Activation of the $\alpha_{1b}$-Adrenergic Receptor Is Initiated by Disruption of an Interhelical Salt Bridge Constraint*  

(Received for publication, June 17, 1996, and in revised form, August 26, 1996)

James E. Porter, John Hwa‡, and Dianne M. Perez§

From the Department of Molecular Cardiology, Research Institute, The Cleveland Clinic Foundation, Cleveland, Ohio 44195

Rhodopsin receptor activation involves the disruption of a salt bridge constraint between glutamic acid 113 on transmembrane 3 and a lysine 296 in transmembrane 7, which forms a Schiff’s base with retinal. Light-induced isomerization of cis-retinal to the all trans form breaks this rhodopsin salt bridge leading to receptor activation. The analogous residues in $\alpha_{1b}$-adrenergic receptors, aspartic acid 125 and lysine 331, also have the potential of forming a constraining salt bridge holding the receptor to an inactive protein configuration. This $\alpha_{1b}$-adrenergic receptor salt bridge constraint is then released upon binding by the receptor agonist. To test this hypothesis, site-directed mutagenesis was used to eliminate the positive charge at position 331 by substitution of an alanine. The expressed $\alpha_{1b}$-adrenergic receptor mutant demonstrated a 6-fold increased epinephrine binding affinity with no alterations of affinity values for selective adrenergic receptor antagonists. Furthermore, an increased epinephrine potency for total soluble inositol phosphate production along with an elevated basal inositol triphosphate level was observed in COS-1 cells transfected with mutant versus wild-type $\alpha_{1b}$-adrenergic receptors. Similar results were obtained for a lysine to a glutamic acid $\alpha_{1b}$-adrenergic receptor mutation. In addition, increased basal inositol triphosphate levels were also observed for two aspartic acid 125 $\alpha_{1b}$-adrenergic receptor mutations, consistent with this residue’s role as the counterion of the salt bridge. Taken together, these $\alpha_{1b}$-adrenergic receptor mutations suggest a molecular mechanism by which the positively charged lysine 331 stabilizes the negatively charged aspartic acid 125 via a salt bridge constraint until bound by the receptor agonist.

Adrenergic receptors (ARs) are part of a larger family of G-protein-coupled receptors that are activated by epinephrine and norepinephrine to mediate the physiological effects of the sympathetic nervous system. The observed physiological response is dependent upon the type of AR expressed in the tissue being innervated. Classification of ARs ($\alpha_{1g}$, $\alpha_{2g}$, and $\beta$) is based upon the primary amino acid sequences and potencies of selective AR ligands (1). Further characterizations describing a heterogeneous population of $\alpha_{1}$-ARs has been accomplished using subtype-selective AR agonists and antagonists (2–5). Most of these pharmacologically identified $\alpha_{1}$-AR subtypes have been cloned and recognized to be unique based on primary amino acid sequences (6–8). In addition, a large amount of data has been catalogued about the cellular biochemistry associated with $\alpha_{1}$-AR activation (9, 10). However, nothing is known about the agonist-dependent molecular mechanisms of $\alpha_{1}$-AR stimulation.

Recently the activation mechanism for a related G-protein-coupled receptor, rhodopsin, has been described (11). Briefly, the visual chromophore, 11-cis-retinal, is covalently bound to the opsin protein by forming a Schiff’s base with the basic amino acid lysine at position 296 in transmembrane (TM) 7. This protonated amine is neutralized by forming an interhelical salt bridge with a negatively charged glutamic acid residue at position 113 in TM 3. This constraining salt bridge holds the rhodopsin receptor in a basal conformational configuration that is unable to interact with the G-protein transducin. Exposure of the rhodopsin receptor to light causes an isomerization of the covalently bound chromophore to the all-trans structure. This ligand isomerization breaks the salt bridge between TM 3 and 7, allowing the rhodopsin receptor to adopt an active conformation that can now signal through transducin.

