Activation of the β2-Adrenergic Receptor Involves Disruption of an Ionic Lock between the Cytoplasmic Ends of Transmembrane Segments 3 and 6*

Juan A. Ballesteros‡‡, Anne D. Jensen§§, George Liapakis****‡‡, Søren G.F. Rasmussen¶¶, Lei Shi**, Ulrik Gether‡‡§§, and Jonathan A. Javitch**††

From ‡‡Novasite Pharmaceuticals, Inc., San Diego, California 92121, the ††Division of Cellular and Molecular Physiology, Department of Medical Physiology, The Panum Institute, University of Copenhagen, DK-2200 Copenhagen, Denmark, the *¶¶Center for Molecular Recognition and the Departments of Psychiatry and Pharmacology, Columbia University College of Physicians and Surgeons, New York, New York 10032, and the **††Department of Pharmacology, School of Medicine, University of Crete, Heraklion 71110, Greece

The movements of transmembrane segments (TMs) 3 and 6 at the cytoplasmic side of the membrane play an important role in the activation of G-protein-coupled receptors. Here we provide evidence for the existence of an ionic lock that constrains the relative mobility of the cytoplasmic ends of TM3 and TM6 in the inactive state of the β2-adrenergic receptor. We propose that the highly conserved Arg-1313.50 at the cytoplasmic end of TM3 interacts both with the adjacent Asp-1303.49 and with Glu-2686.30 at the cytoplasmic end of TM6. Such a network of ionic interactions has now been directly supported by the high-resolution structure of the inactive state of rhodopsin. We hypothesized that the network of interactions would serve to constrain the receptor in the inactive state, and the release of this ionic lock could be a key step in receptor activation. To test this hypothesis, we made charge-neutralizing mutations of Glu-2686.30 and of Asp-1303.49 in the β2-adrenergic receptor. Alone and in combination, we observed a significant increase in basal and pindolol-stimulated cAMP accumulation in COS-7 cells transiently transfected with the mutant receptors. Moreover, based on the increased accessibility of Cys-2853.47 in TM6, we provide evidence for a conformational rearrangement of TM6 that is highly correlated with the extent of constitutive activity of the different mutants. The present experimental data together with the recent high-resolution structure of rhodopsin suggest that ionic interactions between Asp/Glu3.49, Arg3.50, and Glu6.30 may constitute a common switch governing the activation of many rhodopsin-like G-protein-coupled receptors.

The majority of hormones and neurotransmitters exerts its cellular effects by activating cell surface receptors belonging to the superfamily of G-protein-coupled receptors (GPCRs) (1–3). The β2-adrenergic receptor (β2AR) belongs to the subfamily of rhodopsin-like receptors and has been used as a prototype GPCR in numerous studies (1–3). Low-resolution structures of rhodopsin, resolved by Schertler and co-workers (4, 5), have demonstrated the presence of seven membrane-spanning α-helical segments and have provided important insights into the organization of the transmembrane bundle, allowing the development of tertiary structure models of GPCRs (6–8). Importantly, a high-resolution structure of rhodopsin has now become available (9) making it possible to consider the functional roles of individual side chains from the perspective of an atomic resolution structure of a homologous GPCR.

Understanding the function of GPCRs at a molecular level requires an understanding of how agonist binding to the receptor is converted into receptor activation (3). Studies based on EPR spectroscopy, fluorescence spectroscopy, alterations in cysteine accessibility, and engineering of metal-binding sites have altogether pointed to a key role for conformational changes of TM3 and TM6 in receptor activation (10–15). The molecular mechanisms that underlie the movements of TM3 and TM6 and govern the transition of the receptor between its inactive and active states have nonetheless remained unclear.

It has been suggested that the protonation of the aspartic acid in the highly conserved (D/E)RY motif at the cytoplasmic side of TM3 leads to a release of constraining intramolecular interactions, thereby resulting in the movements of TM3 and TM6 and a conversion of the receptor to the active state (7, 14, 16). This hypothesis has been supported by the observation that charge-neutralizing mutations of the aspartic acid (or glutamic acid) in TM3 lead to increased agonist-independent activation of a number of GPCRs (7, 14, 17, 18). Moreover, direct evidence has been obtained indicating that the phototransduction of rhodopsin is accompanied by the uptake of a proton by this residue (16). In β2AR, lowering of the pH has also been shown to...
facilitate the transition of the receptor to the activated state (19). However, the network of constraining intramolecular interactions that maintain the receptor in its inactive state has not been clear. Based on simulations in the \( \alpha \)-adrenergic receptor, Scheer et al. (7) suggested that Arg\(^{3.50} \) in the inactive state is constrained in a “polar pocket” formed by residues in TMs 1, 2, and 7. The specific ionic counterpart of the arginine in the inactive state in this scheme was predicted to be the conserved aspartic acid in TM2 (Asp\(^{3.49} \)) Asp-79 in \( \beta_2 \)AR (7). In contrast, Ballesteros et al. (20) proposed that the ionic counterpart of Arg\(^{3.50} \) in the inactive state could be the adjacent Asp\(^{3.49} \).

