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We examined the role that aromatic residues located in the transmembrane helices of the \( \alpha_1 \)-adrenergic receptor play in promoting antagonist binding. Since \( \alpha_1 \)-antagonists display low affinity binding at \( \beta_2 \)-adrenergic receptors, two phenylalanine residues, Phe-163 and Phe-187, of the \( \alpha_1 \)-AR were mutated to the corresponding \( \beta_2 \)-residue. Neither F163Q nor F187A mutations of the \( \alpha_1 \)-AR had any effect on the affinity of the \( \alpha_1 \)-antagonists. However, the affinity of the endogenous agonist epinephrine was reduced 12.5- and 8-fold by the F163Q and F187A mutations, respectively. An additive loss in affinity (150-fold) for epinephrine was observed at an \( \alpha_1 \)-containing both mutations. The loss of agonist affinity scenario could be reversed by a gain of affinity with mutation of the corresponding residues in the \( \beta_2 \) to the phenylalanine residues in the \( \alpha_1 \). We propose that both Phe-163 and Phe-187 are involved in independent aromatic interactions with the catechol ring of agonists. The potency but not the efficacy of epinephrine in stimulating phosphatidylinositol hydrolysis was reduced 35-fold at the F163Q/F187A \( \alpha_1 \) relative to the wild type receptor. Therefore, Phe-163 and Phe-187 represent novel binding contacts in the agonist binding pocket of the \( \alpha_1 \)-AR, but are not involved directly in receptor activation.

The adrenergic receptors (\( \alpha_1a, \alpha_1b, \alpha_1d, \alpha_2a, \alpha_2c, \beta_1, \beta_2, \) and \( \beta_3 \)) are part of a larger family of membrane proteins commonly referred to as the G protein-coupled receptor family. All of these AR subtypes bind the endogenous catecholamines, epinephrine and norepinephrine and mediate the actions of the sympathetic nervous system (1). The proposed topography adopted by these G protein-coupled receptors in the plasma membrane is modeled to that of bacteriorhodopsin, in which the protein folds to a highly ordered structure comprised of a series of seven hydrophobic transmembrane (TM) \(^1\)-spanning domains, linked by three intracellular and three extracellular loops (2). Comparison of the amino acid sequences of the cloned adrenergic receptors illustrates the greatest residue conservation is found within the transmembrane helix domains. Accordingly, the binding pocket for epinephrine and norepinephrine is localized to this region in all adrenergic receptors, within the circular array of TM helices in the rhodopsin-like core of the receptor (3). Although differences in the agonist binding interactions at different adrenergic receptor subtypes exist, in general, the catecholamine is stabilized in the binding pocket by an ionic interaction involving the protonated amine of epinephrine and an aspartic acid residue in TM3 (4), by hydrogen bonds between the catechol hydroxyl groups of the agonist and serine residues in TM5 (5), and by an aromatic/hydrophobic interaction involving the catechol ring of the agonist and a phenylalanine residue in TM6 (6). The \( \beta \)-hydroxyl of the agonist, which confers stereoselectivity, has been identified in the \( \beta_2 \)-AR as interacting with Asn-293 in TM6 (7), but is not conserved in the \( \alpha_1 \)- and \( \alpha_2 \)-ARs, highlighting the potential differences in agonist binding among family members. For the endogenous agonists, epinephrine and norepinephrine, it is thought that all the point contacts with the receptor have been identified.

Our understanding of the molecular interactions promoting antagonist binding to adrenergic receptors is limited. We have proposed that the plane of the antagonist binding pocket on these receptors lies above that of the agonist binding pocket, since we have identified that the \( \alpha_1 \)-AR-selective antagonists phenotamine and WB4101 make point contacts with three residues in the second extracellular loop of the receptor (8). However, subsequent studies conducted on a series of \( \beta_2 \)/\( \alpha_1 \)-AR chimeric receptors were unable to identify further point contacts between these antagonists and other extracellular loop residues of the \( \alpha_1 \)-AR (9). Two other studies have identified the importance of phenylalanine residues located close to the extracellular surface of the receptor in antagonist binding at adrenergic receptors. In the \( \alpha_2 \)-AR, a single phenylalanine residue (Phe-412) at the top of TM7 promotes high affinity binding of yohimbine (10) and mutagenesis of a phenylalanine residue (Phe-82) in the upper regions of TM2 in the \( \alpha_1 \)-AR accounts for the \( \alpha_1 \)-selectivity of the dihydropyridine antagonists (11). These interactions on their own cannot account for the high affinity binding of all these antagonists to adrenergic receptors. Therefore, the adrenergic receptor antagonists must make additional contact with as yet unidentified residues located within the transmembrane helices of the receptor.