A “constraining factor” has also been postulated for the $\alpha_{1}$-AR subtype, holding the receptor in a basal configuration until bound by a receptor agonist (12). This hypothesis is based upon the characterization of mutant $\alpha_{1}$-ARs where the normal alanine at position 293 in the third cytosolic loop was changed to all possible amino acid combinations. Kjelsberg et al. (12) observed that any amino acid substituted at position 293 caused the $\alpha_{1}$-AR to become constitutively active. This constitutive activity was characterized by higher binding affinity values for AR agonists but not antagonists when compared to the wild-type (WT) $\alpha_{1}$-AR. In addition, a higher AR agonist potency for generating intracellular signals was observed, and more importantly, there was an increased second messenger production in the absence of AR agonist. The characteristics for these constitutively active Ala293 $\alpha_{1}$-AR2 mutations resulted in a revision of the ternary complex model for receptor G-protein activation (13). This revised ternary complex model postulates the isomerization of the receptor between the inactive “R” state and an activated “R” state, before G-protein coupling can occur. In addition, Kjelsberg et al. (12) suggested...
that any amino acid change at position 293 of the $\alpha_{1b}$-AR releases a physical restraint that allows the isomerization of the receptor protein to an active configuration. However, no molecular evidence for this $\alpha_{1b}$-AR “constraining factor” has been presented.

Recently another constitutively active $\alpha_{1b}$-AR has been characterized in our laboratory (14). This mutant was generated by changing the natural cysteine at position 128 in TM 3 to a phenylalanine residue. The attributes of this C128F $\alpha_{1b}$-AR are compatible with the properties of a constitutively active receptor outlined in the revised ternary complex model for G-protein receptor coupling (13). This phenylalanine mutation is located three amino acids away from a highly conserved aspartic acid at position 125, postulated to interact with the protonated amine of endogenous catecholamines (15). In the unbound WT $\alpha_{1b}$-AR, this Asp$^{125}$ has the potential of forming a constraining salt bridge with a lysine residue at position 331 in TM 7, equivalent to what has been established for the rhodopsin receptor. In addition, the proximity of the bulky C128F point mutation to Asp$^{125}$ suggests the potential to disrupt this possible salt bridge and may represent the mechanism of constitutive activity for this mutant $\alpha_{1b}$-AR.

In this report we show that specific amino acid substitutions at position 128 selectively enhanced the constitutively active properties of these $\alpha_{1b}$-AR mutations when compared to the WT or C128F mutant receptor. This suggested that the mechanism of constitutive activity for these Cys$^{128}$ mutations could involve the disruption of the potential $\alpha_{1b}$-AR salt bridge formed by the Asp$^{125}$ and Lys$^{331}$ residues. To further investigate this possible mechanism of $\alpha_{1b}$-AR activation, the natural charges at amino acid positions 331 and 125 were modified using site-directed mutagenesis techniques. The constitutively active properties documented for these mutant receptors substantiated the existence of an interhelical salt bridge that constrains the tertiary protein structure of the $\alpha_{1b}$-AR.

**EXPERIMENTAL PROCEDURES**

**Site-directed Mutagenesis**—Site-directed mutagenesis was performed on a M13mp19 hamster $\alpha_{1b}$-AR construct utilizing the oligonucleotide-mediated double primer method (16). DNA from plaques that hybridized to the mutagenic primer were purified and sequenced by the dideoxymethod to verify the mutation. The mutated $\alpha_{1b}$-AR insert was removed from the phage M13mp19 vector and then subcloned into the eucaryotic expression vector, pMT2. The full-length plasmid DNA was again sequenced to verify the correct $\alpha_{1b}$-AR mutation.

**Cell Culture and Transfection**—COS-1 cells (ATCC) were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 5% fetal bovine serum. COS-1 cells were plated at a density of $10^6$ cells/60-mm dish for transient transfection of WT and/or various $\alpha_{1b}$-AR insertions. The full-length plasmid DNA was again sequenced to verify the correct $\alpha_{1b}$-AR mutation.