Experiments using cysteine cross-linking and engineered metal ion-binding sites (10, 11, 21) suggest that the cytoplasmic ends of TM3 and TM6 are in close proximity. Exploring these proposed proximities in a three-dimensional molecular model of the \( \beta_2 \)AR supports an orientation of the conserved Arg\(^{3.50} \) in TM3 facing a conserved Glu\(^{6.30} \) in TM6. Therefore, we further propose that in the inactive state, Arg\(^{3.50} \) in addition to interacting with Asp\(^{3.49} \) also interacts with the conserved Glu\(^{6.30} \) at the cytoplasmic end of TM6 (Fig. 1), and that this interaction maintains the receptor in the inactive state by holding together the cytoplasmic ends of TM3 and TM6. To test this structural hypothesis, we have generated charge-neutralizing mutations of Glu-268\(^{6.30} \) in the \( \beta_2 \)AR and have studied these mutants alone and in combination with mutations of Asp-130\(^{6.49} \). We find that the disruption of the interaction between TM3 and TM6 produces constitutive receptor activation, and that the extent of constitutive activation is highly correlated with the extent of conformational rearrangement in TM6. Our hypothesis and results are remarkably consistent with the recent high-resolution structure of the inactive state of rhodopsin, which showed that Arg\(^{3.50} \) in this receptor is positioned to form an ionic interaction with both Glu\(^{6.30} \) and Glu\(^{6.30} \) in this receptor.

**MATERIALS AND METHODS**

**Numbering of Residues**—Residues are numbered according to their position in the human \( \beta_2 \)AR sequence. We also index residues relative to the most conserved residue in the TM in which it is located (6). By definition, the most conserved residue is assigned the position index “50”, e.g. Pro-2886.50 and therefore Leu-2876.49 and Phe-2896.51. This indexing simplifies the identification of aligned residues in different GPCRs.

**\( \beta_2 \)-Plasmodia and Site-directed Mutagenesis**—The cDNA sequence encoding the human \( \beta_2 \)AR, epitope-tagged at the amino terminus with a cleavable influenza-hemagglutinin signal sequence followed by the FLAG-epitope (Sigma) and tagged with six histidines at the carboxyl terminus, was a gift from Dr. B. Koblika (Stanford, CA) (22). The mutations were generated by the polymerase chain reaction-mediated mutagenesis using Pfu polymerase (Stratagene). Mutants are named as wild-type residue, residue number, and mutant residue where the residues are given in single letter codes. The polymerase chain reaction-generated DNA fragments containing the mutations were subcloned into pTEJ8 using the LipofectAMINE/Opti-MEM (Life Technologies, Inc.) transfection system, and a stably transfected pool was selected with Geneticin as described previously (13).

**Cell Culture and Transfection**—COS-7 cells were grown at 37 °C in 5% CO\(_2\) in Dulbecco’s modified Eagle’s medium/F-12 supplemented with 10% fetal calf serum, 2 mM glutamine, and 0.01 mg/ml gentamicin. These cells were transfected with 2 µg of the PCIN4 constructs using the LipofectAMINE/Opti-MEM (Life Technologies, Inc.) transfection system, and a stably transfected pool was selected with Geneticin as described previously (13).

