

Identification of Critical Determinants of $\alpha_1$-Adrenergic Receptor Subtype Selective Agonist Binding*

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$\alpha_1$-Adrenergic receptor (AR) subtypes mediate many effects of the sympathetic nervous system. The three cloned subtypes ($\alpha_{1a}$-AR, $\alpha_{1b}$-AR, $\alpha_{1d}$-AR), although structurally similar, bind a series of ligands with different relative potencies. This is particularly true for the $\alpha_{1a}$-AR, which recognizes a number of agonists and antagonists with 5-50-fold higher affinity than the $\alpha_{1b}$- or $\alpha_{1d}$- subtypes. Since ligands bind to receptor-residues that are located in the transmembrane spanning domains, we hypothesize that subtype differences in ligand recognition are due to differences in the binding properties of nonconserved transmembrane residues. Using site-directed mutagenesis, selected putative ligand-binding residues in the $\alpha_{1b}$-AR were converted, either individually or in combination, to the corresponding residues in the $\alpha_{1a}$-AR. Mutation of two such residues (of approximately 172 amino acids in the transmembrane domains) converted the agonist binding profile entirely to that of the $\alpha_{1a}$-AR. Over 80% of this conversion was due to an Ala$^{204}$ → Val substitution; the remainder was due to the additional substitution of Leu$^{314}$ → Met. To confirm that Ala$^{204}$ and Leu$^{314}$ are indeed critical for agonist subtype-selectivity, the equivalent residues in the $\alpha_{1a}$-AR (Val$^{198}$ and Met$^{299}$) were reversed of that of the $\alpha_{1b}$-AR. Correspondingly, the agonist-binding profile of this double $\alpha_{1a}$-AR mutant reverted to that of the $\alpha_{1b}$-AR. From these data, in conjunction with macromolecular modeling of the ligand-binding pocket, a model has been developed, which indicates that the determinants of these two residues for agonist subtype-selectivity are due not only to interactions between their side chains and specific ligand moieties but also to a critical interaction between these two amino acids.

$\alpha_1$-Adrenergic receptors ($\alpha_1$-ARs)$^{1,2}$ are members of the seven transmembrane G-protein-coupled receptor family that are activated by norepinephrine released from sympathetic nerve endings and by epinephrine released from the adrenal medulla. They are a part of a larger subset of related but distinct adrenergic receptors, which include the $\beta_1$, $\beta_2$, and $\beta_3$ adrenergic receptors. $\alpha_1$-ARs are expressed in a wide variety of tissues including the brain, liver, myocardium, and vascular smooth muscle (2, 3). Thus far, three $\alpha_1$-AR subtypes have been cloned and pharmacologically characterized, the $\alpha_{1A}$-AR (4), the $\alpha_{1B}$-AR (2, 5), and the $\alpha_{1D}$-AR (6, 7). The $\alpha_{1A}$-subtype has a 5-50-fold higher affinity for the agonists oxymetazoline and methoxamine and for the antagonists 5-methylurapidil, (−)-niguldipine, and phentolamine when compared with the $\alpha_{1B}$- or the $\alpha_{1D}$-subtype (2, 7). However, there are currently no good subtype-selective drugs readily available that can discriminate between the subtypes, based on 100-1000-fold differences in affinity.

Recent studies have indicated that $\alpha_1$-subtypes can mediate different and sometimes opposing physiological effects. In particular the $\alpha_{1A}$-AR appears to promote automatic and arrhythmias during myocardial ischemia, whereas the $\alpha_{1B}$-AR subtype can activate a Na$^+$/K$^+$ pump leading to cell hyperpolarization, thus decreasing the propensity for abnormal heart rhythms (8). In addition, it is now known that the $\alpha_{1A}$-subtype regulates the predominant dynamic component involved in the development of benign prostatic hypertrophy (9). Hence, development of better $\alpha_1$-subtype-selective drugs to either block or enhance function may help in combating subtype-related disorders.

