Characteristics for a Salt-bridge Switch Mutation of the α₁b Adrenergic Receptor

ALTERED PHARMACOLOGY AND RESCUE OF CONSTITUTIVE ACTIVITY

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Agonist-dependent activation of the α₁-adrenergic receptor is postulated to be initiated by disruption of an interhelical salt-bridge constraint between an aspartic acid (Asp-125) and a lysine residue (Lys-331) in transmembrane domains three and seven, respectively. Single point mutations that disrupt the charges of either of these residues result in constitutive activity. To validate this hypothesis, we used site-directed mutagenesis to switch the position of these amino acids to observe, if possible, regeneration of the salt-bridge reverses that the constitutive activity of the single point mutations. The transiently expressed switch mutant receptor displayed an altered pharmacological profile. The affinity of selective α₁a-adrenergic receptor antagonists for the switch mutant (D125K/K331D) was no different from the wild-type α₁a-adrenergic receptor, suggesting that both receptors are maintaining similar tertiary structures in the cell membrane. However, there was a significant 4–6-fold decrease in the affinity of protonated amine receptor agonists and a 3-6-fold increase in the affinity of carboxylated catechol derivatives for the switch mutant compared with the wild-type α₁b-adrenergic receptor. This pharmacology is consistent with a reversed charge at position 125 in transmembrane domain three. Interestingly, the ability of either a negatively or positively charged agonist to generate soluble inositol phosphates was similar for both types of receptors. Finally, the switch mutant (D125K/K331D) displayed similar basal signaling activity as the wild-type receptor, reversing the constitutive activity of the single point mutations (D125K and K331D). This suggests an ionic constraint has been reformed in the switch mutant analogous to the restraint previously described for the wild-type α₁a-adrenergic receptor. These results strongly establish the disruption of an electrostatic interaction as an initial step in the agonist-dependent activation of α₁-adrenergic receptors.

α₁b-adrenergic receptor (AR) activation initiates a variety of sympathetic nervous system responses by binding the hormone, epinephrine, and the neurotransmitter,norepinephrine (1). Adrenergic receptor subtypes (β₁, β₂, β₃, α₂a, α₂b, α₂c, α₃a, α₁b, and α₅) are members of the G-protein-coupled receptor (GPCR) superfamily typified by seven transmembrane spanning domains. We have postulated that part of the activation mechanism for α₁-ARs paradigm those described for a related GPCR, rhodopsin. Two amino acids in rhodopsin, Glu-113 in transmembrane domain (TMD) three and Lys-296 which forms the protonated Schiff base with 11-cis-retinal in TMD seven, create a salt-bridge constraint that holds the rhodopsin receptor in a basal conformation (2). Light-induced isomerization of retinal to the all trans form disrupts this constraint and allows rhodopsin to adopt an active conformation that can signal through the G-protein, transducin. We have characterized a salt-bridge constraint mechanism of activation for α₁-ARs that is analogous to the rhodopsin receptor system (3). Specifically, a salt-bridge constraint is formed between Lys-331 in TMD seven and Asp-125 in TMD three of the α₁b-AR that is required to partly maintain the inactive state of the receptor. Mutations that disrupt the charges of either residue result in constitutive activity, similar to analogous mutations obtained in rhodopsin. However, unlike the covalently bound retinal in rhodopsin, docking of the adrenergic receptor agonist establishes a competition between Lys-331 and the protonated amine of ligand for neutrality with Asp-125, resulting in salt-bridge breakage. Although salt-bridge disruption does not account for full receptor activation, we believe that it is an initial step in the process of receptor activation (4).

This current study lends support to our molecular model of α₁-AR activation by using site-directed mutagenesis to reverse the amino acids postulated in the salt-bridge constraint. This switch mutation (D125K/K331D) reversed the constitutive activity of the single receptor mutations (D125K or K331D) but, interestingly, was also equally activated as the WT receptor by either positively or negatively charged agonists. The pharmacological profile of this switch mutant was also reversed from the WT α₁b-AR, displaying higher affinity for negatively charged agonists but lower affinity for positively charged agonists. The properties of this switch mutation strongly support our hypothesis of a salt-bridge constraint and its role in agonist-dependent initiation of the α₁-AR activation process.

EXPERIMENTAL PROCEDURES

Site-directed Mutagenesis—Site-directed mutagenesis was performed on a M13mp19 hamster α₁b-AR construct utilizing the oligonucleotide-mediated double primer method (5) and as described previously (3). DNA was purified and sequenced by the dideoxy method to verify the mutation. The mutated α₁b-AR insert was removed from the phage M13mp19 vector then subcloned into the eucaryotic expression vector, pMT2. The full-length plasmid DNA was again sequenced to verify the mutation.