Given that residues in the second extracellular loop connecting TM4 and TM5 have been implicated in binding antagonists at \( \alpha_1 \)-ARs and that \( \alpha_2 \)-AR antagonists contain high aromatic character, we evaluated the role that aromatic residues located at the top of these TM helices may have in binding adrenergic receptor antagonists. Specifically, we identified two phenylalanine residues (Phe-163, Phe-187) located in the upper regions of TM4 and TM5, respectively, of the \( \alpha_1 \)-AR that were obvious.
candidates for mutagenesis. This selection was influenced by neither residue being conserved in the \( \beta_2 \)-AR, a receptor at which these \( \alpha_1 \)-AR selective antagonists possess markedly reduced affinity (10,000-fold lower). Here we unexpectedly report that, although neither mutation altered the pharmacological phenotype with regard to antagonist binding at the \( \alpha_1 \)-AR, these mutations had significant effects on the binding of catecholamine and phenethylamine agonists at this receptor, agonists for which all receptor contacts were thought to have been identified. Since these phenylalanine residues are not conserved in \( \alpha_1 \)-AR and \( \beta \)-ARs, these results stress the differences in the agonist binding pocket of adrenergic receptors despite the fact they all bind the same endogenous ligands.

**EXPERIMENTAL PROCEDURES**

**Materials**—Drugs were obtained from the following manufacturers. Clonidine, dichloroisoproterenol,ephedrine, \( \beta \)-epinephrine, isoproterenol, metaproterenol, methoxamine, oxymetazoline, phenolamine, phenylephrine, prazosin, propranolol, and theophylline were from Sigma; \([^{125}\mathrm{I}]\)HEAT and \([^{3}H]\)mioyinositol were from NEN Life Science Products; \([^{125}\mathrm{I}]\mathrm{ICYP}\) and \([^{3}H]\)AMP were from Amersham Pharma- 

**Membrane Preparation**—Transiently transfected COS-1 cells were harvested 72 h after transfection, collected, and washed in Hanks’ balanced salt solution (HBSS), then pelleted under low speed centrifuga- 

**Site-directed Mutagenesis in the \( \alpha_1 \)-AR**—Site-directed mutagenesis of the \( \mathrm{pMT}^{2} \) rat \( \alpha_1 \)-AR plasmid was performed using polymerase chain reaction technology using commercially synthesized oligonucleotides (Life Technologies, Inc.) specifically designed to code for the desired mutation. A fragment of DNA encoding the F163Q mutation of the rat \( \alpha_1 \)-AR was generated using a sense primer containing the unique EcoRI cloning site before the start site of translation and an antisense primer targeted to the unique Nael site of the rat \( \alpha_1 \)-AR that also encoded the mutation. The F157A mutation was generated using a sense primer containing the mutation that was targeted to the endog- 

**Measurement of Affinity**—The affinity of \([^{125}\mathrm{I}]\)HEAT or \([^{125}\mathrm{I}]\)CYP for \( \alpha_1 \)-ARs was determined in a series of competition binding experi- 

**Aromatic Interactions in the Agonist Binding Pocket**

**Collection**—Rockville, MD) were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% (v/v) fetal bovine serum, 1% (v/v) penicillin and streptomycin. Cells were maintained and passages upon reaching confluence by standard cell culture techniques. Experi- 

**Cell Culture and Transfection**—COS-1 cells (American Type Culture 

**Materials**—Drugs were obtained from the following manufacturers. Clonidine, dichloroisoproterenol,ephedrine, \( \beta \)-epinephrine, isoproterenol, metaproterenol, methoxamine, oxymetazoline, phenolamine, phenylephrine, prazosin, propranolol, and theophylline were from Sigma; \([^{125}\mathrm{I}]\)HEAT and \([^{3}H]\)mioyinositol were from NEN Life Science Products; \([^{125}\mathrm{I}]\)ICYP and \([^{3}H]\)AMP were from Amersham Pharmacia Bio- 