**Membrane Preparation and \( \beta \)-HCGP-12177 Binding**—To increase the expression of the constitutively active mutants, 10 µm sotalol was added to the growth medium of HEK 293 cells stably expressing WT or mutant \( \beta_2 \)AR for 1–2 days. To remove the sotalol, the cells were washed with 10 ml of Dulbecco’s phosphate-buffered saline without Ca-Mg-EDTA and then incubated in 10 µl of Opti-MEM for 30–60 min at 37 °C and 5% CO\(_2\). The cells were washed again with 10 ml of Dulbecco’s phosphate-buffered saline and resuspended in 10 ml of Dulbecco’s phosphate-buffered saline containing 1 mM EDTA. Membranes were prepared as described previously (14), and the membrane pellets from one confluent 100-mm dish for each of the mutants except D130N and D130N/E268A (2–4 dishes) were resuspended in 1 ml of binding buffer (25 mM HEPES, pH 7.4, 5 mM MgCl\(_2\), 1 mM EDTA, 0.006% bovine serum albumin). The membrane suspensions were diluted (typically 1:20–1:40) but 1:6–1:16 for the mutants D130N/E268A, D130N and D130N/E268Q in binding buffer and used for radioligand-binding studies. \( \beta \)HCGP-12177 binding was performed as described previously (13). Data for saturation and competition binding were analyzed by nonlinear regression analysis using GraphPad Prism 3.0 (GraphPad Software, San Diego, CA), and \( K_d \), \( B_{max} \), and IC\(_{50}\) values were determined. \( K_d \) values were determined using the equation \( K_d = I_{50}/(1 + I_{50}/I) \), where \( I \) is the radioligand concentration.

**cAMP Accumulation Assay**—The cAMP accumulation assay was performed essentially as described previously (14, 27). COS-7 cells transiently expressing the WT and mutant receptors were cultured in 12-well plates (2.5 × 10\(^5\) cells/well plate) and incubated overnight with medium containing 2 µCi/mL of \( [\text{H}] \)adenine (TRK311, Amersham Pharmacia Biotech) before carrying out the experiment (14, 27).

**Methanethiosulfonate Reagent Assay**—The methanethiosulfonate agent assay was carried out as described previously (13). Washed dissociated HEK 293 cells stably expressing the mutants were resuspended in 400 µl of buffer (140 mM NaCl, 5.4 mM KCl, 1 mM EDTA, 0.006% bovine serum albumin, 25 mM HEPES, pH 7.4). Aliquots of whole cell suspension (50 µl) were incubated with different concentrations of the methanethiosulfonate ethylammonium (MTSEA) (Toronto Research Biochemicals, Toronto, Canada) for 2 min at room temperature, and after dilution, the binding of \( \beta \)HCGP-12177 was determined as described previously (14).

**Molecular Modeling**—To simulate the bending of TM6 by Pro at 6.50, 100 α-helices of 31 amino acids with a Pro at position 21 from the amino terminus and Ala at other positions were generated using a molecular dynamics simulation with Chemistry at Harvard Macromolecular Mechanics (28) to probe the conformational space of a Pro kink. The simulation was started from the torsion angles taken from average values of Pro kinks (29, 30), and a dielectric constant of 4 was used. The system was heated to 300 K and equilibrated. After the major parameters were stable in the production phase, a structure was taken every 2 picoseconds. The resulting structures (shown in Fig. 5 as blue ribbons) were superimposed from position 1 to 17 onto the corresponding parts of TM6 (6.30–6.46).

To illustrate the interactions at the cytoplasmic ends of TM3 and TM6, a \( \beta_2 \)AR three-dimensional model was made based on the backbone of the rhodopsin structure. The rhodopsin residues were substituted by those of the \( \beta_2 \)AR (or mutants) at aligned positions. The side chain clashes were removed manually.

**RESULTS**

**Constitutive Activation of the \( \beta_2 \)AR by Mutation of Glu-268 and Asp-130**—The conserved glutamic acid at the cytoplasmic end of TM6 (Glu-268\(^{6.30} \)) (Fig. 1) was mutated to glutamine or alanine (E268Q or E268A). The aspartic acid in the conserved DRY motif at 6.50—Asp-130—was mutated to asparagine (D130N) as described previously (14). These mutations were also combined to produce D130N/E268Q and D130N/E268A. As illustrated in Fig. 2 (left panels), these charge-neutralizing mutations caused a clear elevation of basal agonist-independent cAMP accumulation in transiently transfected COS-7 cells as compared with the WT receptor. The basal activity increased linearly with increasing receptor expression. There was a progressive increase in basal activity in the mutants in the following rank order from lowest to highest: WT (slope = 8.7 ± 1.5 CPM × pmol\(^{-1} \) × mg