In order to design selective drugs, an understanding of subtype differences in the ligand binding pockets would be invaluable. Although the residues forming the $\alpha_{1A}$-AR binding-pocket have not been defined, key interactions are likely to be similar to those of the $\beta$-adrenergic receptor, since both receptor families are activated by the catecholamines, norepinephrine, and epinephrine. Based on studies of the $\beta_2$-AR, key points of interaction are believed to occur between Asp$^{113}$ in the third transmembrane helix (TMIII) with that of the protonated amine of the agonists, and between two serine residues (TMV; Ser$^{204}$ and Ser$^{205}$) that hydrogen-bond with the meta- and para-hydroxyl groups of the catechol ring. These critical binding contacts with the natural ligands are well conserved between the subtypes. However, there are 48 amino acid differences in the transmembrane domains between the $\alpha_{1A}$-AR and the $\alpha_{1D}$-AR. We hypothesize that only a few of these nonconserved residues are critical for the ligand binding specificity of each $\alpha_1$-AR subtype since only a subset of these nonconserved residues are designated lower case letters as $\alpha_{1A}$, $\alpha_{1B}$, and $\alpha_{1D}$, respectively (3).
dues occur in the regions of the transmembrane domain thought to be involved in ligand binding. Therefore, rather than sequentially mutating all amino acids that differ between the α_{1a}-subtypes, we have developed simple criteria to select those residues that may be critical for ligand binding and thus are involved in subtype differences in ligand binding profiles. Eight α_{1b}AR residues were mutated individually to the corresponding residue in the α_{1a}AR. Two of these changes, when combined in a double mutant, conferred on the α_{1b}AR the agonist binding properties of the α_{1a}AR. A single change, Ala^{204} → Val^{3} in TMV, made the greatest contribution to the change in ligand binding. Reversal of these two residues in the α_{1b}AR to those in the α_{1a}AR reversed the agonist binding profile back to that of the α_{1a}AR.

EXPERIMENTAL PROCEDURES

Site-directed Mutagenesis—The construct used was the hamster α_{1b}AR cDNA (~3600 bp) (4) including an EcoRI restriction site at the 5’ end and a region encoding an octapeptide tag (D4), at the end of the coding region, which was used to evaluate membrane expression with the monoclonal antibody (anti-D4). The attachment of this epitope after the coding region does not affect protein expression or the function of the receptor. The construct terminates with a stop codon and a Ncol restriction site. The EcoRI/NcoI sites permitted insertion into the modified eukaryotic expression vector pmT2 (7). Site-directed mutagenesis was performed as described previously utilizing the oligonucleotide-mediated double primer M13 method (10). The α_{1b}AR cDNA was divided into two 800-bp fragments by digestion with the restriction endonuclease BamHI. Each of the fragments were inserted individually into a M13 phagemid. The full-length 1600-bp receptor gene was not inserted into M13 since such a large insert would be more susceptible to spontaneous mutations. Mutagenesis utilized a 20-base mutagenic primer encoding the codon mismatches to achieve the desired point mutation(s) and a universal primer (−20) for extension on single-stranded M13 templates. After transformation of the single-stranded products into DH5αE cells, plaques were screened for the mutation on nitrocellulose lifts and probed with the 125I-labeled mutagenic primer. The efficiency of mutagenic incorporation was 5% of total plaques. Positive plaques were purified, and the DNA was isolated and sequenced by the dideoxy chain termination method (Sequenase, Amersham Corp.). The mutated fragment (800 bp) was then ligated with its complementary wild-type fragment (800 bp) into pmT2 for transfection into COS-1 cells. Sequencing was utilized to confirm that the fragments were in the correct orientation and that the appropriate mutation was present. Combination mutations were generated using unique restriction sites located within the α_{1b}AR cDNA. Reverse mutations that incorporated equivalent point mutations into the rat α_{1a}AR were also performed using similar methods.

Transfection of COS-1 Cells—COS-1 cells (ATCC) were grown in Dulbecco’s modified Eagle’s medium containing penicillin, streptomycin, glutamine, and 10% fetal bovine serum. At 60–80% confluency, Dulbecco’s modified Eagle’s medium containing penicillin, streptomycin, and 5% fetal bovine serum. The cells were subjected to nitrogen cavitation at 400 psi for 20 min. The cells were suspended in 0.25 mM sucrose with protease inhibitors and subjected to nitrogen cavitation at 400 psi for 20 min. The cells were then homogenized in a “B” glass Dounce homogenizer for 10 strokes and spun at low speed (1200 × g for 5 min) to remove nuclei and micromeres, and then centrifuged at 30,000 × g for 15 min. After two further washes with HEM (20 mM HEPES, pH 7.5, 1.5 mM EGTA, and 12.5 mM MgCl₂) and recentrifugation at 30,000 × g, the pellet was resuspended in HEM containing 10% glycerol and stored at −70°C. A Bradford protein assay was performed initially on known concentrations of bovine serum albumin followed by membrane preparations of the wild-type and mutant α_{1a/1b}ARs.