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**Quantitation of Intracellular Cyclic 3',5'-Adenosine Monophosphate (cAMP)---**Studies to investigate the signaling capacity of the mutant β,-ARs relative to the wild type receptor were conducted in COS-1 cells transiently transfected to express the wild type or point mutated β,-ARs. Transfected conditions were tiered so that the wild type and mutant receptors were expressed at equal receptor densities. Measurements of the intracellular cAMP levels were made under serum-free conditions in 3 ml of DMEM supplemented with 20 mM HEPES and 5 mM theophylline to prevent phosphodiesterase-mediated breakdown. Agonists (either isoproterenol or ephedrine) were added directly to the medium and incubated at 37 °C for 30 min in a 5% CO2 atmosphere. Concentration-response curves for each agonist were constructed over a suitable range of concentrations performing each data point in duplicate. Incubations were terminated by the removal of the medium containing the agonist and the addition of 200 μl of a 0.1 M HCl solution. The cell lysate was scopped and transferred to microcentrifuge tubes. The residual concentration of cAMP in the lysate of each individual plate was determined by a radioimmunoassay kit, following the manufacturer's directions (Amersham Pharmacia Biotech). Radioactivity was counted using a β-counter (Beckman).

**Fig. 1.** Sequence alignment of TM4 and TM5 helices in adrenergic receptors. Sequence alignment of the TM4 and TM5 residues in adrenergic receptors. Sequences in single-letter code were aligned to maximize homologies within these domains. The residues are shown sequentially running in the extracellular to intracellular direction. The numbers represent the positions of the amino acid in the primary structure of the protein. The point mutations investigated in this study are bold. Serine residues in TM5 that have been shown previously to be important in agonist binding and function are shown in italic. Those residues proposed to constitute each of the transmembrane-spanning domains are underlined.
**Aromatic Interactions in the Agonist Binding Pocket**

The binding affinities of the altered by the combination of these mutations, indicating that the impaired binding of epinephrine at the mutated cells, overexpressed on the binding affinities of agonists at this receptor. In COS-1 1-AR mutations, the affinities of a panel of agonists (Fig. 2) was determined in additional competition binding studies. The affinity of this drug for the 1a-AR exhibiting both phenylalanine mutations did the affinity of oxymetazoline decrease significantly compared to the wild type 1a-AR (Table I). However, the affinity of epinephrine for the F163Q/F187A 1a-AR was found to be over 150-fold lower than its affinity at the wild type 1a-AR (p < 0.001). The additive loss of affinity observed upon combining both mutations in the receptor confirms that each phenylalanine residue makes an interaction with the agonist that is independent of the other residue. A similar additive decrease in affinity at the F163Q/F187A 1a-AR was observed for phenylephrine (32-fold; p < 0.01). We conclude that the effects observed on agonist binding by mutation of the Phe-163 and Phe-187 residues of the 1a-AR identify two distinct and independent interactions between the agonist and the receptor.

The influence of mutating each of these phenylalanine residues on the binding properties of imidazolines at 1a-ARs was also investigated. Imidazolines, e.g. oxymetazoline and clonidine, retain the aromatic characteristics of the phenethylamines; however, their chemical structure differs in that they possess an imidazoline ring containing the protonated amine (Fig. 2). Minimal effects on the binding affinity of oxymetazoline were observed at either the F163Q or the F187A mutation. Only in the 1a-AR exhibiting both phenylalanine mutations did the affinity of oxymetazoline decrease significantly versus wild type (8-fold; p < 0.05; Table I). With clonidine, a minimal decrease in affinity was observed with the F163Q/F187A 1a-AR, which is consistent with the effects observed on oxymetazoline binding. However, the affinity of clonidine for the F163Q/F187A 1a-AR was actually increased 5-fold by the F187A mutation (p < 0.05). Our observations indicate that these F163Q and F187A mutations have greater impact on the binding properties of catecholamines and phenethylamines than on the imidazoline class of agonists, suggesting that there are differences in the three-dimensional geometry of the agonist binding pocket recognized by these two drug classes. Indeed, previous studies have indicated that the imidazolines bind differently in the agonist binding pocket of the 1a-AR. Furthermore, we argue that the impaired binding of epinephrine at the mutated 1a-AR is not the result of changes in the global conformation of the receptor since the affinities of the agonists were not affected by either mutation. Therefore, both the Phe-163 and Phe-187 residues of the 1a-AR constitute novel and specific agonist-receptor point contacts in the binding pocket of the 1a-ARs.

