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Adrenergic receptors (ARs) $^1$ ($\alpha_{1a}$, $\alpha_{1b}$, $\alpha_{2a}$, $\alpha_{2b}$, $\beta_1$, $\beta_2$, and $\beta_3$) are members of the G-protein coupled receptor superfamily of membrane proteins that mediate the actions of the endogenous catecholamines, the neurotransmitter norepinephrine, and the hormone epinephrine. Similar to rhodopsin, these proteins are proposed to traverse the plasma membrane in a series of seven transmembrane-spanning $\alpha$-helical domains linked by three intracellular and three extracellular loops (1). In accordance with the observation that the greatest structural conservation is localized to the transmembrane helical domains of the receptor, the catecholamine binding pocket is also localized to these regions. Mutagenesis studies in our laboratory and others have identified that the endogenous agonist in biogenic amine receptors is stabilized in the binding pocket by ionic, hydrogen bond, and aromatic/hydrophobic interactions involving residues on TM3, TM5, and TM6, although there are various modulations of these interactions between the families (2–5). We have also recently shown that $\alpha_{1a}$-ARs and perhaps some other biogenic amine receptors have additional aromatic/hydrophobic interactions with the endogenous agonist to residues in TM4 and TM5 (6).

However, our knowledge of how antagonists bind to the adrenergic receptor family is limited. Mutagenesis studies in our laboratory have identified that the subtype selectivity of two $\alpha_{1a}$-AR antagonists, phentolamine and WB4101, is conferred by interactions with three consecutive residues of the second extracellular loop (7). Similar results were also obtained in the 5-HT1D receptor (8) and the opioid receptor (9). This observation indicates that in contrast to agonist binding, which is localized to the interior core of the receptor, antagonists interact with residues closer to the extracellular surface of adrenergic receptors, above the plane of the agonist binding pocket. Further point contacts between $\alpha_{1a}$-antagonists and extracellular residues of this receptor could not be identified using a series of $\alpha_{1a}/\beta_2$-AR chimeras. Therefore, the high-affinity binding of these drugs to the receptor must involve additional interactions with residues within the transmembrane domains of the receptor (10). Other previous mutagenesis studies have indicated the importance of phenylalanine residues located close to the extracellular surface of the receptor in antagonist binding at adrenergic receptors. A single phenylalanine residue about two turns into the TM7 domain of the $\alpha_{2a}$-AR (Phe-412) was shown to promote high-affinity binding of yohimbine (11). Mutagenesis of a phenylalanine residue about two turns into the TM7 domain of the $\alpha_{2c}$-AR (Phe-310 of the $\alpha_{2c}$-AR) in TM6 has been identified as being important not only for agonist binding but also for the binding of certain $\alpha_1$-agonists (13). In light of these studies and given that the $\alpha_1$-agonists contain high aromatic/hydropho-
bic character, we continued our investigation into the role of aromaticity/hydropobicity in antagonist binding at \(\alpha_2\)-ARs. We now report the importance of two conserved phenylalanine residues near the extracellular surface of TM7 involved in nonselective binding for several \(\alpha_1\)-antagonists. Our studies suggest that \(\alpha_1\)-antagonists bind the receptor in an elevated pocket from agonist binding that is also skewed toward TM7.

**EXPERIMENTAL PROCEDURES**

**Materials**—Drugs were obtained from the following manufacturers: (a) alprenolol, (-)-ephedrine, oxymetazoline, phenylephrine, methamphetamine, phentolamine, propranolol, clonidine, and propranolol, Sigma; (b) \(^{125}\)I-HEAT, PerkinElmer Life Sciences; and (c) 5'-methylurapidil, BMY7378, (+) nimodipine, cilastatin, and WRB101, Research Biochemicals Inc. (Natick, MA). Chemicals for buffered solutions (HEPES (free acid), EGTA, and MgCl\(_2\)) were obtained from Mallinckrodt Baker (Phillipsburg, NJ).