Ligand Binding—The ligand binding characteristics of the expressed receptors were determined in a series of radioligand binding studies using [125I]HEAT, an α_{1b}-specific antagonist as the radioprobe (7). In brief, for competition studies, the procedure involved duplicate tubes containing (total volume, 250 μl) 200 pm [125I]HEAT, HEM buffer, COS-1 membranes, and increasing amounts of unlabelled competing ligand (at 10 or different dilutions). Nonspecific binding was determined by the addition of phentolamine (10 μM). For saturation binding studies, 200-1600 pm [125I]HEAT was used. After 1 h of incubation at room temperature, the reactions were stopped by the addition of ice-cold HEM buffer and were filtered onto Whatman GF/C glass filters with a Brandel cell harvester. The filters were washed 5 times with ice-cold HEM. They were then analyzed for bound radioactivity using a Packard Auto γ 5000 counter. Data were analyzed using the interactive program LIGAND. IC_{50} values were converted to K_i using the Cheng-Prusoff equation and a K_i for [125I]HEAT of 90 pm. K_i values were expressed as a mean ± S.E. An analysis of variance and Student’s t test was used to determine significant differences (p < 0.05). To detect small but significant differences, sets of mutations along with the wild-types α_{1b}AR and α_{1a}AR were evaluated simultaneously (i.e. in the same assay).

Molecular Modeling—The coordinates of the α-carbon positions were determined by an overlay of the putative α_{1a}AR transmembrane residues with the transmembrane coordinates of bacteriorhodopsin (11). Data files were generated with the Insight II molecular modeling software from Biosym Technologies. The boundaries of the putative transmembrane domains were determined by an algorithm based upon the weighted pairwise comparisons of adjacent residues (12). The model was then minimized, and conflicts were adjusted as described previously (13). Assumptions of key interactions of specific amino acids with agonist are based upon previous mutagenesis work and proposed models with the p-AR (14). Results of our mutagenesis were then used to refine the structure of the α_{1b}AR ligand binding pocket.

Materials—Drugs were obtained from the following manufacturers. 1-epinephrine, (−)-norepinephrine, oxymetazoline, methoxamine, phenylephrine, and phentolamine were from Sigma; Cirazoline was a gift from Pfizer. [125I]HEAT, was from DuPont NEN. 5-Methylurapidil and WB4101 were from Research Biochemicals International (Natick, MA).

RESULTS AND DISCUSSION

The α_{1b}AR and the α_{1a}AR, although structurally similar in their transmembrane domains, have some significant differences in their ligand-binding affinities for a number of agonists and antagonists. Determining the critical amino acids responsible for these differences in binding properties may assist in the design of better subtype selective drugs. We hypothesized
that there are only a few nonconserved transmembrane amino acids that distinguish the \(\alpha_{1A}\)-AR agonist-binding pocket from that of the \(\alpha_{1B}\)-AR.

Selection Criteria—Our choice of the \(\alpha_{1A}\)-AR and the \(\alpha_{1B}\)-AR as the initial targets for mutagenesis was 2-fold. First, as already described, there are some important differences in the responses mediated by these two subtypes, which may be of pathophysiological and therapeutic relevance. Second, the \(\alpha_{1A}\) has a 5–50-fold greater affinity for a number of agonists and antagonists than the \(\alpha_{1B}\)-AR. This allowed us to detect even small but significant changes in affinity (eg, 2–5-fold) with our individual mutations in converting the \(\alpha_{1B}\)-AR to the \(\alpha_{1A}\)-AR.