**Table I**

| Agonists          | F163Q | F187A | F163Q/F187A |
|-------------------|-------|-------|-------------|
| **Crystalllographic** | pKᵢ ± S.E. (n-fold change) | pKᵢ ± S.E. (n-fold change) | pKᵢ ± S.E. (n-fold change) |
| Agonists          |       |       |             |
| Epinephrine       | 5.81 ± 0.20 | 4.71 ± 0.24** (12.5) | 4.89 ± 0.08** (8) |
| Phenylephrine     | 5.32 ± 0.18 | 4.55 ± 0.30* (6) | 4.78 ± 0.23* (3.5) |
| Methoxamine       | 4.72 ± 0.15 | 3.64 ± 0.11** (12) | 4.05 ± 0.19* (4.5) |
| Oxymetazoline     | 7.91 ± 0.21 | 8.09 ± 0.29 (1.5) | 7.69 ± 0.18 (1.65) |
| Clonidine         | 5.75 ± 0.24 | 5.45 ± 0.31 (2) | 6.30 ± 0.06* (3.5) |
| Agonists          |       |       |             |
| Prazosin          | 9.54 ± 0.24 | 9.50 ± 0.12 | 9.79 ± 0.21 |
| Phenolamine       | 8.01 ± 0.18 | 8.07 ± 0.18 | 8.11 ± 0.13 |
| 5’-Methyurapidil  | 8.94 ± 0.05 | 8.75 ± 0.24 | 9.05 ± 0.42 |
| WB4101            | 10.29 ± 0.06 | 9.96 ± 0.43 | 10.30 ± 0.04 |
| [125I]HEAT (pKᵢ) | 9.57 ± 0.19 | 9.53 ± 0.17 | 9.70 ± 0.11 |
| Expression (pmol/mg) | 2.101 | 0.35 | 0.21 |

α₁a-AR mutations, the affinities of a panel of agonists (Fig. 2) was determined in additional competition binding studies. Kᵢ values for five α₁-AR agonists are shown in Table I. Unlike the results obtained with the antagonists, mutation of either phenylalanine residue in the α₁-AR had considerable effects on the binding affinities of agonists at this receptor. In COS-1 cells, overexpressed α₁-ARs display single-site competition curves with Hill coefficients near unity. The addition of GTP analogues does not change the slope significantly and were not used in these competition studies. The affinity of the endogenous agonist epinephrine was reduced 12.5-fold by the F163Q mutation (p < 0.01) and by a factor of 8-fold by the F187A mutation (p < 0.01), suggesting that each phenylalanine residue is directly involved in stabilizing the catecholamine in the agonist binding pocket of the α₁-AR. Furthermore, we argue that the impaired binding of epinephrine at the mutated α₁-AR is not the result of changes in the global conformation of the receptor since the affinities of the antagonists were not affected by either mutation. Therefore, both the Phe-163 and Phe-187 residues of the α₁-AR constitute novel and specific agonist-receptor point contacts in the binding pocket of the α₁-ARs.

Like epinephrine, the affinities of the synthetic phenethylamines, phenylephrine and methoxamine (Fig. 2), were also reduced by each phenylalanine mutation. A 6-fold decrease in affinity for phenylephrine (p < 0.05) and a 12-fold decrease in the affinity of methoxamine (p < 0.01) were observed at α₁-ARs displaying the F163Q mutation (Table I). The F187A mutation had greater effects on the binding of the endogenous agonist epinephrine than on either of the phenethylamines. Phenylephrine and methoxamine binding was reduced by factors of just 3- and 4-fold, respectively, by the F187A mutation (p < 0.05), as opposed to the greater than 8-fold decrease in affinity for epinephrine induced by the same mutation.

Single mutagenesis of either the Phe-163 or Phe-187 residue of the α₁-AR illustrates that both amino acids provide significant contributions to the binding and stabilization of the agonist in the binding pocket of the receptor. To prove that these interactions between each phenylalanine residue and the catechol ring of the agonist are independent of one another, we constructed a mutant receptor expressing both mutations (F163Q/F187A α₁-AR). As observed with the single mutations, the binding affinity of the α₁-AR-selective antagonists was not altered by the combination of these mutations, indicating that the global conformation of the receptor remains comparable to the wild type α₁a-AR (Table I). However, the affinity of epinephrine for the F163Q/F187A α₁-AR was found to be over 150-fold lower than its affinity at the wild type α₁-AR (p < 0.001). The additive loss of affinity observed upon combining both mutations in the receptor confirms that each phenylalanine residue makes an interaction with the agonist that is independent of the other residue. A similar additive decrease in affinity at the F163Q/F187A α₁-AR was observed for phenylephrine (32-fold; p < 0.01). We conclude that the effects observed on agonist binding by mutation of the Phe-163 and Phe-187 residues of the α₁-AR identify two distinct and independent interactions between the agonist and the receptor.