**Radioligand Binding**—Transfected COS-1 cell membranes were prepared as described previously (7). The pharmacological profile of expressed $\alpha_{1b}$-ARs was determined by saturation and/or competition binding experiments using the selective $\alpha_{1b}$-AR antagonist [3H]3-[4-(2-thienyl)butyryl]-L-glutamate (125I-HEAT) as the radiolabel. All binding experiments were performed at 25°C in a 0.25-ml total volume of buffer (20 mM HEPES, pH 7.5, 1.4 mM EGTA, 0.25-ml total volume of buffer (20 mM HEPES, pH 7.5, 1.4 mM EGTA, 12.5 mM MgCl$_2$) using 0.1 mM phentolamine to determine nonspecific binding. Bicinchoninic acid (BCA) protein assay [25°C for 5 min to pellet the precipitate. The supernatant was applied to a 0.8-ml packed AG1-X8 column (Bio-Rad, 100–200–mesh, formate form) and washed with 8 ml of 0.1 M formic acid. Soluble inositol phosphates were then eluted from the column with 2 ml of 1 M ammonium formate, 0.1 M formic acid. The amount of [3H]inositol phosphate eluted from the column was counted and a concentration-response curve generated using iterative nonlinear regression analysis (17). From this curve, the EC$_{50}$ of the AR agonist used to increase total soluble inositol phosphates in transfected COS-1 cells was calculated.

In separate and more sensitive studies, basal measurements for phospholipase C (PLC)-dependent inositol 1,4,5-trisphosphate (IP$_3$) production was determined by a [3H]IP$_3$ radioreceptor assay (DuPont) using the same pre-assay condition as described above.

**Statistical Analysis**—The runs test was used to determine if the data differed significantly from a linear relationship ($p < 0.05$). For each individual experiment, the fitted iterative nonlinear regression curve that best represented the data was determined using a partial $t$ test ($p < 0.05$). Significance among groups was tested using a paired $t$ test or one-way analysis of variance ($p < 0.05$). All values are reported as the mean ± S.E. of $n$ experiments, each performed in duplicate.

**RESULTS AND DISCUSSION**

**Saturation Mutagenesis at Position 128 of the $\alpha_{1b}$-Adrenergic Receptor**—Site-directed mutagenesis of the natural Cys$^{128}$ in TM 3 to a phenylalanine generated a mutated $\alpha_{1b}$-AR with documented properties of constitutive activity (14). To further investigate the molecular mechanism for this constitutive activation, saturation mutagenesis was performed at position 128 of the $\alpha_{1b}$-AR. These $\alpha_{1b}$-AR mutations were expressed on COS-1 cell membranes and their constitutively active properties examined. The amount of agonist-independent IP$_3$ produced in these transfected COS-1 cells was quantified and normalized to $\alpha_{1b}$-AR density (Fig. 1A). The introduction of charged amino acids at position 128 of the $\alpha_{1b}$-AR generated no PLC-dependent intracellular signal. This most likely was caused by improper protein folding, since the epinephrine binding affinity for these poorly expressed $\alpha_{1b}$-AR mutations was dramatically reduced (Fig. 1B). In addition, similar inactive $\alpha_{1b}$-ARs were produced when substitution of amino acids that have $\beta$-substituents, such as proline or isoleucine, were introduced at this same position. The inability of these $\alpha_{1b}$-AR mutations to express functional proteins could be the result of unsuitable TM packing caused by either the early branching of these amino acid side chains or the inability of the TM to accommodate unpaired charges. However, greater amounts of basal IP$_3$ were generated when tyrosine, threonine, leucine, methionine, and tryptophan were substituted at position 128 of the $\alpha_{1b}$-AR. The amounts of basal IP$_3$ generated for these $\alpha_{1b}$-AR mutants was greater than the original phenylalanine mutation. This increased agonist-independent second messenger production correlated with a higher epinephrine binding affinity for these same $\alpha_{1b}$-AR mutations. These results suggest that substitution of long, bulky, or hydrophobic amino acids at position 128 are disturbing an intrinsic “constraining factor” of the $\alpha_{1b}$-AR. Similar results were noted for the characteristics of rhodopsin receptor activation via mutagenesis of a charged amino acid at position 128 of the rhodopsin receptor (11). This suggests a comparable mechanism may be conserved to the $\alpha_{1b}$-AR.

