Pharmacophore mapping of adrenergic receptors indicates that the phenyl ring of catecholamine agonists is involved in receptor binding and activation. Here we evaluated Phe$^{310}$, Phe$^{303}$, and Phe$^{303}$ in transmembrane VI (TMVI), as well as Tyr$^{348}$ in TMVII of the $\alpha_{1B}$-adrenergic receptor ($\alpha_{1B}$-AR), which have been implicated in a catechol-ring interaction. Neither catecholamine docking studies nor mutagenesis studies of Phe$^{310}$, Phe$^{303}$, or Tyr$^{348}$ supported a role for these residues in catechol-ring binding. By contrast, docking studies indicated that the Phe$^{310}$ side chain is well positioned to interact with the catechol-ring, and substituted cysteine accessibility method studies revealed that the side chain of the 310, but not 311 residue, is both solvent accessible and directed into the agonist-binding pocket. Also, saturation mutagenesis of both Phe$^{310}$ and Phe$^{303}$ revealed for the former, but not for the latter, a direct relationship between side chain volume and agonist affinity, and that aromaticity is essential for wild-type agonist binding, and for both wild-type agonist potency and efficacy. Moreover, studies of Phe$^{310}$ mutants combined with a previously described constitutively active $\alpha_{1B}$-AR mutant, A293E, indicated that although not required for spontaneous receptor isomerization from the basal state, R, to a partially activated conformation $R'$, interaction of Phe$^{310}$ with catecholamine agonists is essential for isomerization from $R'$ to the fully activated state, $R^*$. 

$\alpha_1$-Adrenergic receptors ($\alpha_1$-AR)\textsuperscript{3} are members of the heptahelical superfamily that share a common structural motif of seven putative $\alpha$-helical transmembrane spanning regions linked by three extra- and three intracellular loops, an extracellular N terminus and intracellular C-terminal tail. Transmembrane signaling by all $\alpha_1$-AR subtypes ($\alpha_{1A}$, $\alpha_{1B}$, and $\alpha_{1D}$) in response to the natural catecholamine agonists, norepinephrine and epinephrine, is mediated by G-proteins of the $G_\alpha$ family or in some instances, the $G_\beta$ family of tissue transglutaminases (1, 2). Based on certain key structural features, $\alpha_1$-ARs are more closely related to rhodopsin or the type A subfamily of GPCRs that includes $\beta$-ARs, than to the calcitonin (type B) or metabotropic (type C) subfamilies.

Binding of catecholamines by both $\alpha_1$- and $\beta$-ARs involves the formation of a salt bridge between the basic aliphatic nitrogen atom common to all sympathomimetic amines and an aspartate (Asp$^{125}$ in the hamster $\alpha_{1B}$-AR; Asp$^{113}$ in the hamster $\beta_2$-AR) in the third transmembrane segment (TMIII) (3, 4). With rhodopsin, light induced isomerization of the retinal chromophore leads to deprotonation of a Schiff base linking the chromophore to Lys$^{296}$ in TMVII (5). In the ground state the protonated Schiff base is ionically bonded to a TMIII acidic residue (Glu$^{113}$) that is equivalent to Asp$^{125}$ and Asp$^{113}$ in the $\alpha_{1B}$ and $\beta_2$-ARs, respectively (5). With the $\alpha_{1B}$-AR there is evidence that receptor activation also is due to disruption of an ionic interaction between the TMIII aspartate and a TMVII lysine (Lys$^{327}$), due to competition between the catechol protonated amine and the TMVII lysine, for binding to the TMIII aspartate (3). The TMIII aspartate thus serves as a counterion and most likely is an important residue for agonist binding and activation of all adrenergic receptors.