The influence of mutating each of these phenylalanine residues on the binding properties of imidazolines at α₁-ARs was also investigated. Imidazolines, e.g. oxymetazoline and clonidine, retain the aromatic characteristics of the phenethylamines; however, their chemical structure differs in that they possess an imidazoline ring containing the protonated amine (Fig. 2). Minimal effects on the binding affinity of oxymetazoline were observed at either the F163Q or the F187A mutation. Only in the α₁-AR exhibiting both phenylalanine mutations did the affinity of oxymetazoline decrease significantly versus wild type (8-fold; p < 0.05; Table I). With clonidine, a minimal decrease in affinity was observed with the F163Q mutation. However, the affinity of this drug for the α₁-AR was actually increased 5-fold by the F187A mutation (p < 0.05). Our observations indicate that these F163Q and F187A mutations have greater impact on the binding properties of catecholamines and phenethylamines than on the imidazoline class of agonists, suggesting that there are differences in the three-dimensional geometry of the agonist binding pocket recognized by these two drug classes. Indeed, previous studies have indicated that the imidazolines bind differently in the α₁-AR agonist pocket (18) and confer differential desensitization of the receptor (19), suggesting that the phenethylamines and imidazolines may promote agonist trafficking by coupling to different signaling pathways.

The F163Q-, F187A-, and F163Q/F187A-mutated α₁-ARs all displayed phenotypes showing a reduced affinity for the endogenous agonist epinephrine. To confirm and validate these phenotypes, we constructed mutations of the β₂-AR that now introduced phenylalanine residues to the corresponding positions of this receptor. The aim of this mutagenesis strategy was to
demonstrate a gain of function manifested by an increased affinity of agonists at the phenylalanine-substituted β2-ARs relative to the wild type receptor. Therefore, the Q170F and A202F mutations were individually inserted into the coding sequence of the β2-AR by cassette oligonucleotide replacement and the mutant receptors expressed in COS-1 cells to study their agonist binding properties.

Incorporation of either the Q170F or the A202F mutation to the β2-AR did not appear to affect the global conformation of the receptor since the affinities of several antagonists were not found to be different from the observed wild type values (Table II). However, as we predicted, both mutations did alter the binding properties of agonists to the β2-AR. Small but statistically significant increases in the affinity of both epinephrine and isoproterenol were observed at both the Q170F or A202F mutations relative to their affinity at the WT β2-AR (Table II). The affinity of isoproterenol was increased by 1.6-fold (p < 0.05) and by 2-fold (p < 0.05) at the Q170F and A202F mutations, respectively. Likewise, the affinity of epinephrine was increased 2.4-fold at the Q170F mutation (p < 0.05) and by 1.65-fold at the A202F mutation. In order to validate these small increases in affinity at the individual mutations, a β2-AR that coded for both phenylalanine mutations was constructed. Competition binding studies at this Q170F/A202F receptor revealed a larger 4.2-fold increase in affinity for both isoproterenol (p < 0.05) and epinephrine (p < 0.05) (Table III). The observed additivity of the response at the double mutation validates the increased affinities of these agonists at each phenylalanine residue. Therefore, incorporation of the phenylalanine residues to the fourth and fifth transmembrane helices of the β2-AR results in small gains in the affinity of agonists.

The ternary complex model of agonist interactions at these receptors predicts that agonists bind to the G protein-coupled and uncoupled forms of the receptor with different affinity (20). The small increases in agonist affinity observed at the Q170F- and A202F-β2-ARs may therefore be due to an enhanced coupling of the mutant receptors to a G protein. To address this concern, the affinities of these agonists at both of the single mutation constructs (Table II) and the double mutation construct (Table III) of the β2-AR were determined in the presence of GTPγS, a stable GTP analog that “locks” the receptor in the uncoupled state. Under these experimental conditions, both epinephrine and isoproterenol displayed affinity increases at the single and double mutations that were of similar magnitude to those observed in the absence of GTPγS. The results of these binding experiments were confirmed by investigating the basal signaling properties of these mutant receptors. Measurement of the cAMP signal transduction pathway in COS-1 cells transiently transfected to express either the wild type or a mutant β2-AR revealed that the basal cAMP stimulation associated with either the Q170F or A202F β2-AR was not different from the wild type receptor (data not shown). We conclude that neither of these mutations to the β2-AR induces conformational
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TABLE II