**Radioligand Binding Properties for Lys$^{331}$ Mutations of the $\alpha_{1b}$-Adrenergic Receptor**—If an $\alpha_{1b}$-AR salt bridge exists to hold
the receptor protein in a basal conformation, then abolishing this ionic bond would allow the α1b-AR to adopt an active conformation that would have properties of a constitutively active receptor. To test this hypothesis, site-directed mutagenesis was performed to individually eliminate this charged amino acid pair by mutating the Lys331 to a neutral alanine or a negatively charged glutamic acid. Substitution of these amino acids should destroy the association between position 331 and the Asp125 postulated to occur in the WT α1b-AR. This suggests that no gross changes in the receptor’s tertiary structure are caused by these point mutations. Competition experiments revealed no changes in the affinity of 125I-HEAT for these mutant α1b-ARs compared to WT receptor (Table I). However, for both Lys331α1b-AR mutants, no binding affinity differences for selective α1-AR antagonists were noted when compared to the WT α1b-AR (Table I).

**FIG. 1. Properties of mutant α1b-ARs generated by saturation mutagenesis at position 128.** Site-directed mutagenesis was used to change the natural cysteine expressed at position 128 of the α1b-AR to all possible amino acid combinations. Panel A, moles of IP$_3$ generated in transfected COS-1 cells was quantified by a radioreceptor assay kit and normalized to the number of expressed α1b-ARs calculated from parallel saturation binding assays. Single-letter amino acid codes are used. α1b-AR expression (pmol/60-mm plate) was as follows: WT, 0.36; G, 0.15; A, 0.3; F, 0.19; L, 0.43; M, 0.08; W, 0.11; Y, 0.85; P, 0.001; I, 0.009; S, 0.13; T, 0.25; T, 0.11; N, 0.022; Q, 0.027; E, 0.065; D, 0.066; H, 0.125; K, 0.026; R, 0.064. Linearity is presumed between basal IP$_3$ activity and α1b-AR expression as demonstrated in Figs. 3 and 4. Results are represented as the mean ± S.E. for n = 3 experiments performed in duplicate. Panel B, the change from WT α1b-AR of the epinephrine binding affinity value for Cys128 mutant receptors. Epinephrine binding affinity values are calculated from competition binding experiments using transfected COS-1 cell membranes. Results are presented as the mean ± S.E. of individual mutant receptor values after subtraction of the mean WT α1b-AR epinephrine affinity for n = 2–5 experiments performed in duplicate.

In addition, similar AR agonist binding affinity changes were estimated for the K331E α1b-AR mutation (Table I). The essential property for a constitutively active receptor is the capacity to generate intracellular second messenger in the absence of bound agonist. Agonist-independent production of IP$_3$ in COS-1 cells transiently transfected with either the WT α1b-AR or one of the Lys331 mutations was quantified by a sensitive radioreceptor assay. This amount of IP$_3$ was determined at various levels of α1b-AR density calculated from parallel saturation binding studies (Fig. 3A). The linear regression lines generated through the data points is an indication for the amount of IP$_3$ produced per α1b-AR. The slope of the regression line calculated from these data points shows a greater increase in the amount of IP$_3$ generated per Lys331α1b-AR mutant receptor compared to the WT α1b-AR (Fig. 3B). The amount of IP$_3$ produced for the K331A (6.5 ± 0.9 pmol/fmol) and the K331E (8.0 ± 0.8 pmol/fmol) α1b-AR mutations was significantly larger when compared to the WT receptor (2.9 ± 0.2 pmol/fmol). These results demonstrate the central distinctive element of agonist-independent receptor constitutive activity. In addition, these Lys331α1b-AR mutants strongly suggest the importance of the natural residue for maintaining a basal configuration of the α1b-AR.

**Radioligand Binding Properties for Asp125 Mutations of the α1b-Adrenergic Receptor—The pharmacological characteristics of the Lys331α1b-AR mutations described previously suggest that a disassociation with the Asp125 counterion could be the mechanism of constitutive activation. If this hypothesis is correct, then eliminating the negative charge at position 125 should also generate constitutively active α1b-ARs. To investigate this possibility, the Asp125 of the WT α1b-AR was changed to a neutral alanine or a positively charged lysine using site-directed mutagenesis. These mutant α1b-ARs were then examined for properties of receptor constitutive activity. Unlike previous studies where this conserved aspartic acid was mutated in the α$_{2}$- or β$_{2}$-ARs, the Asp125 α1b-AR mutations did demonstrate specific radioligand binding (19, 20). Specific radioligand binding has also been shown for similar aspartic acid counterion mutations in the serotonin receptor (21). The ligand binding properties for these Asp125 α1b-AR mutations showed no significant changes in the $K_{d}$ of 125I-HEAT when compared to WT receptor (Table I). This implies no gross changes in the structure of the ligand binding pocket for these
expressing the WT mutations. And potency changes are no longer valid parameters for assessing amine of epinephrine, determining AR agonist affinity in the produced was quantified and used to generate membrane protein for the K331A receptor mutation. The amount of $K_i$ values are presented as the mean ± S.E. for $n = 3–5$ experiments performed in duplicate.