**Site-directed Mutagenesis in the \(\alpha_1\)-AR**—Site-directed mutagenesis of the pMT2' \(\alpha_1\)-AR plasmid (14) was performed using polymerase chain reaction technology and commercially synthesized oligonucleotides (Life Technologies, Inc.) specifically designed to code for the desired mutation. A fragment of cDNA encoding either the F312A or F312N mutation of the rat \(\alpha_1\)-AR was generated using sense primers containing the unique EcoRI cloning site before the start site of transcription and an antisense primer targeted to the unique AhoI III site of the rat \(\alpha_1\)-AR that also encoded the mutation. The F308A or F308L mutations were generated using a sense primer containing the mutation targeted to the endogenous AhoI restriction site and an antisense primer containing the NofI restriction site after the stop codon. Using the Expand High Fidelity polymerase chain reaction protocol (Roche Molecular Biochemicals), these specific fragments of the rat \(\alpha_1\)-AR were generated using 1 \(\mu\)g of pMT2'\(\alpha_1\)-AR plasmid, 300 nm sense and antisense primers, 200 nm each deoxynucleotide triphosphate, and 2.6 units of Taq and Pwo DNA polymerase in a 20 mM Tris-HCl, pH 7.5, buffer containing 100 mM KCl, 1 mM dithiothreitol, 0.1 mM EDTA, 0.05% (w/v) Tween 20, and a final MgCl\(_2\) concentration of 1.5 mM. All polymerase chain reactions were conducted in 10% (v/v) glycerol. The amplification reactions, which were repeated for 40 cycles, consisted of a denaturation at 95 °C for 3 min and an annealing and elongation phase at 72 °C for an additional 3 min. The polymerase chain reaction-generated fragments resulting from each of the reactions were isolated and purified, followed by either EcoRI/AhoI III (Phe-312 mutations) or AhoI/NofI (Phe-308 mutations) restriction enzyme digestion. The polymerase chain reaction products were ligated with their respective WT fragment and subcloned into the pMT2' expression vector to yield the full-length \(\alpha_1\)-AR construct containing single mutations. Mutations were confirmed by full-length sequence analysis of the construct by the dideoxy method (Cleveland Clinic Sequencing Core Facility).

**Cell Culture and Transfection**—COS-1 cells (American Type Culture Collection, Manassas, VA) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) bovine serum, penicillin, and streptomycin. Cells were maintained and passaged upon subconfluence. The cells were transfected with either wild type or mutated \(\alpha_1\)-AR plasmid, 300 nM sense and antisense primers, 200 nM each deoxynucleotide triphosphate, and 2.6 units of Taq and Pwo DNA polymerase in a 20 mM Tris-HCl, pH 7.5, buffer containing 100 mM KCl, 1 mM dithiothreitol, 0.1 mM EDTA, 0.05% (w/v) Tween 20, and a final MgCl\(_2\) concentration of 1.5 mM. All polymerase chain reactions were conducted in 10% (v/v) glycerol. The amplification reactions, which were repeated for 40 cycles, consisted of a denaturation at 95 °C for 3 min and an annealing and elongation phase at 72 °C for an additional 3 min. The polymerase chain reaction-generated fragments resulting from each of the reactions were isolated and purified, followed by either EcoRI/AhoI III (Phe-312 mutations) or AhoI/NofI (Phe-308 mutations) restriction enzyme digestion. The polymerase chain reaction products were ligated with their respective WT fragment and subcloned into the pMT2' expression vector to yield the full-length \(\alpha_1\)-AR construct containing single mutations. Mutations were confirmed by full-length sequence analysis of the construct by the dideoxy method (Cleveland Clinic Sequencing Core Facility).