**Constitutive Activation of the \( \beta_2 \)AR by Mutation of Glu-268 and Asp-130**—The conserved glutamic acid at the cytoplasmic end of TM6 (Glu-268\(^{6.30} \)) (Fig. 1) was mutated to glutamine or alanine (E268Q or E268A). The aspartic acid in the conserved DRY motif at 6.50—Asp-130—was mutated to asparagine (D130N) as described previously (14). These mutations were also combined to produce D130N/E268Q and D130N/E268A. As illustrated in Fig. 2 (left panels), these charge-neutralizing mutations caused a clear elevation of basal agonist-independent cAMP accumulation in transiently transfected COS-7 cells as compared with the WT receptor. The basal activity increased linearly with increasing receptor expression. There was a progressive increase in basal activity in the mutants in the following rank order from lowest to highest: WT (slope = 8.7 ± 1.5 CPM × pmol\(^{-1} \) × mg
of protein), E268Q (slope = 137 ± 29 CPM × pmol⁻¹ × mg of protein), E268A (slope = 540 ± 103 CPM × pmol⁻¹ × mg of protein), D130N (slope = 938 ± 170 CPM × pmol⁻¹ × mg of protein), D130N/E268Q (slope = 1,390 ± 372 CPM × pmol⁻¹ × mg of protein), and D130N/E268A (slope = 997 ± 441 CPM × pmol⁻¹ × mg of protein). It was difficult to differentiate the very high basal activity of the latter three mutants because of our inability to express sufficiently high amounts of receptor to generate an accurate slope. This low level of expression is consistent with previous observations and with the decreased stability of constitutively active receptors (14, 18, 31).

Stimulation of COS-7 cells transiently expressing the WT or mutant receptors with a saturating concentration of the partial agonist pindolol (1 μM) caused a clear increase in cAMP accumulation that rose with increasing expression of the individual receptor constructs (Fig. 2, right panels). Similar to the effects observed for the basal activity, the increase in cAMP accumulation was most significant for D130N/E268A (slope = 34,500 ± 23,900 CPM × pmol⁻¹ × mg of protein) followed by D130N/E268Q (slope = 6030 ± 993 CPM × pmol⁻¹ × mg of protein) and D130N (slope = 6050 ± 964 CPM × pmol⁻¹ × mg of protein), E268A (slope = 2590 ± 490 CPM × pmol⁻¹ × mg of protein), and WT (slope = 384 ± 97 CPM × pmol⁻¹ × mg of protein). The data indicate an increased efficacy of pindolol at all the mutant receptors. An increased efficacy of partial agonists has previously been described for other constitutively active mutants and is consistent with an enhanced ability of the mutant receptors to assume an activated state (32). Because the full agonist isoproterenol significantly increased CAMP levels in untransfected COS-7 cells by stimulating endogenous β₂-adrenergic receptors present at low levels (data not shown), it was not possible to investigate the response of the mutant receptors to full agonists. However, the partial agonist pindolol did not affect CAMP production in untransfected cells (data not shown).

Binding Characteristics of Mutant Receptors—Saturation-binding experiments with the antagonist [³H]CGP-12177 showed a slightly decreased affinity for D130N/E268A as compared with the WT with all the other mutants having an affinity within ~2-fold of WT (Table I). However, the receptor expression was markedly reduced for D130N and especially for D130N/E268A (Table I). E268A and E268Q, which were less constitutively active, expressed at near WT levels. Surprisingly, D130N/E268Q was found to express well in the HEK 293 cells despite its high constitutive activity. However, as it is clear from Fig. 2, the apparent expression level was much lower than the WT and comparable to D130N/E268A upon transient expression in COS-7 cells (Table I and Fig. 2). It should be noted that whereas the Bₘax determinations for the COS-7 cells in Fig. 2 represent many individual transfections, the...
Bmax determinations in the HEK 293 cells are based on the analysis of pool clones from single stable transfections. Consistent with the elevated basal activities, isoproterenol competition binding showed a very small increase in the affinity for E268Q relative to WT, a 3-fold increase in affinity for E268A, and a 30–100-fold increase in affinity for D130N and the two double mutants (Table I). Thus, the charge-neutralizing mutations of Glu-268 and Asp-130 lead to an increase in apparent agonist affinity, consistent with a higher propensity of the receptors to assume the activated R* state (32). Competition-binding experiments with pindolol demonstrated unchanged affinities for the mutant receptors as compared with the WT (Table I). This finding is consistent with the unchanged affinities previously observed for weak partial agonists such as pindolol in another constitutively active mutant b2AR (32).