Asp$^{125}$ α$_{1b}$-AR mutants. In addition, no significant differences in the $K_i$ of selective α$_{1b}$-AR antagonists were demonstrated (Table 1). However, there was a significantly lowered epinephrine affinity for these Asp$^{125}$ mutant receptors when compared to the WT α$_{1b}$-AR (Table 1). This decrease in the epinephrine binding affinity is likely to the elimination of the conserved negative charged at position 125, shown in the β-AR system to be responsible for docking with the protonated amine of epinephrine (15). However, these data are the first to demonstrate in α$_{1b}$-ARs the significance of this conserved TM 3 aspartic acid as a contact point for epinephrine binding. Since this Asp$^{125}$ is the counterion necessary for receptor docking with the protonated amine of epinephrine, determining AR agonist affinity and potency changes are no longer valid parameters for assessing the constitutively active properties of these Asp$^{125}$ α$_{1b}$-AR mutations.

**Table I**

| Agonist          | WT max (fmol/mg of membrane protein) | K331A | K331E | D125A | D125K |
|------------------|-------------------------------------|-------|-------|-------|-------|
| (-)-Epinephrine  | 231                                 | 94    | 143   | 50    | 21    |
| (-)-Norepinephrine | 101                                 | 84    | 77    | 47    | 38    |
| Methoxamine      | 492                                  | 327   | 325   | 210   | 123   |
| Phentolamine      | 52.8                                 | ND    | 17.6  | ND    | ND    |
| Prazosin          | 6.3                                 | 0.33  | 0.23  | 0.13  | 0.34  |

**Fig. 2.** Potency of epinephrine to produce soluble inositol phosphates by activating the WT or K331A α$_{1b}$-ARs. COS-1 cells expressing the WT α$_{1b}$-AR (●) or the mutant K331A receptor (○) were stimulated with increasing concentrations of epinephrine to generate soluble inositol phosphates. Similar levels of α$_{1b}$-AR expression were achieved by varying the plasmid construct concentration used for the COS-1 cell transfection. Representative expression levels were 112 fmol/mg of membrane protein for the WT α$_{1b}$-AR and 94 fmol/mg of membrane protein for the K331A receptor mutation. The amount of $[^3H]$inositol phosphates produced was quantified and used to generate concentration-response curves for calculating an epinephrine EC$_{50}$ value. A half-maximal response for the mutant K331A receptor (381 ± 101 nM) was achieved at a significantly lower epinephrine concentration ($p < 0.05$) when compared with the WT α$_{1b}$-AR (1849 ± 537 nM). EC$_{50}$ values are presented as the mean ± S.E. for $n = 6–7$ experiments performed in duplicate.

**Fig. 3.** Agonist-independent production of IP$_3$ by Lys$^{331}$ mutations of the α$_{1b}$-AR. Panel A, the amount of IP$_3$ generated for 10$^5$ COS-1 cells/60-mm plate, transfected with the WT α$_{1b}$-AR (●), K331A (○), or the K331E mutant receptor (△), was quantified at various receptor densities determined from parallel saturation binding experiments. Data points did not depart from a linear relationship, as determined by a runs test ($p > 0.05$). The linear regression line is an indication for the amount of IP$_3$ produced per α$_{1b}$-AR. Data points are presented as the mean ± S.E. for $n = 3–5$ experiments performed in duplicate. Panel B, the slope of the regression line generated through the data points presented in panel A demonstrates a significant increase ($p < 0.05$) in the amount of IP$_3$ generated per receptor for the K331A (6.5 ± 0.9 pmol/fmol) and K331E mutant receptors (8.3 ± 0.8 pmol/fmol) compared to the WT α$_{1b}$-AR (2.9 ± 0.2 pmol/fmol).