**Membrane Preparation**—Membranes were prepared as described previously (6). Briefly, transfected COS-1 cells were scraped 72 h after transfection, collected, washed in Hank's balanced salt solution, and then pelleted under low-speed centrifugation. The cell pellet was resuspended in a 0.25 M sucrose solution and centrifuged. The pellet was resuspended in water containing a mixture of protease inhibitors and frozen at −70 °C for 30 min. Pellets were dounced from a B glass Dounce homogenizer. Nuclear debris was removed by a low-speed centrifugation step. Membranes in the supernatant were washed with HEM buffer and pelleted by high-speed centrifugation. The membrane pellet was washed twice in HEM buffer, and the final pellet was reconstituted in HEM buffer containing 10% (v/v) glycerol and stored at −70 °C until use. The protein concentration was determined by a Bradford method using the known standard of bovine serum albumin.

**Measurement of Ligand Binding Affinities**—Saturation binding experiments were performed on competition binding binding performed as described previously (6). The binding affinities of various adrenergic receptor agonist and antagonists were determined in a series of competition binding experiments performed as described previously (6). All binding assays were performed in duplicate in HEM buffer, in a total assay volume of 250 μL. Nonspecific binding was defined as the amount of radioactivity that remained bound to the filters in the presence of 10 μM phenotamine.

**RESULTS**

Fig. 1 shows the comparison of residues located in TM7 for the \(\alpha_1\)-ARs as well as all other adrenergic receptors. The biogenic amine family has a consensus domain of WXGXSNNXX-PXY that is thought to be involved in signal transduction and sequestration but not directly involved in binding per se. (17). Therefore, we focused our attention on aromatic residues located closer to the extracellular border. TM7 has two aromatic residues; both are absolutely conserved in both the \(\alpha_1\)- and \(\alpha_2\)-AR subtypes but not in the \(\beta\)-AR subtypes. These two residues, Phe-308 and Phe-312 in the \(\alpha_1\)-AR, were generated as described previously using Insight II molecular modeling software from Biosym Technologies (4, 7). The coordinates of the a-carbon positions were determined by an overlay of the \(\alpha_1\)-AR residues with the TM coordinates of rhodopsin (16).

**Binding Phenotype at Phe-308 Mutations**—Binding results for a series of \(\alpha_1\)-AR, \(\alpha_2\)-AR, and \(\beta\)-AR antagonists are presented in Table I. All binding curves resulted in one-site fits. In saturation binding studies, the radiolabeled antagonist \(^{125}\)I-HEAT bound to each of the mutated receptors with comparable affinity and density as determined for the wild type \(\alpha_1\)-AR. To determine changes in \(\alpha_1\)-antagonist affinity, a number of ligands were tested with each ligand tested with the known standard affinity for a- or \(\beta\)-ARs. We then tested both \(\alpha_1\)-AR and \(\beta\)-AR antagonists to address cross-family selectivity issues. All ligands tested but alprenolol showed no changes from WT
Antagonist Binding at $\alpha_1$-Adrenergic Receptors

Aromaticity/hydrophobicity has been shown to be important in the binding of agonists and antagonists to the various subtypes of adrenergic receptors. Recently, we have identified two novel aromatic interactions promoting agonist binding that involve two phenylalanine residues located at the extracellular surface of TM4 and TM5 of the $\alpha_1\beta$-AR (6). These residues were modeled to form contacts from above the plane of the agonist pocket. Until recently, determination of residues involved in antagonist binding at $\alpha_1$-ARs was limited to two domains. One is located on the second extracellular loop of the receptor. These consecutive residues (Gln-177, Ile-178, and Asn-179) are involved only in $\alpha_1\alpha$-AR versus $\alpha_1\beta$-AR selectivity issues and then for only three ligands, phenotamine, WB4101, and partial effects on 5-methylurapidil (7). Another group has shown that mutagenesis of a phenylalanine residue (F86M) at the surface of TM2 in the $\alpha_2\alpha$-AR accounts for the $\alpha_1\alpha$ versus $\alpha_1\beta$ selectivity of dihydropyridine antagonists such as nifedipine (12). This aromatic residue is also modeled to be in the first helical turn of TM2. Nevertheless, both these previous interactions involve selectivity issues and separate domains of the receptor and involved only certain antagonists. A major antagonist docking site has never been reported in any adrenergic receptor family member, let alone any G protein-coupled receptor; thus, it has been thought that these ligands are very diverse in their binding parameters because of the diversity of their chemical structures.