For the $\beta_2$-AR, two serine residues, Ser$^{204}$ and Ser$^{207}$, which are conserved in most adrenergic receptors, have been proposed to hydrogen bond with the meta- and para-hydroxyls, respectively, of the catechol ring (6). Mutation of either serine to an alanine results in a 30-fold decrease in affinity for catecholamine agonists, and each serine contributes about 50% to receptor activation. Thus, binding of both catechol hydroxyls is required for full agonist activity. By contrast, agonist binding to the $\alpha_{1A}$-AR involves an interaction between the meta-hydroxyl and Ser$^{186}$ (equivalent to Ser$^{203}$, not Ser$^{203}$ in the $\beta_2$-AR) that plays a major role in receptor activation, being responsible for 70–90% of the wild-type response. An interaction between the para-hydroxyl and Ser$^{192}$ (equivalent to Ser$^{207}$ in the $\beta_2$-AR) on the other hand, contributes minimally to receptor activation (7). Moreover, since the interacting serines in the $\alpha_{1A}$-AR are separated by four residues, whereas those in the $\beta_2$-AR are separated by only three residues, docking of the catecholamine ring is in a more planar orientation in the $\alpha_{1A}$-AR, and is rotated by about 120° to that in the $\beta_2$-AR.

This altered catechol ring orientation may also contribute to other agonist docking differences between $\alpha_1$- and $\beta$-ARs. For example, stereoselectivity of binding and activation has been attributed, in part, to a hydrogen bond interaction between the chiral benzylic hydroxyl group attached to the $\beta$-carbon atom of catecholamines, and Asn$^{294}$ in TMVI of the $\beta_2$-AR (8). Although stereoselectivity of catecholamine binding and activation is preserved with $\alpha_1$-ARs, the determinants of stereoselectivity
interaction with the phenyl ring involves Phe310 in TMVI and, in hamster α1B-AR, are shaded and in bold type, while Phe290, which is highly conserved among all G protein-coupled receptors is boxed. The dashed line at the top delineates the transmembrane residues of helix VI.

have not been defined, and the Asn290 equivalent is replaced by a residue (leucine or methionine) lacking hydrogen-bonding potential. This finding again provides evidence that some of the catecholamine-binding and activation residues in α1-A-Rs are distinct from those in β-ARs.

Previous studies of the hamster β2-AR suggested that a phenylalanine in TMVI (Phe290, equivalent to Phe311 in the α1B-AR, see Fig. 1), which is conserved only in biogenic amine-binding GPCRs, is involved in forming an aromatic-aromatic interaction with the phenyl ring of catecholamines (9). This conclusion was based on the finding of a 10-fold decrease in agonist, but not antagonist, binding with mutation of Phe290 to methionine. However, no additional studies were performed to exclude a nonspecific global or local change in receptor structure with this Phe290 mutation, or to evaluate the role of the potential aromatic-aromatic interaction in receptor activation.

In addition, substitution of an adjacent TMVI phenylalanine (Phe289; equivalent to Phe310 in the α1B-AR) to alanine, resulted in a 1000-fold decrease in agonist affinity with no change in antagonist binding. Finally, Tyr256 in TMVI was also suggested to potentially be involved in a catechol ring interaction, since substitution of this residue with leucine decreased agonist, but not antagonist binding by 10-fold (9).

Here we show, based on macromolecular modeling studies, in which the planar orientation of the phenyl ring in α1-A-Rs was taken into consideration when docking catecholamines, that interaction with the phenyl ring involves Phe311 in TMVI and not Phe291 in TMVI, or Tyr254 in TMVII (equivalent to Tyr256 in the β2-AR). Furthermore, based on mutagenesis studies coupled with the evaluation of group-specific catecholamine analogs, as well as SCAM (substituted cysteine accessibility method) studies, we provide evidence that Phe310 is critically involved both in forming an aromatic-aromatic interaction with the catecholamine phenyl ring and in receptor activation.

**EXPERIMENTAL PROCEDURES**

**Materials**—(−)-Epinephrine, (−)-norepinephrine, (−)-symphephrine, (−)-halostachine, dopamine, phenethylamine, prazosin, phenolamine, hydrochloride, lithium chloride, and dl-propranolol were purchased from Sigma. 5-Methylurapidil was from Research Biochemicals International.