**Fig. 4.** Agonist-independent production of IP$_3$ by Asp$^{125}$ mutations of the α$_{1b}$-AR. Panel A, the amount of IP$_3$ generated for 10$^5$ COS-1 cells per 60-mm plate, transfected with the WT α$_{1b}$-AR (●), D125A (○), or the D125K mutant receptor (△), was quantified at various receptor densities determined from parallel saturation binding experiments. Data points did not depart from a linear relationship as determined by a runs test ($p > 0.05$). The linear regression line is an indication for the amount of IP$_3$ produced per α$_{1b}$-AR. Data points are presented as the mean ± S.E. for $n = 3–5$ experiments performed in duplicate. Panel B, the slope of the regression line generated through the data points presented in panel A demonstrates a significant increase ($p < 0.05$) in the amount of IP$_3$ generated per receptor for the D125A (15.4 ± 2.1 pmol/fmol) and D125K mutant receptors (36.3 ± 5.0 pmol/fmol) compared to the WT α$_{1b}$-AR (2.9 ± 0.2 pmol/fmol).
agonist-independent properties of constitutive activity.

The increased AR agonist binding affinities calculated for the Ly8331-α1b-AR mutants, as well as the elevated basal activity observed for both Ly8331 and Asp1125 receptor mutations, could alternately suggest that their apparent constitutive activity might be the result of higher levels of receptor occupancy due to the higher affinity for catecholamines present in the serum-containing culture media. However, two lines of reasoning dismiss this notion. First, the IP₃ assays are performed in serum-free media after extensive washing of the transfected COS-1 cells. Second, epinephrine displays a lower binding affinity for these Asp1125α1b-AR mutations when compared to WT or the Ly8331 receptor mutants. Therefore, the higher basal IP₃ activity documented for these Asp1125α1b-AR mutations is not dependent upon the possibility of tightly binding to any catecholamines that may be present. In summary, these results satisfy an important criteria in identifying these Asp1125α1b-AR receptor mutants as constitutively active α1b-ARs. In addition, this constitutively active property of these Asp1125α1b-AR mutations strongly suggests the existence of an Asp1125-Lys331 salt bridge in the WT α1b-AR.

Molecular Mechanism of the α1b-Adrenergic Receptor Salt Bridge—A molecular mechanism for α1b-AR activation can now be hypothesized based upon the unique pharmacological properties of these salt bridge receptor mutations. When the α1b-AR ligand binding pocket is empty, a salt bridge between Lys331 in TM 7 and the Asp1125 in TM 3 constrains the receptor in a basal activation state. This is analogous to what has been described for the rhodopsin receptor in the absence of light. However, unlike rhodopsin the endogenous agonist epinephrine is not covalently bound to the receptor and must dock in the ligand binding pocket. Upon docking, the protonated amine of epinephrine competes with the protonated amine of Lys331 for the Asp1125 counterion’s negative charge. The neutrality that results from competing with this Asp1125 occurs because the basicity of epinephrine’s protonated amine (pKᵣ = 10) is similar to the alkaline properties of Lys331 (pKᵣ = 10). Destruction of this salt bridge releases an α1b-AR structural constraint that allows a translational movement of the third TM helix toward the protonated amine of epinephrine. This TM 3 translational movement brings the Asp1125 in closer proximity to the protonated amine of epinephrine, promoting the higher binding affinity observed for these α1b-AR salt bridge mutations. In addition, this translational movement of TM 3 enhances α1b-AR/G-protein interactions, causing an increased production of second messengers that leads to a physiological response. This molecular mechanism is also supported by recent work in rhodopsin, where a site-directed spin label study was employed to demonstrate a rigid body movement of the TM 3 helix during receptor activation in relationship to the other six TM bundles.

The charged amino acid pairs that make up the rhodopsin and α1b-AR salt bridge are conserved in other G-protein-coupled receptors that are activated by biogenic amines. The negatively charged aspartic acid is highly conserved among all aminergic receptors and is analogous in both position and charge to the glutamic acid residue found in TM 3 of rhodopsin. In addition, the positive counter ion is also preserved between these aminergic and rhodopsin receptors, suggesting that a constraining salt bridge could be a common mechanism among these types of receptors.