Candidate residues potentially involved in subtype selectivity were selected for mutation from the 48 nonconserved transmembrane amino acids, based on the following criteria: 1) location in the upper half of the transmembrane domain, where previous studies indicate that small ligands are bound; 2) exclusion of the first transmembrane domain since, based on models of bacteriorhodopsin and rhodopsin, it appears not to be directly involved in forming the ligand binding pocket (11); 3) location on the transmembrane \(\alpha\)-helices such that their side-chains are oriented toward the putative binding pocket or adjacent transmembrane domains rather than toward the surrounding phospholipid bilayer (orientation of the side-chains was determined based on consideration of models of the \(\alpha_{1A}\)-AR developed in our laboratory (13), and on the model of rhodopsin developed by Baldwin (15)); 4) lack of conservation due merely to interspecies differences, since there is conservation of \(\alpha_{1A}\)-subtype ligand-binding profiles across species.

Seven amino acids (Fig. 1) of the \(\alpha_{1A}\)-AR were identified that fulfilled the criteria, and these residues were mutated to the corresponding residues on the \(\alpha_{1B}\)-AR. An eighth residue Leu\(^{182}\) \(\rightarrow\) Phe was also mutated. Although this residue was predicted to be facing the hydrocarbon environment, it was selected because the corresponding \(\alpha_{1A}\)-AR residue represented a significant change in size and functional group. Moreover, if our prediction of its orientation was correct, mutation of this residue should not alter ligand binding and, thus, this mutant would serve as a negative control.

Analysis of Single Mutations in Converting the \(\alpha_{1A}\)-AR to the \(\alpha_{1B}\)-AR—The eight point mutations were initially analyzed by their ability to bind a panel of agonists. \(K_I\) values for six \(\alpha_{1B}\)-AR agonists are shown in Table I and Fig. 2. The Ala\(^{204}\) \(\rightarrow\) Val mutation had a 5–10-fold increased binding affinity over the wild-type \(\alpha_{1A}\)-AR (WT) for oxymetazoline (p < 0.001), cirazoline (p < 0.001), and methoxamine (p < 0.001), a ligand binding profile that is more consistent with that of the \(\alpha_{1B}\)-AR (Fig 2A). Paradoxically, this mutation also had increased affinity (3–5-fold) for the natural ligands epinephrine (p < 0.01) and norepinephrine (p < 0.001) as well as phenylephrine (p < 0.001), which all show no selectivity between these two \(\alpha_{1A}\)-AR subtypes (Fig. 2B). The Leu\(^{314}\) \(\rightarrow\) Met mutant also demonstrated a significant increase in its affinity for methoxamine (p < 0.001), cirazoline (p < 0.001), and oxymetazoline (p < 0.05) (Fig. 2B) but not for epinephrine, norepinephrine, or phenylephrine (see Fig. 5). The other six point mutations including the Leu\(^{182}\) \(\rightarrow\) Phe mutation showed no significant change from an \(\alpha_{1A}\)-AR (WTa) along with the mutations that were statistically different (by a Student’s t test) from the WTb. The x-axis represents the difference in \(K_I\) (log) from the WTb.

Our results indicate that with agonist binding, individual
amino acids, particularly in the 5th and 6th transmembrane domains, are critical in defining the agonist binding pocket between the $\alpha_{1b}$-AR and the $\alpha_{1a}$-AR. Each agonist is similarly affected by changes in critical residues but not to the same degree. The Ala$^{204}$ → Val mutation appears to explain most of the higher affinity for agonists as seen with the $\alpha_{1a}$-AR. The increased affinity observed with the substitution of valine for alanine may be related to an increased hydrophobic interaction between the valine and the aromatic ring of the ligands. The increase in binding affinity with the Leu$^{314}$ → Met may be due to an increased interaction as a result of the extended chain length of the methionine residue with the ortho hydrophobic group found with many of the synthetic agonists. Supporting this is the progressive increase in affinity conferred by Leu$^{314}$ → Met with a progressive increase in size and hydrophobicity of the ortho side chain. The descending order of binding affinities (Fig. 2A) are cirazoline > methoxamine > oxymetazoline and their respective side chains are a cyclopropyl, a methoxy, and a methyl group.