Evidence for a Conformational Rearrangement of TM6—The treatment of cells expressing the WT b2AR with the charged sulfhydryl-specific agent MTSEA did not affect the binding of the radioligand [3H]CGP-12177 to the b2AR (13). High concentrations of MTSEA, however, inhibited the binding of [3H]CGP-12177 to the constitutively active mutant a constitutively active mutant (L266S/K267R/H269K/L272A) (13) and to D130N (14). In both cases, the mutation of the endogenous cysteine, Cys-2656.47 in TM6 (see Fig. 1), eliminated the inhibition, suggesting that during the activation this Cys in a constitutively active mutant becomes partially accessible to

*Fig. 3. Effects of MTSEA treatment on specific [3H]CGP-12177 binding to the mutants. HEK 293 cells transiently expressing WT, E268Q, E268A, D130N, D130N/E268Q, and D130N/E268A were treated with (A) 25 mM or (B) 75 mM MTSEA for 2 min before determination of specific [3H]CGP-12177 binding. The data are given as fractional inhibition compared with control binding activity without MTSEA treatment (means ± S.E., n = 2, 5, 3, 4, 3, and 8 for WT, E268Q, E268A, D130N, D130N/E268Q, and D130N/E268A, respectively, at 25 mM, and n = 2, 4, 3, 4, 3, and 2, respectively at 75 mM.).

**Table I**

| Receptors   | CGP-12177 | Mean [S.E. interval] |
|-------------|-----------|---------------------|
| WT          | 66 [53–79] | 0.06 [0.05–0.06] |
| E268Q       | 110 [87–134] | 0.12 [0.10–0.14] |
| E268A       | 61 [49–74] | 0.14 [0.13–0.16] |
| D130N       | 6 [4–8] | 0.06 [0.05–0.06] |
| D130N/E268Q | 41 [30–53] | 0.15 [0.12–0.18] |
| D130N/E268A | 0.5 [0.4–0.7] | 0.24 [0.22–0.25] |

D2 AR binding properties of WT b2AR and mutants

Saturation and competition binding studies were performed on membrane preparations from HEK 293 cells stably expressing WT or mutant receptors as described under "Materials and Methods." The Ki values, which determine the affinity of the ligand for the receptor, were determined by the method of Cheng and Prusoff (26). The IC50 values, which determine the concentration of the ligand required for 50% inhibition of binding, were used for calculations of Ki values. The IC50 values, used for calculations of Ki values, were obtained from the means of logIC50 values and the S.E. intervals from log IC50 values. The Ki and Bmax values represent the means of Ki and Bmax values, respectively, and their S.E. intervals represent Ki and Bmax values, respectively. Values are from 3–8 independent experiments.
the intracellular end of the TMs. Aidues at the cytoplasmic ends of TM3 and TM6 (9).

Asn (6.30) from position 1 to 17 onto the corresponding parts of TM6 (6.30–6.46). The simulated (see under "Materials and Methods") structures (blue ribbons) are superimposed from position 1 to 17 onto the corresponding parts of TM6 (6.30–6.46). The blue arrow indicates the conformational space that a Pro kink can assume relative to TM3. C, a closer view of the interactions of Glu-268 6.30–Asp3.49 in a model of the β2AR based on the rhodopsin structure. In C, the wild-type interactions are shown with the closest contacts between Glu-6.30, Asp-3.49, and Arg3.50–Asp3.49, which are within the distance range of an ionic interaction shown with dashed lines. D and E, the same view as C but with alterations in putative interactions after the mutation of Glu-6.30 to Ala (D) or of Glu-6.30 to Gln and of Asp3.49 to Asn (E).

MTSEA in the water-accessible binding-site crevice (13, 14).

MTSEA in the water-accessible binding-site crevice (13, 14).

FIG. 4. Correlation of fractional inhibition. The correlation of fractional inhibition of specific [3H](CGP-12177 binding produced by MTSEA (from Fig. 3) with the natural log of the slope of the pindolol-activated cAMP changes observed in the mutants/receptor density (CPM × pmol-1 × mg of protein) (from Fig. 2). The data from 25 mM MTSEA are shown in open squares, and the data of 75 mM MTSEA are shown in solid squares. The p values and r² values are given in the text.