We also hypothesize that the constitutive activity documented for the C128Fα1b-AR mutation is due to an enhanced translational movement of TM 3. Substitution of large, bulky or hydrophobic amino acids for the Cys128 residue were best for constitutively activating the α1b-AR (Fig. 1). For the C128F mutation, the proximity of this phenylalanine residue in the TM domain may be sufficient to physically disrupt the endogenous α1b-AR salt bridge, moving the Asp1125 counterion toward the protonated amine of epinephrine. This may result from direct translational movement of TM 3 or could be indirectly due to the increased packing of this substituted phenylalanine against the second TM helix.

These data also suggest that the activation mechanism for α1b-ARs may be different than what has been defined for β-ARs. Substituting a serine or asparagine for the conserved Asp1125 in TM 3 of the β₁AR resulted in a 10,000-fold decreased affinity value for selective AR agonists and antagonists (15). Since a 10,000-fold affinity difference equates to a free energy change of 9 kcal/mol, linkage to a single ligand-receptor contact point is unlikely. However, this evidence is used to support the role of the TM 3 aspartic acid in AR agonist binding. The 3-fold decreased epinephrine binding affinity for α1b-AR Asp1125 mutations described in this report are not comparable to these β₁-AR mutants. This differential affinity loss assigned to the elimination of the ionic bond between the protonated amine of epinephrine and the conserved TM 3 aspartic acid could be masked due to the constitutive activity documented for the Asp1125α1b-AR but not the β₁-AR mutations. Therefore, a better estimate of the free energy change due to elimination of this ionic bond would be to compare epinephrine’s Kᵣ value for the K331A and the D125A α1b-AR mutants. This affinity difference is actually 17-fold (329 ± 53 nM versus 5636 ± 963 nM), which equates more reasonably to a typical salt bridge interaction of 3 kcal/mol that would be formed between Asp1125 and the protonated amine of epinephrine. The discrepancies between these analogous AR mutagenesis studies may be explained by the type of amino acids substituted for the conserved aspartic acid counterion. For example, the asparagine or serine substituted for Asp1125 of the β₁-AR may still hydrogen-bond with Lys331 in TM 7, mimicking the constraining properties of a WT receptor salt bridge. However, in the β₁-AR studies, no definitive experiments were performed that would establish any constitutively active properties for these receptor mutants.

Recent work has documented important interactions between AR agonists and specific serine residues in TM 5 that cause a functional response in α1b-ARs (29). This suggests that a change in the position of TM 5 may also be important for α₁-AR activation. Therefore, disruption of the interhelical salt bridge that leads to a translational movement of TM 3 is not the only agonist-dependent mechanism for α₁-AR activation. However, disruption of this Asp1125-Lys331 salt bridge may represent the first event in α₁-AR activation initiated by docking of the endogenous ligand. This would imply that epinephrine takes an active role to change the conformation of the α₁-AR by unlocking a physical constraint on the receptor structure similar to what is established for rhodopsin. This dynamic function of the AR agonist is in contrast to a postulated passive role that would “trap” a naturally isomerizing α₁-AR in the activated protein configuration (see Ref. 24 for review). In addition, this salt bridge also represents the molecular explanation for at least part of the “constraining factor” suggested by
others that holds the α1b-AR in a basal configuration until bound by the receptor agonist (12).

The ability to constitutively activate α1b-ARs, by substituting many types of amino acids at multiple and diversified positions in the protein, suggests the importance of maintaining the basal conformation of the WT receptor (12, 14, 25). Epinephrine may in fact be a molecular switch releasing the stored potential energy of the α1b-AR salt bridge constraint. This allows the α1b-AR to adopt the activated "lowest energy" configuration, therefore, suggesting that epinephrine is not involved in stabilizing or selecting for the activated (R*) state of the receptor. The maintenance of basal protein structure by the α1b-AR salt bridge may regulate the physiological response of receptor activation until authorized by chemical signals from the sympathetic nervous system. Hence, the important protein configuration to understand in α1b-AR activation is not the easily attainable "activated state" but instead the highly regulated "inactive state" of the receptor. This understanding would serve to provide insights into receptor structure-function and further investigations in drug development.

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