Effects of Ser$^{208}$ → Ala ($\alpha_{1a}$) on the Agonist Binding Properties of $\alpha_{1}$-ARs—The serine involved in the Ser$^{208}$ → Ala ($\alpha_{1b}$) mutation corresponds to one of the proposed serines that binds to the catechol ring hydroxyls. Previous studies have shown that mutation of the equivalent serine residue in the $\beta_{2}$-adrenergic receptor (residue 204) to alanine, decreases binding affinity by 10-fold (14) and reduces the intrinsic activity of full agonists. Thus, it was proposed that for full agonist activity in the $\beta_{2}$-AR, both hydroxyls on the catechol ring hydrogen bonded to the two serines, Ser$^{204}$ and Ser$^{207}$. Site-directed mutagenesis on the $\alpha_{2a}$-AR at the equivalent positions Ser$^{208}$ and Ser$^{204}$ suggested a role for Ser$^{204}$ in hydrogen bonding, whereas Ser$^{208}$ appeared not to be necessary in catecholamine binding (16). In the $\alpha_{1}$-AR, the serine at position 208 (equivalent to Ser$^{204}$ in the $\beta_{2}$-AR and Ser$^{207}$ in the $\alpha_{1b}$-AR) appears not to be required for hydrogen bonding to the catechol ring of the natural ligands since the binding affinity of the Ser$^{208}$ → Ala ($\alpha_{1b}$) mutant was unaltered from that of the wild-type $\alpha_{1b}$-AR (Table I). Consistent with this hypothesis, phenylephrine has only a single hydroxyl at the metaposition and is a full agonist for both the $\alpha_{1b}$-AR and the $\alpha_{1a}$-AR. Thus the methyloxy is likely to hydrogen bond to Ser$^{211}$ ($\alpha_{1b}$), the serine equivalent to Ser$^{208}$ on the $\beta_{2}$-AR and Ser$^{204}$ on the $\alpha_{1b}$-AR. This indicates that determinants of agonist binding and intrinsic activity in the $\beta_{2}$-AR are likely not entirely conserved among the $\alpha$-adrenergic receptors and that $\alpha_{1}$-ARs display a phenotype for hydroxyl binding similar to the $\alpha_{2}$-ARs.

Effects of the Point Mutations on Antagonist Affinity—Analysis of the eight $\alpha_{1b}$-AR mutants for alterations in antagonist binding revealed no significant changes particularly for Ala$^{204}$ → Val and Leu$^{314}$ → Met, which were shown to significantly alter agonist binding affinities (Table II). This indicates that the determinants for agonist binding may be quite distinct from those for antagonist binding. Antagonists, as a result of their larger size, may extend to both the first and seventh transmembrane domain and perhaps bind below the upper half of the transmembrane domain. Our selection criteria for mutagenesis precluded the first transmembrane domain and the lower half of the binding pocket. Thus our selection criteria may have favored residues involved in agonist interactions.

Analysis of Combined $\alpha_{1b}$-AR Mutations—Combination mu-
tations were made to assess if the individual mutations were independent and/or additive in their effects on changing the binding-profiles to that of the $\alpha_{1a}$-AR. The initial combination (Ser95→Thr/Phe96→Ser, Leu182→Phe, and Ser208→Ala), involved mutations that by themselves had no major effect on ligand binding. The binding-profile of this combination was not different from that of the wild-type $\alpha_{1a}$-AR (Table III, Fig. 3).

When the two mutants that alone showed a significant change toward the $\alpha_{1a}$-AR (Leu314→Met/Ala204→Val) were combined, the ligand binding profile of this double mutant was now similar to that of the wild-type $\alpha_{1a}$-AR (Table III, Fig. 3). Interestingly, this double mutant showed affinity changes that were additive for methoxamine, oxymetazoline, and cirazoline but inhibitory for epinephrine, norepinephrine, and phenyleph-

### Table IV

Ligand-binding profiles wild-type and mutant $\alpha_{1a}$-ARs and wild-type $\alpha_{1b}$-AR

| $\alpha_{1a}$-AR | $\alpha_{1b}$-AR |
|------------------|------------------|
| Oxymetazoline    | 6.05 ± 0.11      | 6.91 ± 0.08     |
| Cirazoline       | 5.64 ± 0.04      | 6.14 ± 0.01     |
| Methoxamine      | 3.16 ± 0.06      | 3.76 ± 0.06     |
| (-)Epinephrine   | 5.29 ± 0.04      | 6.09 ± 0.03     |
| Phenylephrine    | 4.69 ± 0.06      | 6.58 ± 0.03     |
| $B_{max}$ (pmol/mg protein) | 3.00 | 1.17 | 1.66 | 1.26 | 1.01

