation.

Rather, the increased expression of CAM in the presence of ligand may be due to biochemical stabilization of an inherently unstable protein. Of interest, an even more dramatic up-regulation is observed in transgenic mice expressing CAM in response to antagonist treatment (21). Most likely, these data also reflect ligand stabilization of an inherently labile protein.

The instability of CAM can be demonstrated by observing the rate of denaturation at 37 °C as shown in Fig. 2A. Assuming an exponential decay, $t_{1/2}$ for degradation of CAM was 12.3 min versus 49.9 min for the wild type receptor (Fig. 2A). The decrease in binding activity for both wild type and CAM could be partially prevented by both the agonist, isoproterenol, and the inverse agonist, ICI 118,551 (22, 23) with isoproterenol being slightly less effective than ICI 118,551 (Fig. 2B). Western blotting of partially purified receptor before and after exposure to 37 °C for 3 h revealed essentially no changes in the intensity of the receptor band and no evidence of proteolysis (Fig. 2A, inset).

The cysteine-reactive fluorescent probe IANBD can be used as a sensitive molecular reporter of ligand-induced conformational changes in the $\beta_2$ adrenoreceptor (16). Agonist stimulation of purified IANBD-labeled $\beta_2$ receptor leads to a dose-dependent, reversible decrease in fluorescence, indicating that one or more cysteines labeled with IANBD are exposed to a slightly more polar environment upon agonist binding. By systematic mutation of cysteines we have localized the responsible residues to the third and sixth transmembrane domain.2 There was a linear correlation between the magnitude of the fluorescence change in response to a series of adrenergic agonists and the intrinsic efficacy of the compounds in adenyl cyclase assays implying that the agonist-mediated decrease in fluorescence ensues from a conformational change involved in receptor activation and G protein coupling (16). In contrast to agonists, we found that inverse agonists caused a relative increase in fluorescence emission (16). Emission scans of IANBD-labeled CAM and wild type receptor both showed maximal fluorescence at an emission wavelength of 523–526 nm (data not shown). Time course analysis revealed that stimulation of IANBD-labeled CAM with the full agonist, isoproterenol, and the partial agonist, salbutamol, elicited substantially greater decreases in fluorescence emission than in the IANBD-labeled WT receptor (Fig. 3, A–E). In addition, the ratio of the salbutamol response relative to the isoproterenol response increased from 0.40 in the WT to 0.76 in CAM (Fig. 3E). This agrees with

2 U. Gether, S. Lin, and B.K. Kobilka, unpublished observation.
CAM receptor protein indicates that a larger percentage of this protein was applied to the gel. The larger amount of immunoreactive 37°C for 3 h. The same amount of WT and CAM binding activity (0.1 pmol) was chromatography purified WT and CAM before and after incubation at 37 °C without ligand (No ligand), with $10^{-4}$ M of the agonist isoproterenol (ISO), or with $10^{-3}$ M of the inverse agonist ICI 118,551 (21, 22) (ICI). Data are remaining binding activity in the percentage of control binding at $t = 0$ (mean ± S.E., n = 3).

The well described increase in the efficacy of partial agonists at constitutively activated receptor mutants in biological assays (Refs. 3 and 4 and data not shown). The changes were fully reversible by antagonist for both receptors (Fig. 3, A–D). As with the WT receptor, the inverse agonist ICI 118,551 induced an increase in fluorescence of the IANBD-labeled CAM receptor; however, the magnitude of the response was greater for the CAM receptor (Fig. 3E). The larger changes in fluorescence induced by both agonists and inverse agonists in the IANBD-labeled CAM receptor are even more impressive considering that the specific activity of CAM was lower than that of the WT (CAM, 3 nmol/mg protein; WT, 8 nmol/mg protein).

Taken together, our findings delineate two novel properties of a constitutively activated receptor: structural instability and an exaggerated conformational response to drug binding. Our findings, in particular the larger changes in fluorescence observed immediately before the addition of ligand. The experiments shown are representative of three identical experiments. E, bar diagram of changes in fluorescence in response to the full agonist isoproterenol (ISO), the partial agonists salbutamol (SAL), and the inverse agonist ICI 118,551 (ICI). Data are given as the percentage of change in fluorescence (mean ± S.E., n = 3). For isoproterenol and salbutamol, the change was determined by the amplitude of the reversal by alprenolol. For ICI 118,551 the percentage of change was calculated as the change in fluorescence relative to the extrapolated baseline at $t = 15$ min after the addition of ligand.