Aromaticity/hydrophobicity should be more important in antagonist binding than in agonist binding, given the higher degree of these contacts contained in adrenergic antagonists and their decreased aqueous solubility. Agonists typically have one aromatic ring and hydrophilic substituents, whereas $\alpha_1$-AR antagonists are larger molecules with multiple aromatic/hydrophobic ring components. However, little is known of specific residues involved in antagonist binding, even among other adrenergic receptor family members. Chimeras between the $\beta_1$- and $\beta_2$-AR failed to reveal discrete residues (18, 19). However, a single residue in TM7 (either Phe-412 in the $\alpha_2$ or Asn-385 in the 5-HT1d) seemed responsible for certain $\beta$-AR antagonist selectivity (11, 20). Therefore, to explore additional residues involved in antagonist binding, we postulated that in addition to the antagonist pocket being closer to the extracellular surface than agonists, the pocket must also be skewed but sufficient in overlap to maintain competitive interactions. Because we and others have found agonist interactions in TM3 through TM6 for the $\alpha_1\beta$-AR (4–6, 13, 21) and an antagonist-binding residue in TM7 in $\alpha_2$/$\beta_2$-ARs (11) and in TM6 for a few $\alpha_1$-antagonists (6), the antagonist pocket must also be skewed toward TM6 and TM7. We therefore focused our attention on TM7 of the $\alpha_1$-AR for mutagenesis studies.

As shown in the sequence alignment (Fig. 1), the phenylalanine residue at position 308 of the $\alpha_1\alpha$-AR is conserved across all the cloned adrenergic receptor subtypes except the $\beta_2$-AR, where the aromaticity is conserved as a tyrosine residue. Two mutations of this residue were made: (a) a F308A mutation in which the side chain packing and the potential for Van Der Waal’s interactions were minimized, and (b) a F308L mutation in which the aromaticity of the side chain was removed, but hydrophobicity was maintained. The phenylalanine at position 312 was mutated in expectation of cross-family selectivity issues such as those found in the $\alpha_1\alpha$-AR, $\beta_2$-AR, and 5-HT1a receptors (11, 20). Therefore, to reproduce this same system, we mutated Phe-312 to Asn, the same corresponding residue found in the $\beta$-ARs and the 5-HT1a. This mutation would also test hydrophobicity. We also made the F312A substitution to ex-
pKᵦ values for drugs were determined in competition binding as described under "Experimental Procedures." All competition binding isotherms were best fit to a single-site model. Values in parentheses represent the fold changes in drug affinity relative to wild type. Statistically significant differences in affinities (bold) at the mutant receptors versus wild type were determined by Student’s t test. Data are reported as the mean ± S.E.M. of three to five experiments.

### Table I

| α₂-AR antagonists       | WT       | F312N  | F312A  | F308A  | F308L  |
|-------------------------|----------|--------|--------|--------|--------|
| Phenolamine             | 8.1 ± 0.2| 8.1 ± 0.3 | 8.0 ± 0.3 | 8.6 ± 0.3 | 8.3 ± 0.1 |
| Prazosin                | 9.5 ± 0.2| 9.9 ± 0.3 | 7.6 ± 0.2 (85) | 9.5 ± 0.2 | 9.3 ± 0.3 |
| 5-Methylurapidil        | 9.0 ± 0.1| 8.9 ± 0.3 | 7.7 ± 0.1 (21) | 9.7 ± 0.5 | 8.8 ± 0.2 |
| WB4101                  | 10.2 ± 0.1| 9.8 ± 0.2 | 9.5 ± 0.2 (5) | 9.2 ± 0.1 (10) | 8.8 ± 0.2 (25) |
| BMY7378                 | 6.8 ± 0.1| 7.2 ± 0.4 | 7.0 ± 0.2 | 6.2 ± 0.1 (4) | 6.4 ± 0.1 (3) |
| (+) Niguldipine         | 9.3 ± 0.4 (1200) | 6.3 ± 0.1 (1000) | 7.8 ± 0.5 (30) | 8.5 ± 0.3 (8) |
| ¹²⁵I-HEAT                | 9.7 ± 0.1| 9.9 ± 0.2 | 9.6 ± 0.2 | 9.7 ± 0.2 | 9.7 ± 0.1 |
| B_max (pmol/mg)         | 0.25     | 0.15    | 0.17    | 0.27    | 0.21    |