Agonist and antagonist binding affinities at single mutations of the β2-AR

|                       | - GTPyS          | + GTPyS (100 μM) |
|-----------------------|------------------|------------------|
|                       | pK<sub>B</sub> ± S.E. (-fold change) | pK<sub>B</sub> ± S.E. (-fold change) |
| Agonists              |                  |                  |
| Isoproterenol         | 7.01 ± 0.03      | 6.99 ± 0.05      |
| Epinephrine           | 6.38 ± 0.06      | 6.42 ± 0.03      |
|                        |                  |                  |
| Antagonists            |                  |                  |
| Propranolol           | 9.60 ± 0.07      | ND               |
| ICI 118,551           | 9.45 ± 0.11      | ND               |
| [125I]CYP             | 10.43 ± 0.13     | ND               |
| Expression (pmol/mg)  | 1.65             | 1.89             |

TABLE III

Affinities of agonist and antagonist binding at the double mutation of the β2-AR

|                       | - GTPyS          | + GTPyS (100 μM) |
|-----------------------|------------------|------------------|
|                       | pK<sub>B</sub> ± S.E. (-fold change) | pK<sub>B</sub> ± S.E. (-fold change) |
| Agonists              |                  |                  |
| Isoproterenol         | 6.30 ± 0.16      | 6.57 ± 0.02      |
| Epinephrine           | 6.45 ± 0.04      | 6.53 ± 0.04      |
| Dichloroisoproterenol | 6.25 ± 0.04      | 6.26 ± 0.04      |
| Ephemidine            | 4.89 ± 0.03      | 4.97 ± 0.08      |
| Metaproterenol        | 5.25 ± 0.07      | 4.96 ± 0.07      |
| Albuterol             | ND               | ND               |
| Nylidrin              | ND               | ND               |
|                        |                  |                  |
| Antagonists            |                  |                  |
| Propranolol           | 9.47 ± 0.04      | ND               |
| ICI 118,551           | 9.47 ± 0.04      | ND               |
| [125I]CYP             | 10.15 ± 0.10     | ND               |
| Expression (pmol/mg)  | 1.87             | 0.302            |

Changes that render the receptor to be constitutively active. Rather, these binding and signaling studies indicate that the observed increases in agonist affinity at the phenylalanine-substituted β2-ARs are independent of G protein coupling effects and that the increases in affinity are due directly to interactions between the agonist and the phenylalanine residues.

Phenylalanine residues in TM6 of biogenic amine receptors have been shown to be important for agonist binding and activation in the β2-AR (6), the dopamine D<sub>2</sub> receptor (21), serotonin receptors (22), and recently in the α<sub>1D</sub>-AR (16). We have now demonstrated that two additional and novel phenylalanine residues found in the TM4 and TM5 helices are also involved in agonist binding at the α<sub>1D</sub>-AR receptor. The α<sub>1D</sub>-AR and α<sub>1A</sub>-AR subtypes also conserve TM5 aromaticity but conserve TM4 hydrophobicity. Therefore, these agonist point contacts may be conserved in the other α<sub>1</sub>-AR subtypes, but with subtle differences in the characteristics of the interaction.

In addition, these two aromatic residues are also conserved in the dopamine D<sub>2</sub> receptor, while only the TM5 phenylalanine residue is conserved in the serotonin receptor. Based on the other known ionic and hydrogen bond interactions between the agonist and receptor, we know that the catechol ring group of the agonist is projected toward the closed end of the receptor that is formed by the TM4, TM5, and TM6 helices, the same helices upon which the important phenylalanine residues that we and others (16) have identified. Consequently, these phenylalanine residues may assist in binding epinephrine at the α<sub>1D</sub>-AR as a result of stacking interactions formed between the respective π-electron clouds of the agonists catechol ring and the aromatic side chain of the phenylalanine residue. Alternatively, the phenylalanine residues may interact with the catechol ring group of the agonist via edge to face aromatic interactions. These aromatic interactions occur as a result of dipole-dipole interactions that develop due to an unequal local electron distribution within the respective aromatic rings (23). The strength of these interactions is dependent upon the strength of the dipole moments in the aromatic rings, the distance between the two aromatic rings (1/β<sup>2</sup>), and the angle of orientation between the rings.