Fig. 4. A model of the Ala204→Val and Leu314→Met ($\alpha_{1b}$) mutations showing proposed interactions in the agonist binding pocket. The graph represents the individual mutations followed by the combination of Leu314→Met and Ala204→Val. The y-axis shows Kd (log) differences from the WTh. Below each graph is a model that describes the data. Oxymetazoline is used to represent an agonist with a hydrophobic ortho group and phenylephrine, an agonist without such a group. Only transmembranes 5 (TMV) and 6 (TMVI) are shown. Only the phenyl ring and the ortho group of the ligand is represented. Arrows indicate the putative sites of interactions. A, for oxymetazoline, Leu314→Met interacts with the ortho methyl group, and Ala204→Val interacts with the phenyl ring. In combination the 314 position is pulled away by the ortho substituent from the 204 position allowing both residues to interact freely with the ligand, thus having an additive effect. B, for phenylephrine, the lack of an ortho group results in steric inhibition of the 204 position by the bulkier methionine, leading to an inhibitory effect when they are combined.
Thus, for epinephrine, norepinephrine, and phenylephrine, the additional change of the Leu314 to methionine decreased the affinity observed for these ligands with the single Ala204Val mutant. Fig. 4, a and b, represents a model that explains this phenomenon. All three agonists that showed an additive effect when Leu314Met and Ala204Val were combined have a hydrophobic group at the ortho position of the phenyl ring. Thus, the bulkier methionine residue may allow a hydrophobic interaction with this ortho substituent on the phenyl ring. As a result, the ortho group would no longer interact with the valine of the Ala204Val mutation, and the valine side chain would be free to interact with the hydrophobic phenyl ring (Fig. 4A). Since both residues interact with different components on the agonist and assist each other, their interaction is additive and cooperative. In the absence of an ortho group on the ligand, the bulkier methionine may interact with the Val204 and inhibit this valine from interacting with the phenyl ring, due to the proximity of these two residues on the fifth and sixth transmembrane domains. Therefore, the double mutant (Leu314Met and Ala204Val) of the a1b-AR revealed an a1b-AR agonist pharmacology. Shown for comparison is the a1b-AR double mutant Ala204Val/Leu314Met.

Reversing the Mutations in the a1b-AR—We performed the equivalent reverse mutations in the a1a-AR to confirm that the Leu314Met and Ala204Val (a1b-AR) was performed in the a1a-AR (Met293Leu and Val185Ala, respectively). Removal of the inhibitory effect of methionine (Met293Leu) resulted in an increased affinity for agonists that lacked an orthohydrophobic group (epinephrine, norepinephrine, and phenylephrine). This is equivalent to the Ala204Val in the a1b-AR. B, the combination of Met293Leu and Val185Ala in the a1a-AR revealed an a1b-AR agonist pharmacology. Shown for comparison is the a1b-AR double mutant Ala204Val/Leu314Met.

FIG. 5. A, the reverse mutations of Leu314Met and Ala204Val (a1b-AR) was performed in the a1a-AR (Met293Leu and Val185Ala, respectively). Removal of the inhibitory effect of methionine (Met293Leu) resulted in an increased affinity for agonists that lacked an orthohydrophobic group (epinephrine, norepinephrine, and phenylephrine). This is equivalent to the Ala204Val in the a1b-AR. B, the combination of Met293Leu and Val185Ala in the a1a-AR revealed an a1b-AR agonist pharmacology. Shown for comparison is the a1b-AR double mutant Ala204Val/Leu314Met.