### Table II

| Phenylethylamine versus imidazoline agonist binding |
|-----------------------------------------------|
| Phenylethylamines                        | WT       | F312N  | F312A  | F308A  | F308L  |
| Epinephrine                              | 5.8 ± 0.2| 6.2 ± 0.4 | 5.8 ± 0.5 | 6.3 ± 0.8 | 6.4 ± 0.7 |
| Phenylephrine                            | 5.0 ± 0.02| 5.8 ± 0.3 | 5.0 ± 0.3 | 5.3 ± 0.1 | 5.3 ± 0.8 |
| Methoxamine                              | 4.2 ± 0.1| 4.0 ± 0.4 | 3.6 ± 0.2 | 3.9 ± 0.1 | 3.8 ± 0.1 |
| Imidazolines                             |          |        |        |        |        |
| Oxymetazoline                            | 8.0 ± 0.2| 7.8 ± 0.6 | 5.8 ± 0.2 (150) | 7.5 ± 0.03 | 6.9 ± 0.01 (13) |
| Cirazoline                               | 6.0 ± 0.1| 5.6 ± 0.3 | 5.1 ± 0.2 (8) | 6.1 ± 0.1 | 5.8 ± 0.1 |
| Clonidine                                | 6.1 ± 0.1| 4.7 ± 0.4 (25) | 4.7 ± 0.1 (25) | 5.8 ± 0.3 | 5.5 ± 0.04 (4) |

### Footnotes

• p < 0.01.
• p < 0.001.
• p < 0.05.
• p < 0.1.

### Exploration

Potential packing interactions.

Mutation of either Phe-308 or Phe-312 did not change the affinity of ¹²⁵I-HEAT, the radiolabeled antagonist (Table I). This supports the position that the overall global conformation of the receptor was maintained, and the changes in affinity we do not see with other ligands are specific. Likewise, there are no significant differences in antagonist receptor expression as measured by significant differences in receptor expression as measured by Student’s t test. Data are reported as the mean ± S.E.M. of three to five experiments.

In modeling these interactions and using the current coordinates of rhodopsin (16), the binding orientation of WB4101 is illustrated in Fig. 3A. This model incorporates the residues involved in subtype-selective affinity changes in WB4101 (30-25) that we described previously that are located in the second extracellular loop (7). According to the structure of rhodopsin, the second extracellular loop folds down into the binding pocket of retinal and likewise in our model folds down sufficiently to allow interactions with WB4101. The portion of the loop near TM5 was previously found to contain charged interactions with WB4101, and thus these moieties are oriented toward the charged portions of the antagonist. The extracellular loop interactions still allow aromatic interactions with Phe-308 and Phe-312 that are likely due to the phenyl ring. This docking of WB4101 is consistent with its placement above the plane of the agonist pocket (note the orientation of Ser-188 and Ser-192, which are involved in phenethylamine agonist binding, (4)). Interaction of Asp-106 and the amine group of the antagonist is...
FIG. 3. Molecular modeling of the rat $\alpha_1$-AR antagonist binding pocket. A, the binding of WB4101. $\alpha$-Carbon coordinates are taken from rhodopsin (17). Residues mutated in this study are represented in purple (Phe-308) and blue (Phe-312). The second extracellular loop (EX loop 2) folds into the binding pocket and interacts with the charged moieties of WB4101 as described previously (7). This orientation allows a phenyl ring of WB4101 to interact with the Phe residues in TM7. Agonist-binding residues (Asp106, Ser188, and Ser192) are represented to illustrate the depth of the agonist binding pocket. B, the binding of oxymetazoline, an imidazoline partial agonist. Orientation is rotated counterclockwise from A. To account for TM7 residues involved in binding interactions and the orientation of nitrogens on the imidazoline ring toward TM3, the aromatic ring of oxymetazoline must be oriented toward TM7, which is in sharp contrast to ephedrine binding. Color coding on the ligands is consistent with the traditional nomenclature: oxygen, red; nitrogen, blue; hydrogen, white; and carbon, green.
still possible but would be a weak interaction due to a greater separation than agonist binding. However, this result is consistent with previous mutagenesis of Asp-125 in the \( \alpha_2 \)- AR, in which antagonist binding was not affected as greatly as agonist binding (5). Modeling of the other antagonists produced similar results, with one end of the molecule interacting with Phe-308 and Phe-312, whereas the other end was orientated more toward TM6 than TM4–5.