To determine whether these TM4 and TM5 phenylalanine residues of the α<sub>1A</sub>-AR interact with the catechol ring of the agonist via aromatic interactions, a further series of radioligand binding experiments were conducted. In these experiments, a series of agonists with a variety of electron donating (i.e., CH<sub>3</sub>, OCH<sub>3</sub>, OH ranked in order of increasing electron-
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donating strength) and/or electron withdrawing (i.e., Cl) substituents in the catechol ring of the agonist were used. Since the strength of an aromatic interaction is dependent upon the magnitude of the charge separation in the ring, we hypothesized that these substituents would influence the dipole strength sufficiently such that the magnitude of the affinity increase or decrease would be altered accordingly with electron-donating groups increasing aromaticity and electron-withdrawing groups decreasing aromaticity. The affinities of albuterol, metaproterenol, dichloroisoproterenol, nylidrin, and ephedrine at wild type and Q170F/A202F are listed in Table III. As shown in Fig. 2, dichloroisoproterenol is a chemical analog of the agonist isoproterenol that differs only in respect that both catechol hydroxyl groups on the ring are substituted with chlorine groups. The strong electron-negative character of the chlorine substituents when compared with ring hydroxyl groups, acts to draw the electron density out from the center of the catechol ring to the extremities, weakening the strength of the charge separation in the ring. Consistent with our hypothesis, the affinity of dichloroisoproterenol was only marginally increased by a factor of 1.4-fold while the affinity of isoproterenol was increased by 4.2-fold (p < 0.05) at the double mutant receptor (Table III). Albuterol, metaproterenol, and nylidrin, which all have intermediate electron-donating properties as compared with isoproterenol, displayed intermediate increases in affinity ranging from 2- to 3-fold. Ephedrine was the only drug tested that displayed a greater gain in affinity (6-fold; p < 0.01) than that of isoproter enol at the Q170F/A202F $\beta_2$-AR. Relative to isoproterenol, the structure of ephedrine has no substituents in the ortho, meta, or para positions of its aromatic ring. As a consequence, the absence of anchoring substituents in the aromatic ring of ephedrine may permit greater rotational freedom that optimizes its interaction with the phenylalanine residues causing ephedrine to bind differently in the pocket as compared with epinephrine. Based on the binding profiles of dichloroisoproterenol (1.4-fold increase) and isoproterenol (4.2-fold increase) at the Q170F/A202F $\beta_2$-AR being proportional to the magnitude of the theoretical dipole moment in the catechol ring, we conclude that the gain in affinity displayed for agonists at this receptor is provided by aromatic interactions between the catechol ring of the drug and the phenylalanine residues substituted into TM4 and TM5 of the $\beta_2$-AR. Analysis of aromaticity in the $\alpha_1$-agonists is also consistent with the results in the $\beta_2$-AR, where a rank order of the loss of affinity (methoxamine < phenylephrine < epinephrine) correlates to the increasing aromatic character of the catechol ring (OCH$_3$ < OH) (Table I).

Based on the changes in drug affinity at the F163Q/F187A $\alpha_1a$-AR, we can calculate that the two aromatic interactions in the $\alpha_1a$-AR contribute a total free energy equivalent of 2.95 kcal/mol toward the binding of epinephrine. Since the theoretical bond energy of a single aromatic-aromatic interaction lies in the range of 1.5–2 kcal/mol (23), our observed change in free energy at the double mutant is consistent with each phenylalanine residue participating in an independent aromatic-aromatic interaction with the catechol ring of the agonist. Since neither of these phenylalanine residues are conserved in the TM4 and TM5 helices of the $\beta_2$-AR, we expected to observe increases of similar magnitude in the affinity of epinephrine affinity for the Q170F/A202F $\beta_2$-AR as a result of the receptors potential to bind the agonist by two novel aromatic interactions. Contrary to our expectations, the observed change in free energy resulting from epinephrine binding at the Q170F/A202F $\beta_2$-AR was calculated to be only 0.75 kcal/mol. In theory, the greatest energy contribution between two aromatic rings occurs when the rings are aligned such that the $\delta^+$ edge of one ring is projected toward the $\delta^-$ face of the other ring. As the angle of orientation moves away from this perpendicular edge-to-face alignment, the force of the interaction decreases exponentially. In a previous study, we have reported that the catechol ring of epinephrine may be aligned differently in the agonist binding pockets of the $\alpha_1a$-AR and the $\beta_2$-AR, a consequence of the unique hydrogen bond interactions between the meta and para catechol hydroxyl groups of the agonist and the serine side chains and the number of amino acids separating these serine residues in TM5 of these receptors (15). As a result, the relative orientation of the catechol ring in epinephrine between these receptors is altered by an angle of 120°. The binding data in this study suggest that the alignment of epinephrine at the $\alpha_1a$-AR is oriented so that the interaction of its catechol ring with the TM4 and TM5 phenylalanine residues is favorable; thus, the proposed planar orientation of epinephrine results in an edge to face orientation of the phenylalanines to the catechol ring. Previous structural studies on the dopamine D$_3$ receptor (24) and density maps in the rhodopsin receptor (2) suggest that this region of TM5 exists as a loop and not a true
α-helix, therefore permitting Phe-187, Ser-188, and Ser-192 to remain accessible in the binding crevice of the receptor in order that contact with the agonist is maintained. Conversely, the angle of orientation of the catechol ring of epinephrine in the binding pocket of the β2-AR is such that it minimizes the aromatic interactions with the Q170F or A202F mutations in this receptor, consistent with the proposed skewed orientation of epinephrine in the pocket and the resulting and less favorable edge to edge orientation of the phenylalanines to the catechol ring. Therefore, this study suggests that the unique nature of the serine interactions in the α1a-AR is also important in terms of the receptor’s ability to enhance agonist binding by promoting aromatic-aromatic interactions.