**Cell Culture and Transfection**—COS-1 cells (American Type Culture Collection, Manassas, VA) were cultured and transiently transfected with the indicated constructs using the DEAE-dextran method, as described previously (2, 12, 13). The transfection efficiency was 30–40%, as determined by in situ staining of cells transfected with pSV- LacZ, a plasmid encoding the reporter, β-galactosidase, and treatment of the cells with 0.2% 5-bromo-4-chloro-3-indoyl-β-D-galactoside. Cells were harvested 72 h post-transfection.

**Membrane Preparation**—Membranes were prepared from transfected COS-1 cells, as described previously (2, 10). The membranes were resuspended in HEM buffer (20 mM HEPES, pH 7.5, 1.5 mM EGTA, 12.5 mM MgCl2) containing 10% (v/v) glycerol, and stored at 70 °C. Protein concentration was determined by the Bradford method (14).

**Western Blotting**—Membranes (50 μg of protein) were dissolved in 1% CHAPS and SDS sample buffer overnight at 4 °C, and then subjected to SDS-polyacrylamide gel electrophoresis, as described previously (2, 12). The resolved proteins were electroblotted onto Immobilon-P membranes and then immunostained for detection using the ECL chemiluminescence system (Amersham), as described previously (2). In some experiments (2), α1B-AR was detected using a monoclonal antibody against the 1D4 epitope at the C-terminal of α1B-AR.

**Site-directed Mutagenesis**—The construct used was the hamster α1B-AR cDNA with an octapeptide tag (ID4) at the end of coding region in the modified eukaryotic expressing vector, pMT2 (10). The presence of the ID4 epitope at the C-terminal of α1B-AR does not affect its expression and function (data not shown). Site-directed mutagenesis was performed as described previously (2, 11). Briefly, two primers, one carrying the nucleotide change(s) to produce the desired amino acid mutation in the α1B-AR sequence, the other carrying the nucleotide changes to convert a unique restriction site (ClaI) to another restriction site (NarI) in a non-essential region of the vector, pMT2, were simultaneously annealed to the denatured template and a new competitor DNA containing both primers was synthesized by treatment with T4 polynucleotide kinase. The DNA was then digested with ClaI to linearize annealed parental plasmid and the reaction mixture used to transform Escherichia coli cells (BMH 71–18 mutS cells). Transformants were grown en masse in liquid media and used to isolate plasmid DNA. The resulting DNA was digested with ClaI again to lineerize remaining parental plasmid and the reaction mixture used to transform

**Ligand Binding**—The ligand binding characteristics of the membrane expressed receptors were determined in a series of radioligand binding studies performed exactly as described previously (2, 10), using [125I]HEAT, an α1-specific antagonist, as the radioligand. For saturation binding studies, the concentrations of [125I]HEAT used ranged from 10-fold below to 10-fold above the Ki value, whereas for competition studies, a concentration near the Ka of the radioligand was used. Ki values were determined using the Cheng-Prusoff equation (15). The membrane concentration used in these studies was selected to allow binding of less than 10% of the total radioligand added. To avoid interassay differences replicate studies with the wild-type α1B-AR were performed with the analysis of each mutant. Binding data were analyzed using a linear, curve-fitting program, Prism. For comparison of the fit to a one-site or two-site model, the F test was used; p < 0.05 was considered to be statistically significant.

**Reaction with MTSEA and Binding Assays in Intact Cells**—Transfected COS-1 cells were harvested by trypsinization. After washing with phosphate-buffered saline, cells were resuspended in 1.4 ml of HEPES buffer (140 mM NaCl, 5.4 mM KCl, 1 mM EDTA, 0.006% bovine serum albumin, 25 mM HEPES, pH 7.4) as described previously (16). Aliquots were prepared from transfected COS-1 cells, as described previously (2, 10). The membranes were resuspended in HEM buffer (20 mM HEPES, pH 7.5, 1.5 mM EGTA, 12.5 mM MgCl2) containing 10% (v/v) glycerol, and stored at 70 °C. Protein concentration was determined by the Bradford method (14).