Cell Culture and Transfection—COS-1 cells (ATCC) were grown in DMEM supplemented with 10% fetal bovine serum and antibiotics. COS-1 cells (5 × 10⁵) were transfected with the plasmid pMT2-α₁b-AR using lipofectamine (Gibco/BRL). Transfected cells were analyzed 48 h later by whole cell, [35S]GTPγS binding assay, or electrophysiological recordings.

Legend:

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**RESULTS AND DISCUSSION**

Recent studies have described a salt bridge constraint mechanism of activation for the rhodopsin receptor system involving amino acids Glu-113 and Lys-296 in TMDs three and seven of the receptor (2). Constitutively active rhodopsin receptors generated by site-directed mutagenic disruption of the electrostatic interaction between these two amino acids were used to support this activation model. In further experiments, these amino acids were switched in a double mutation (E113K/K296E) of the rhodopsin receptor to substantiate this ionic constraint hypothesis. Results of these experiments were inconclusive. Regeneration of the ionic pair in the switch mutant reversed the constitutive activity of the single mutant K296E. However, functionality of the double and E113K mutations could not be verified because they could not reconstitute with the chromatophore. This is because of the strict structural requirements for 11-cis retinal to covalently attach with Lys-296 suggesting that these mutants were misfolded or just incapable of attaching retinal (2). To validate our α1b-AR salt-bridge constraint hypothesis, we used site-directed mutagenesis to generate a double mutation of the wild type (WT) receptor. This mutant α1b-D125K/K351D-AR switches the amino acids that constitute the previously described receptor salt-bridge constraint (3). However, unlike the rhodopsin receptor system, there is no requirement for the adrenergic ligand to become covalently bound to form a functional α1-AR.

This switch mutant AR mutation could be transiently expressed on α1-AR negative COS-1 cell membranes at a level of 22 ± 4 fmol/mg of protein (data not shown). In addition, there were no differences in binding affinity of the competitive receptor antagonists 125I-HEAT, prazosin, or phentolamine for the switch mutation (110 ± 31 pm, 299 ± 99 pm, and 29 ± 20 nm, respectively; Table I) when compared with the WT α1a-AR (83 ± 10 pm, 243 ± 30 pm, and 36 ± 10 nm; Table I). A negative charge at position 125 on TMD three is thought to be important for agonist as well as antagonist affinity at β- and α2-ARs (9). Competitive α1-AR antagonists are larger molecules when compared with epinephrine, although they may have common binding

contacts with receptor agonists in the ligand binding pocket. Therefore, the Asp-125 binding contact may contribute less to the overall binding affinity of competitive α1-AR antagonists than for agonists. Alternately, Asp-125 may not participate in antagonist binding to the α1-ARs. For example, an α1b-D125A-AR mutant does not have a significantly different antagonist affinity value when compared with the WT receptor (3). Previous studies have also characterized amino acid residues on the extracellular loop as α1-AR binding contacts for competitive receptor antagonists (10). Molecular modeling suggests that competitive receptor antagonists may contact the α1-AR above or near the surface of the agonist binding pocket involving amino acid residues near the top of the TMD. Nevertheless, similarities in antagonist binding between WT and the switch mutant suggest that the overall tertiary structure of these two receptors is comparable.

In contrast, AR agonists such as epinephrine, phenylephrine, and oxymetazoline, which all contain a protonated amine (Fig. 1), significantly (p < 0.05) displayed 4–6-fold decreases in affinity for the switch mutation (6.2 ± 0.7 μM, 77.6 ± 1.5 μM, and 5.0 ± 1.0 μM respectively; Table I) compared with WT α1b-AR (1.4 ± 0.3 μM, 12.9 ± 0.6 μM, and 0.9 ± 0.2 μM; Table I). Conversely, carboxylated catechol derivatives such as 3-(2-hydroxyphenyl) propionic acid and 3-methoxyphenyl acetic acid (Fig. 1), which replace the protonated amine with an acid group, displayed a significant (p < 0.05) 3–6-fold higher binding affinity for the switch receptor mutant (0.5 ± 0.4 mM and 1.9 ± 0.4 mM, respectively; Table I) when compared with WT α1b-AR (2.9 ± 0.9 mM and 5.7 ± 1.4 mM; Table I). Although the carboxylated derivatives display poor overall binding affinity, they are not the exact cogners of epinephrine. Both derivatives have altered distances of the charged head group to the aromatic ring, a crucial feature of agonist binding affinity. However, regardless of the low affinity, the switched binding profile of the double mutant is consistent with a positively charged amino acid now substituted at position 125 in TMD three of the receptor. The degree of decreased affinity of protonated amine agonists (4–6-fold) for the switch mutant is consistent with agonist affinity losses (3–17-fold) for the D125A α1a-AR mutation (3).