Sequence alignment of the TM4 and TM5 helices reveals that the two phenylalanine residues in TM4 and TM5 involved in agonist binding at the α1a-AR receptor are also conserved in the dopamine D2 receptor but only the TM5 phenylalanine residue is conserved in the serotonin receptor and the α1b-AR and α1d-AR subtypes. In the two additional α1-AR subtypes, the corresponding TM4 residue is a leucine, suggesting that agonist binding to this site on TM4 is promoted by hydrophobicity rather than aromaticity in these subtypes. Our observations comparing α1a-AR and β2-AR agonist binding interactions and the potential for hydrophobic interactions at TM4 at the α1a-AR and α1d-AR subtypes emphasizes the diversity of interactions in the agonist binding pocket among the adrenergic receptor subtypes, even though they all bind the endogenous agonist with similar affinities.

The conserved phenylalanine residue in TM6 is involved in both binding and the efficacy of signaling in biogenic amine receptors (6, 16, 21, 22). Therefore, the importance of the Phe-163 and Phe-187 residues in facilitating agonist-induced receptor activation in the α1a-AR was studied by determining the ability of the mutant receptor to stimulate phosphatidylinositol hydrolysis in response to challenge with epinephrine. Experiments were conducted in COS-1 cells that were transiently transfected to express either the wild type α1a-AR or the F163Q/F187A α1a-AR at equal receptor densities. As shown in Fig. 3, the concentration-response curve for epinephrine in stimulating IP at the F163Q/F187A α1a-AR (EC50 = 4.89 ± 1.1 μM) is shifted dramatically to the right of the wild type receptor response (EC50 = 0.13 ± 0.08 μM). This shift represents a 36-fold decrease in the potency of epinephrine in stimulating IP at the F163Q/F187A α1a-AR (p < 0.01), consistent with its change in affinity. The efficacy (maximal response) of the epinephrine was not altered with respect to the wild type receptor by either of the F163Q and F187A mutations of the α1a-AR. In the β2-AR, the equivalent paradigm was held. Ephedrine, which demonstrated the greatest increase in affinity (6-fold) at the double mutant, Q170F/A202F receptor, also produced a 4-fold leftward shift in potency (EC50 = 2.4 ± 1.1 μM) when compared with the WT receptor (EC50 = 8.8 ± 1.4 μM) with no change in the maximal response (Fig. 4). We conclude that the decreased potency of epinephrine at the F163Q/F187A α1a-AR results from the reduced occupancy of the receptor that is attributable to the phenylalanine residues acting as important binding contacts.

In conclusion, we have identified two novel aromatic-aromatic interactions that promote the high affinity binding of epinephrine and the phenethylamines to the α1a-AR. These interactions involve two phenylalanine residues located in the upper regions of TM helix 4 and TM helix 5 of the α1a-AR, respectively. Each phenylalanine residue interacts independently with the catechol ring of the agonist. The free energy change in binding affinities resulting from the mutagenesis of these residues is consistent with the reported energetic contributions provided by aromatic-aromatic interactions in proteins. Neither Phe-163 nor Phe-187 appear to be directly involved in promoting agonist induced stimulation of phosphatidylinositol hydrolysis. Rather, the results indicate the importance of aromaticity to agonist binding and may have important consequences for future therapeutic drug design of selective α1-AR agonists. Differences in binding as observed upon incorporation of the corresponding aromatic residues in the β2-AR stress the differences in the agonist binding pocket between the members of the adrenergic receptor family, even though they all bind the endogenous agonists with similar affinity.

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