FIG. 6. A, model of the a1b-AR showing the docking of oxymetazoline (oxy) in the mutated ligand binding pocket (Leu314Met and Ala204Val), which mimics that of the a1b-AR. The orthomethyl group interacts with Met314, the phenyl ring with Val204, and the metahydroxyl with Ser211. B, with phenylephrine (PH) the lack of an ortho substituent allows steric interaction of Leu314Met and Ala204Val and therefore no difference is seen in agonist binding affinity. TMI, -II, -V, -VI, and -VII are shown; TMIII and -IV have been removed to allow visualization of the binding pocket.

showed that a Val185Ala mutation (corresponding residue to the Ala204Val mutation in the a1b-AR) of the a1a-AR reversed the binding affinity of the wild-type receptor toward that of the a1b-AR, particularly for oxymetazoline and methox-
amine. Supporting our theory that a methionine (Leu\textsuperscript{314} → Met in the α\textsubscript{1b}-AR) is involved in inhibiting the interaction of valine with the phenyl ring of the agonists, Met\textsuperscript{293} → Leu (α\textsubscript{1a}-AR) relieved this inhibition, thus increasing the binding affinity for the agonists lacking ortho-substituents (epinephrine, norepinephrine, and phenylephrine) (Fig. 5A). In combination (Val\textsuperscript{185} → Ala and Met\textsuperscript{293} → Leu (α\textsubscript{1a})) they had α\textsubscript{1a}-AR pharmacology, however there were still some residual differences with methoxamine and cirazoline (Fig. 5B). This indicates that there still may be other minor conformational differences in the agonist ligand binding pocket of the α\textsubscript{1a}-AR due to other non-conserved amino acids. However, the reversal of these mutations supports our hypothesis that the Leu\textsuperscript{314} and Ala\textsuperscript{204} in the α\textsubscript{1b}-AR and their corresponding α\textsubscript{1a}-AR residues Val\textsuperscript{185} and Met\textsuperscript{293} are the most critical determinants of the differences in agonist binding between these two subtypes.

Modeling the Agonist Ligand Binding Pocket of the Two Subtypes—Previous work analyzing subtype specificity between the β\textsubscript{1} and β\textsubscript{2}-AR (17) indicated that residues in the middle portion of the β-AR sequence, particularly around transmembrane regions 4 and 5, contribute predominantly to the sub-type-specific binding of agonists. However, no single residue substitution appeared to be capable of altering the subtype specificity of the receptor. Analysis of an extensive number of chimeric β\textsubscript{1} and β\textsubscript{2} receptors functionally expressed in Escherichia coli (18) demonstrated that each of 11 selective ligands appeared to define its own ligand binding subsite. In contrast, our results indicate that at least for agonist binding, discrete amino acid interactions can account for subtype selectivity.

From our data we were able to model the subtype differences between their agonist binding pockets (Fig. 6, A and B). It appears that when an agonist is docked into the binding pocket, the presence of a hydrophobic ortho substituent on the phenyl ring will interact with the bulkier methionine residue at position 293 on the α\textsubscript{1a}-AR (Leu\textsuperscript{314} on the α\textsubscript{1b}-AR) thus increasing binding affinity significantly. Similarly the larger hydrophobic valine residue in the α\textsubscript{1b}-AR at position 185 (Ala\textsuperscript{204} in the α\textsubscript{1a}-AR) interacts directly with the phenyl ring. For agonists with an ortho hydrophobic substituent as with cirazoline, oxymetazoline, and methoxamine, the effect of these two residues is additive conferring upon the α\textsubscript{1a}-AR increased binding affinity. In the absence of the ortho group (phenylephrine, norepinephrine, and epinephrine) Met\textsuperscript{293} (α\textsubscript{1b}) sterically hinders Val\textsuperscript{185} (α\textsubscript{1a}) from interacting with the phenyl ring, thus there is no increase in affinity. These two residues in combination differentiate the agonist binding pocket of these two α\textsubscript{1}-AR subtypes. The methahydroxyl group appears to interact with the Ser\textsuperscript{211} as there was no difference in binding affinity between the wild-type α\textsubscript{1b}-AR and Ser\textsuperscript{211} (α\textsubscript{1a}) for phenylephrine, which contains only a methahydroxyl group. Thus each agonist docks into the ligand binding pocket in a tilted manner.

From our data we have defined two important residues that are critical in differentiating the ligand binding pocket between two α\textsubscript{1}-AR receptor subtypes. We are able to model the interaction between these two residues and in so doing have provided a rational basis for the design of better subtype-selective agonists to allow optimal binding and receptor activation.

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