coupled receptors (3, 23–27) can be considered a high energy, intermediate state that can be stabilized by the G protein and/or the agonist (Fig. 4). However, in the absence of agonist or G protein, the expected lifetime of the excited $R^*$ state for both CAM and the WT receptor would be short due to its high energy. Receptor in $R^*$ will either rapidly return to the ground state or denature. Therefore, at any given time the fraction of both CAM and WT receptor in $R^*$ should be rather low in absence of agonist and G protein ($[R^*_WT] < < [R^*CAM]$) (Fig. 4). Our model proposes that agonists stabilize both $R^*_WT$ and $R^*_CAM$, however, because of the smaller energy difference between the $R$ and $R^*$ state of CAM, agonists would cause a higher proportion of CAM molecules than WT molecules to undergo the transition from $R$ to $R^*$. For example, agonist occupancy of the wild type receptor in the absence of G protein might cause a change in the $R^*_WT \text{ fraction}$ from 5 to 20%, whereas the change could be from 10 to 35% for CAM. This greater change in the fraction of $R^*$ for CAM than for the wild type receptor in response to agonists is consistent with the larger changes in fluorescence, because the change in fluorescence most likely reflects receptor molecules undergoing transition from $R$ to $R^*$ (16). Moreover, it explains the super-activity of agonist-activated CAM as compared with agonist-activated WT measured in GTPase and adenyl cyclase assays (3). Finally, the model can explain the larger increase in fluorescence over time in response to the inverse agonist ICI.
Instability of Constitutively Active $\beta_2$ Adrenoceptor

Fig. 4. A simplified, thermodynamic energy state diagram for activation of G protein-coupled receptors. The diagram represents a system at equilibrium. The abundance of any single state at equilibrium is determined by its relative energy. Receptor activity for both WT and the CAM receptor is determined by the ability of the receptor to overcome the energy difference between the R and R* states. The relative energies among the receptor states in this qualitative energy diagram can be inferred from known properties of the receptor. First, the energy difference between R and R* is smaller for CAM than for WT. This is indicated by the dramatic increase in basal and agonist induced activity of CAM compared with WT, which implies that it is energetically easier to reach R* CAM from R CAM than it is to reach R* WT from R WT. Second, the energy of an active R* state (in the absence of agonist or G protein) is higher than that of the R state. This is indicated by the low basal activity levels of WT and the ability of agonists (A) to increase the population of activated receptors for both WT and CAM. Third, for the agonist-bound receptor, the energy of an active receptor bound to agonist (AR*) (in the absence of G protein) is higher than for the inactive receptor bound to agonist (AR). This inference comes from the known effect of GTP on agonist affinity in the presence of G protein. GTP abolishes (or significantly decreases) high affinity binding by reducing G protein coupling; thus the high affinity binding component is taken to represent AR*. Our studies are done in the absence of G protein, and only low affinity agonist binding is observed suggesting that the AR* state is sparsely populated relative to AR and must be of higher energy. As discussed in the text, the smaller energy difference between AR and AR* for CAM as compared with the WT receptor predicts a larger change in the fraction of CAM in R* following exposure to agonist and thus a larger change in fluorescence.

118,551. ICI 118,551 stabilizes the receptor in the ground state R and, as discussed above, the fraction of CAM molecules in R* would always be higher than for the WT receptor in the absence of ligands; therefore, the population of receptor going from R* to R upon the addition of the inverse agonist would be greater, and a larger increase in fluorescence would be expected.

An alternative explanation for our observations would be that CAM has an altered structure with an altered environment around the fluorophore, and the active state (RCAM) is structurally distinct from the active state of the WT (R*WT). Thus, CAM may accommodate a superactivated state that interacts more efficiently with the G protein and causes the more dramatic changes in fluorescence. Our data cannot distinguish between these two explanations. However, the model we have outlined in Fig. 4 represents the simplest hypothesis, because it does not propose any changes in the tertiary structure of R* for CAM as compared with R* for the WT receptor. Thus it is compatible with the prevailing two-state model for activation of G protein-coupled receptors (3, 23–27). It is also highly unlikely that the environment around the fluorophore is markedly changed in CAM, because the mutant has the same stoichiometry of IANBD labeling (see “Experimental Procedures”) and the emission maxima of IANBD-labeled CAM and WT are indistinguishable (data not shown).

The structural instability should also be expected according to the model outlined in Fig. 4. Due to the higher energy, the excited R* state is predicted to be structurally more unstable; therefore it follows that CAM is more susceptible to denatur-