We also examined the ability of these agonists to activate transiently expressed WT or switch mutant α1a-ARs. Demonstrating that the switch mutation is not signaling defective, both WT and switch mutant displayed a similar ability to invoke release of soluble IPs in a concentration-response to phenylephrine (Fig. 2). While the maximum IP response of the switch mutant was not significantly different from the WT α1a-AR, the EC50 of phenylephrine for the switch mutation was

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**Table I**

| Agonists        | WT     | D125K/K351D-AR |
|-----------------|--------|----------------|
| **Equilibrium dissociation binding constants for the WT and switch mutant α1b ARs** |        |                |
|                 |        |                |
| **K** values for the radiolabel, selective AR ligands and carboxylated catechol derivatives are calculated from binding curves generated from saturation or competition binding experiments. An asterisk indicates significance (p < 0.05) from the WT α1b-AR. Values are expressed as the mean ± S.E. for N = 3–6 experiments performed in duplicate. |        |                |
| **Antagonists** |        |                |
| **125I-HEAT**   | 83 ± 10pm | 110 ± 31 pm    |
| **Prazosin**    | 243 ± 30 pm | 299 ± 99 pm    |
| **Phentolamine** | 36 ± 10 nm | 29 ± 20 nm     |
| **Agonists**    |        |                |
| **Epinephrine** | 1.4 ± 0.3 μM | 6.2 ± 0.7 μM* |
| **Phenylephrine** | 12.9 ± 0.6 μM | 77.6 ± 1.5 μM* |
| **Oxymetazoline** | 0.9 ± 0.2 μM | 5.0 ± 1.0 μM*  |
| **3-(2-Hydroxyphenyl)propionic acid** | 2.9 ± 0.9 mM | 0.5 ± 0.4 mM*  |
| **3-Methoxyphenyl acetic acid** | 5.7 ± 1.4 mM | 1.9 ± 0.4 mM*  |

**Materials**—(--)-Epinephrine, phenylephrine, oxymetazoline, 3-(2-hydroxyphenyl) propionic acid, 3-methoxyphenyl acetic acid, prazosin, phentolamine were from Sigma; [3H]inositol and 125I-HEAT was from NEN Life Science Products.
significant 10-fold lower (2.0 ± 0.8 versus 0.2 ± 0.1 μM, p < 0.05) than the WT receptor. This lowered potency is consistent with the 6-fold decrease in affinity of phenylephrine for the switch mutation when compared with the WT α1b-AR. Low expression levels and the poor affinity of the carboxylated catechol derivatives for the switch α1b-AR mutation did not allow for the accurate generation of concentration-response curves in the presence of these ligands. However, when expression levels of WT α1b-AR were titrated to equivalent receptor densities as the switch mutation, a single ligand concentration that occupied at least 50% or greater of these transiently expressed receptors was used to increase soluble IPs in COS-1 cells (Fig. 3). Interestingly, either charged agonist was able to pressurize receptors was used to increase soluble IPs in COS-1 cells (10^6 cells) transiently expressing either the WT, D125K, K331D, or D125K/K331D α1b-ARs was quantified and normalized to receptor number determined from parallel saturation binding experiments. There was a significant increase, p < 0.05 (*) in the basal amount of IP3 generated per receptor for the D125K (52.2 ± 3.0 pmol/mg of receptor) and K331D (55.2 ± 3.6 pmol/mg of receptor) mutant receptor when compared with either the switch mutation (19.3 ± 2.1 pmol/mg of receptor) or WT (18.8 ± 1.8 pmol/mg of receptor) α1b-AR. The density of expression was 59 ± 7 fmol/mg membrane protein for the WT, 17 ± 6 fmol/mg for D125K, 45 ± 7 fmol/mg for K331D, and 21 ± 4 fmol/mg for the D125K/K331D α1b-ARs. Data is presented as the mean ± S.E. for n = 3 transfections of ten replicates each.

The amount of basal IP3 generated from WT α1b-AR was quantified and used to generate concentration-response curves for calculating an epinephrine EC50 value. A half-maximal response for the WT basal IP3 (0.05) for the D125K mutant (52.2 ± 3.0 pmol/mg of receptor) or WT (18.8 ± 1.8 pmol/mg of receptor) α1b-AR (55.2 ± 3.6 pmol/mg of receptor) was calculated for the newly characterized α1b-AR (55.2 ± 3.6 pmol/mg of receptor; Fig. 4). A similar 3-fold increase in basal IP3 production over the WT receptor was calculated for the switch α1b-AR (55.2 ± 3.6 pmol/mg of receptor). Finally, when both single mutations were generated in the same receptor (switch mutation), the amount of agonist-independent IP3 produced (19.3 ± 2.1 pmol/mg of receptor) or WT (18.8 ± 1.8 pmol/mg of receptor) α1b-AR was quantified and normalized to receptor number determined from parallel saturation binding experiments. There was a significant increase, p < 0.05 (*) in the basal amount of IP3 generated per receptor for the D125K (52.2 ± 3.0 pmol/mg of receptor) and K331D (55.2 ± 3.6 pmol/mg of receptor) mutant receptor when compared with either the switch mutation (19.3 ± 2.1 pmol/mg of receptor) or WT (18.8 ± 1.8 pmol/mg of receptor) α1b-AR. The density of expression was 59 ± 7 fmol/mg membrane protein for the WT, 17 ± 6 fmol/mg for D125K, 45 ± 7 fmol/mg for K331D, and 21 ± 4 fmol/mg for the D125K/K331D α1b-ARs. Data is presented as the mean ± S.E. for n = 3 transfections of ten replicates each.