The model is also consistent with the reason why phenolamine did not change affinity with either TM7 mutation. All of the ligands that changed affinity in this report have long and extended structures, whereas phenolamine is more compact and is likely confined to TM5–6 and the loop regions and not extended to TM7. Consistent with this interpretation, phenolamine displayed an 8-fold loss of affinity at the second extracellular loop mutations (7) and a 10-fold loss at the Phe-310 mutation in TM6 (13) but did not do so with either TM7 mutation.

Interestingly, we found that all imidazoline-type agonists also decreased affinity with Phe-308 and Phe-312 substitutions. The affinity of oxymetazoline, a weak partial agonist, was decreased at F308L (13-fold) and F312A (150-fold). Decreases in affinity were also found for other imidazolines such as cirazoline (8-fold) and clonidine (25-fold). Imidazolines have been proposed to bind differently in the agonist pocket than phenylethylamine agonists such as epinephrine because many mutants that affect phenylethylamine binding do not change imidazoline binding (6, 22). In fact, in earlier studies, the Eason-Stedman hypothesis predicted that imidazolines bind differently than phenethylamines (23). This is also supported by signaling studies in which imidazolines lack cross-desensitization with phenylethylamine agonists, suggesting different activation and signaling parameters (24). Because the imidazoline ring has protonated nitrogens that are involved in binding to Asp-106 in TM3 and is required for agonism (5), modeling of this ligand (Fig. 3B) requires that the imidazoline ring must be pointed toward TM3, but the second aromatic ring analogous to epinephrine must then orient toward TM7. This is in sharp contrast to epinephrine binding, in which the aromatic ring is oriented toward TM5 (4). Therefore, the binding of imidazolines is also skewed toward TM7 and located higher in the pocket, much like antagonist binding. Mutation of Phe-412 to Asn (analogous to Phe-312 in the \( \alpha_1 \)- AR) in TM7 of the \( \alpha_2 \)- AR also resulted in lower binding affinity for \( \alpha_1 \)-AR agonists, which were recognized previously to bind differently than epinephrine-like agonists, also bind to these two Phe residues in TM7, suggesting that imidazolines bind with some antagonist characteristics. This antagonist-like binding may explain their generally poorer agonism at \( \alpha_2 \)-ARs. A consensus of the results of numerous mutagenesis studies suggests that the agonist pocket lies deeper in the hydrophobic core of the receptor and involves TM3 through 6, whereas antagonist binding is located above the plane of agonist binding but is skewed toward TM7. This study represents the first report of a major docking site for \( \alpha_1 \)-AR antagonists and for a G protein-coupled receptor in general. These studies suggest that whereas antagonists generally have diverse structures, there are conserved pharmacophores that recognize a common site on the receptor. These studies may be helpful in the synthesis of selective ligands for these receptors and may represent conserved paradigms for antagonist binding in other adrenergic receptors or other nonpeptide G protein-coupled receptors.

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