FIG. 5. Molecular three-dimensional representations of the interaction of TM3 and TM6 at their cytoplasmic ends and the effects of 6.30 mutations. The Ca traces are taken from the high-resolution structure of bovine rhodopsin (9). Except in A, the top of each panel shows the extracellular end, and the bottom of each panel shows the intracellular end of the TMs. A, an extracellular view of the high-resolution structure of rhodopsin showing the interaction between residues at the cytoplasmic ends of TM3 and TM6 (9). B, the Ca ribbons of TM3 and TM6 of rhodopsin are shown in pink. The simulated (see under "Materials and Methods") structures (blue ribbons) are superimposed from position 1 to 17 onto the corresponding parts of TM6 (6.30–6.46). The blue arrow indicates the conformational space that a Pro kink can assume relative to TM3. C, a closer view of the interactions of Glu-268 6.30–Asp3.49 in a model of the β2AR based on the rhodopsin structure. In C, the wild-type interactions are shown with the closest contacts between Glu-6.30, Asp-3.49, and Arg3.50–Asp3.49, which are within the distance range of an ionic interaction shown with dashed lines. D and E, the same view as C but with alterations in putative interactions after the mutation of Glu-6.30 to Ala (D) or of Glu-6.30 to Gln and of Asp3.49 to Asn (E).

MTSEA in the water-accessible binding-site crevice (13, 14).

MTSEA at 25 and 75 mM inhibited the binding to varying degrees with the following rank order from the lowest to highest inhibition, E268Q, E268A, D130N, D130N/E268Q, and D130N/E268A (Fig. 3). The fractional inhibition at both 25 and 75 mM MTSEA correlated significantly with ln(slope) for basal activity (r² = 0.69, p < 0.03, and r² = 0.73, p < 0.05, for 25 mM and 75 mM MTSEA, respectively) and with ln(slope) for maximal pindolol-stimulated activity (Fig. 4) (r² = 0.96, p < 0.001, for both concentrations). Because it is reasonable to assume that these constitutively active mutants spontaneously assume an activated state of the receptor, this change in accessibility most probably reflects a conformational change associated with receptor activation with greater inhibition in the mutants that are more constitutively active.

DISCUSSION

It is well established that TM3 and TM6 play a role in GPCR activation, but the underlying mechanism has remained unclear. The DRY motif (Fig. 1) at the cytoplasmic side of TM3 is highly conserved throughout the subfamily of rhodopsin-like GPCRs. Glu-268 6.30 at the cytoplasmic side of TM3 is also highly conserved among multiple rhodopsin-like GPCRs including most neurotransmitter receptors, the glycoprotein hormone receptors, the opsins, and rhodopsin (Fig. 1). Mutation of the arginine (Arg3.50) has established an important role of this residue in G-protein activation (7, 20, 33, 34). Previous studies have also indicated a critical function of the adjacent aspartic acid (Asp3.49) for receptor activation (7, 14, 18, 20). In this study, we establish an important and interrelated role also of Glu-268 6.30. Not only do we find that the charge-neutralizing mutations of Glu-268 6.30 alone and together with Asp-130 3.49 lead to spontaneous activation of the receptor, we can also demonstrate a clear correlation between the extent of constitutive receptor activation and conformational rearrangements of TM6. Altogether, the present experimental data together with the recent high-resolution structure of rhodopsin (9) suggest that a network of interactions among Asp/Glu3.49, Arg3.50, and Glu6.30 maintains the cytoplasmic ends of these two helices immobilized in the inactive state of the receptor and, thus, may constitute a switch mechanism that is important for the activation of many rhodopsin-like GPCRs. Although this interpretation is satisfying given its consistency with the rhodopsin structure (see below), it is also possible that the mutations of Asp3.49 and Glu6.30 produce an additive effect by disrupting separate intramolecular interactions with other residues not identified here or even with bound water.

Based on simulations in the α1b-adrenergic receptor, Scheer et al. (7) suggested that the Arg3.50 in the inactive state is constrained in a polar pocket formed by residues in TM1, 2, and 7. Upon protonation (or mutation) of the adjacent Asp3.49, the arginine was proposed to shift out of the polar pocket leading to long range conformational changes. The ionic counterpart of the arginine in the inactive state was predicted to be the conserved aspartic acid in TM2 (Asp2.50), Asp-79 in β2AR (Fig. 1) (7). Ballesteros et al. (120) proposed that the ionic counterpart of Arg3.50 in the inactive state could be the adjacent Asp3.49, and that in the active state Asp3.49 is protonated, and Arg3.50 interacts instead with Asp2.50. We here now further hypothesized that in the inactive state, Arg3.50 not only interacts with Asp3.49 but also with Glu6.30, and that this ionic interaction between the cytoplasmic ends of TM6 and TM3 is an ionic lock maintaining the receptor in the inactive state. The recent high-resolution structure of rhodopsin represents the inactive species as it was obtained in the dark with retinal bound. In agreement with our hypothesis, the structure reveals an optimal placement of Arg3.50 for interaction with Glu3.49 and with Glu6.30 (Fig. 5) in a position where it would allow our
An Ionic Lock between TM3 and 6 of \( \beta_2 \)-Adrenergic Receptor