This manuscript characterizes a switch mutation of the α1b-AR in which the amino acids of a postulated ionic pocket for the WT receptor have been reversed. This switch mutation receptor has comparable characteristics to the WT α1b-AR (i.e. affinity for receptor antagonists, basal production of IP3, and agonist-induced activation), suggesting that the overall tertiary structure and function of these receptors in the membrane bilayer is similar. However, there are distinct differences in the pharmacological properties of the agonist binding pocket between the switch mutant and the WT receptor.
Interestingly, both WT and the switch receptor mutation are bound to and activated by either catecholamines or carboxylated catechol derivatives, suggesting some flexibility in the docking of agonists in $\alpha_{1b}$-AR binding pocket. This is in contrast to the more stringent structural requirements of the opsins receptor ligand binding pocket for reacting with 11-cis-retinal to generate rhodopsin although alterations in the chromophores length and ability to form a Schiff’s base has been shown to generate viable rhodopsin receptors with limited ability to activate transducin (11, 12). Furthermore, the ability to activate transducin or WT receptor ligand binding pocket for reacting with 11-cis-retinal to generate soluble IPs mediated by $\alpha_{1b}$-AR should be dependent on the effectiveness of a ligand to compete for the appropriate counterion that forms the ionic constraint of the receptor. Therefore, it is not unexpected to observe activation of the WT or switch mutant $\alpha_{1b}$-AR by phenylephrine or carboxylated catechol ligands, even though the orientation of the charged ligand to the salt-bridge constraint on the receptor may not be optimal in some combinations. This is evident in the reduced potency of phenylephrine to generate soluble IPs mediated by the switch $\alpha_{1b}$-AR mutation. If we were able to accurately generate a concentration-response curve for the carboxylated catechol derivatives, predicted changes in potency that correlated to binding affinity differences would likely have become apparent.

Although Asp-125 is a highly conserved amino acid in biogenic amine receptors, evidence for the conservation of this mechanism to other family members is still unresolved. Previous mutation of Asp-113 in the $\beta_2$-AR resulted in a dramatic loss of agonist and antagonist affinity (13). The receptor was still capable of signaling although at reduced potency. The constitutive behavior of this mutation was not explored at that time because of the field’s lack of knowledge for active state receptors. Analysis is also complicated because of the poor expression of these mutations and the endogenous expression of $\beta$-ARs in most cell types. Mutations of Asp-113 in the $\alpha_{2a}$-AR also resulted in a similar phenotype to the $\beta_2$-AR (14). However, recent $\beta_2$-AR work in which a histidine replaced Asp-113 in TMD three and a cysteine replaced Asn-312 in TMD seven, followed by chelation with zinc, resulted in a constitutive phenotype (15). The altered distance between these two helices introduced by the zinc is thought to be responsible for the active state stabilization of this mutant $\beta_2$-AR. This suggests that these two helices and/or residues are linked in the WT $\beta_2$-AR activation process. Analogous amino acids in the $\delta$ opioid receptor have also generated constitutively active receptors with mutations at Asp-128 in TMD three and Tyr-308 in TMD seven (16). It has been proposed that these two residues participate in hydrogen bonding forming a constraint similar to our salt-bridge hypothesis for the $\alpha_{1b}$-AR. This suggests that similar constraining paradigms may be part of a universal activation mechanism among GPCRs.

To summarize, disruption of this ionic constraint has been suggested to be an initial but partial event that is required for full activation of the WT $\alpha_1$-AR (4). In addition, pH-dependent binding studies resulting in $pK_a$ shifts for the WT and Lys-331 mutations have implied that Asp-125 and Lys-331 are in close proximity to each other in the unbound WT $\alpha_{1b}$-AR. Finally, reversing the constitutive properties of D125K and K331D by combining these mutations in a single receptor and activation of this switch mutant by either type of charged agonist strongly supports our original hypothesis for a salt-bridge constraint holding the WT $\alpha_{1b}$-AR in a basal conformation (3).

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