predicted network of interactions to serve the proposed ionic
lock function (9). However, the distance between Arg\(^{3.50} \) and Asp\(^{2.50} \) in the high-resolution structure is \( \sim 20 \) Å, a distance that is inconsistent with the proposed interaction of these residues in the inactive state (9). Even in the activated state, it seems improbable that Arg\(^{3.50} \) and Asp\(^{2.50} \) interact, because it would require large conformational changes to bring these side chains into proximity.

In rhodopsin, receptor activation is accompanied by the uptake of two protons at cytoplasmic sites (16). One site of uptake was inferred to be Glu\(^{3.49} \), which interacts with Arg\(^{3.50} \). The second protonated residue could not be identified but was hypothesized to be Glu\(^{3.28} \) (35), although this position is not an acidic residue in most other GPCRs. Based on our proposed interaction of Arg\(^ {3.50} \) with Glu\(^ {6.30} \), it is tempting to suggest that Glu\(^ {6.30} \) represents the second site of protonation. A protonation of these two acidic residues would disrupt their ionic interaction with Arg\(^{3.50} \), thereby disrupting the ionic lock holding TM3 and TM6 together in the inactive state. Accordingly, we predicted that disrupting the lock by the mutation of Asp\(^ {3.49} \) and/or Glu\(^ {6.30} \) in the \( \beta_2 \)AR would lead to constitutive activation of the receptor. We found that glutamine can substitute reasonably well for Glu\(^ {6.30} \), presumably because it can still hydrogen bond to Arg\(^ {3.50} \) and maintain an interaction, albeit more weakly, based on the small degree of enhanced basal and pindolol-stimulated cAMP accumulation. E268A is substantially more constitutively active as judged by basal and pindolol-stimulated activity. Similar to other findings that we have demonstrated previously, D130\(^ {3.49} \)N is highly constitutively active as well (14), presumably because the altered interaction of Asn\(^ {3.49} \) with Arg\(^ {3.50} \) in turn affects its interaction with TM6. Although it was difficult to reliably assess the increases in basal activity in double mutants because of their low levels of expression, the pindolol-stimulated cAMP stimulation suggested that the combination of the D130N and E268A mutants was even more active than D130N was by itself, consistent with this receptor adapting the active state more easily. It has recently been shown that the mutation of Arg\(^ {3.50} \) to His in \( \beta_2 \)AR did not prevent agonist-dependent accumulation of cAMP (36). Consistent with our hypothesis, this mutant had a lower EC\(_{50} \) for isoproterenol activation, a lower \( K_i \) for agonist binding, and an increased basal accumulation of cAMP, all characteristic hallmarks of constitutive activation.

Consistent with our observations in the \( \beta_2 \)AR, the mutations of the aspartic acid residue in position 6.30 of glycoprotein hormone receptors were also constitutively activating (37–39). Glu/Asp\(^ {6.30} \) is not universally conserved among all the rhodopsin-like receptors, although it is nearly 100% conserved among the neurotransmitter receptors, the glycoprotein hormone receptors, and the opsins. Thus, the ionic interaction between Arg\(^ {3.50} \) and Glu\(^ {6.30} \) cannot be the universal mechanism stabilizing the inactive state of all GPCRs, and accordingly it would be expected that other interactions might serve this function in receptors without this acidic residue. It is of notable interest that the originally published constitutively activating mutant of the \( \beta_2 \)AR was not mutated in position 6.30 but was mutated in four neighboring positions (L266\(^{6.28} \)S/K267\(^ {6.29} \)P/H269\(^ {6.31} \)K/L270\(^ {6.34} \)A) (32). It is conceivable that these mutations indirectly destabilize the interactions among Glu\(^ {6.30} \) , Asp\(^ {3.49} \) and Arg\(^ {3.50} \). For example, it may be possible that Arg\(^ {6.29} \) and/or Lys\(^ {6.31} \) might interact with Glu\(^ {6.30} \) and thereby play a stabilizing role. In the rhodopsin structure, Thr\(^ {6.34} \) also appears to contribute to the interaction with Arg\(^ {3.50} \) (9), and other constitutively activating mutations have been made at the 6.34 position in amine GPCRs as well (7, 40).

To assess the conformational rearrangements of the mutated receptors, we used an assay based on the accessibility to a charged sulfhydryl-specific reagent, MTSEA, of an endogenous cysteine in TM6 of the \( \beta_2 \)AR (Cys-285\(^ {6.47} \)). The reaction with MTSEA covalently couples SCH\(_2\)CH\(_2\)NH\(_2\) to the cysteine sulfhydryl (41). Methanethiosulfonate derivatives such as MTSEA react more than a billion times faster with the thiolate anion than with the thiol (42), and only water-accessible cysteines are likely to ionize to a significant extent. The binding site of the \( \beta_2 \)AR and homologous receptors for small-water-soluble agonists is contained within a water-accessible binding-site crevice formed by the seven-membrane-spanning segments (13, 43). Accordingly, a cysteine facing the water-accessible binding-site crevice should react much faster with charged MTSEA than should a cysteine facing the lipid bilayer or another transmembrane segment. We have shown previously that Cys-285\(^ {6.47} \) becomes partially accessible to MTSEA in the binding-site crevice in response to constitutively activating mutations (13, 14). In the present series of mutants, the extent of inhibition of binding produced by MTSEA was significantly correlated with the extent of constitutive activation based on both basal and pindolol-stimulated activity (Fig. 4), suggesting a progressive increase in the accessibility of Cys-285 with increasing activation.

According to a model of the \( \beta_2 \)AR, Cys-285\(^ {6.47} \) is pointing toward TM7 and is located in a boundary zone between the lipid bilayer and the water-accessible binding-site crevice (6, 8). The position of Cys\(^ {6.47} \) in the rhodopsin structure is consistent with such an orientation (9). Our data are consistent with a counterclockwise rotation (as seen from the extracellular side) or tilting of TM6 in the activated state bringing Cys-285\(^ {6.47} \) more toward the margin of the binding-site crevice. A similar movement is predicted both from EPR spectroscopy analyses in rhodopsin (10) and from fluorescence spectroscopy analyses of agonist-induced conformational changes in the \( \beta_2 \)AR (12, 15).

Experimental and simulation studies indicate that conformational switches in transmembrane \( \alpha \)-helices can be generated via proline-containing motifs that form flexible molecular hinges (44). Although Farrens et al. (10) predicted that a rigid body motion of TM6 relative to TM3 was associated with the activation of rhodopsin, the movements crucial to activation may involve flexibility around the hinge formed by the highly conserved proline in TM6 (Pro-288\(^ {6.50} \) in \( \beta_2 \)AR. In rhodopsin and presumably in the \( \beta_2 \)AR, TM6 exists in a highly bent configuration with its cytoplasmic end near the cytoplasmic end of TM3 (Fig. 5). Shown also in Fig. 5 are the results of a molecular dynamics simulation in which we examined the conformations explored by a helix containing a proline. Most of the simulated structures shown as fine ribbons are less kinked than the rhodopsin structure, and thus in the absence of an interaction constraining the cytoplasmic ends of TM6 and TM3, TM6 would be expected on average to be substantially less bent. This hypothesis has been supported by fluorescence labeling of TM6 residues at the cytoplasmic interface of the \( \beta_2 \)AR where such a movement around the Pro kink in TM6 could explain the observed fluorescence changes upon receptor activation (15). We propose that this straightening of the bent angle of TM6 is a critical feature of activation, and that this conformational change also exposes Cys-285\(^ {6.47} \) at the margin of the binding site crevice where it can react with MTSEA.

Acknowledgments—We thank Arthur Karlin and Harel Weinstein for helpful discussion and Dorte Frederiksen for technical assistance.
Activation of the β2-Adrenergic Receptor Involves Disruption of an Ionic Lock between the Cytoplasmic Ends of Transmembrane Segments 3 and 6
Juan A. Ballesteros, Anne D. Jensen, George Liapakis, Søren G.F. Rasmussen, Lei Shi, Ulrik Gether and Jonathan A. Javitch

J. Biol. Chem. 2001, 276:29171-29177.
doi: 10.1074/jbc.M103747200 originally published online May 25, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M103747200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 44 references, 21 of which can be accessed free at http://www.jbc.org/content/276/31/29171.full.html#ref-list-1