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(80 μl) of the cell suspension were incubated with 20 μl of freshly prepared MTSEA at the stated concentrations at room temperature for 2 min. Cell suspensions were then diluted 100-fold, and 100-μl aliquots were used to assay for [125I]HEAT (600 pm) binding in the presence or absence of 0.1 mM phentolamine in a total volume of 250 μl in triplicate. The result was analyzed as described above.

Construction of an α1B-AR Molecular Model and Catecholamine Docking—The coordinates of the α-carbon positions were determined by overlay of putative α1-AR transmembrane residues with the transmembrane coordinates of bacteriorhodopsin (17), with data files generated using the Insight II molecular modeling software from Biosym Technologies. The boundaries of the putative transmembrane domains were determined using an algorithm based on the weighted pairwise comparisons of adjacent residues (18). The positioning of each helix with respect to the adjacent helices was based where possible on data from α1B-AR mutagenesis studies (10, 18, 19). The projections of the helices proposed by Baldwin (21) were used to determine the tilt of each helix. The model was minimized and conflicts adjusted to remove steric clashes based on dynamic runs, as described previously (22).

RESULTS AND DISCUSSION

A model of the α1B-AR developed previously (22) was refined to accommodate the findings of recent mutagenesis studies and used to dock the agonist, (−)-epinephrine, into the binding pocket. In particular the refined model takes into account an interhelical stacking interaction we have identified between Alα204 in TMV and Leu314 in TMVI (10, 20), as well as the planar orientation of the catechol ring. As shown in Fig. 2A, from this model it is evident that the TMVI Phe310 side chain is well positioned to interact with the catechol ring in a parallel stacked and displaced conformation, which is an energetically favored structure for benzene dimers (23). The TMVI Phe311 side chain, on the other hand, is directed toward TMV, or is projecting into the lipid bilayer, and Tyr348 is located toward the intracellular end of TMVII, well below the plane of the catecholamine ligand.

To more directly evaluate the involvement of Phe310 in catechol ring binding, it was mutated to alanine or leucine, and the resulting mutants (F310A and F310L), as well as the wild-type α1B-AR, were then evaluated in terms of membrane expression, ligand binding, and stimulation of PI hydrolysis. As controls for the potential Phe310-catechol ring interaction, alani ne and leucine mutants of two other TMVI phenylalanines, Phe311 and Phe303, and a leucine mutant of Tyr348 in TMVII, were also constructed and similarly evaluated (Fig. 2B). Saturation binding and immunoblotting studies indicated that the F303A, F303L, and Y348L mutants were expressed in the plasma membrane and processed (glycosylation) at levels almost equal to the wild-type α1B-AR (Table I and Fig. 3). All three mutants showed only small decreases (1.7–2.2-fold) in affinity for the antagonist radioligand, [125I]HEAT (Table I) and no change (Y348L) or an increase (5–20-fold, F303A and F303L) in affinity for the agonists, norepinephrine, epinephrine, and phenylephrine (Fig. 4). As will be reported in a subsequent paper, the increased agonist affinities observed with the Phe303 mutants are due to the fact that these substitutions result in constitutive receptor activation. Taken together, therefore, these findings do not support involvement of Phe303 or Tyr348 in an interaction with the catechol ring.

As shown in Table II, the Phe310 mutants showed binding affinities for the antagonists, phentolamine, prazosin, and 5-methy lurapidil that were 1.4–44-fold less than observed with the wild-type receptor. However, their affinity for the radioligand [125I]HEAT was largely unchanged (Table I) and their plasma membrane expression was equivalent to that of wild-type receptor (Table I and Fig. 3).

The Phe311 mutants also displayed decreased antagonist binding (5–94-fold, Table II). However, in contrast to the Phe310 mutants, plasma membrane expression was markedly impaired with both F311A and F311L, and could not be enhanced by increasing the amounts of plasmid transfected (Table I and Fig. 3). These findings suggest that whereas substitution of Phe310 with alanine or leucine results in the loss of receptor interactions involved in the binding of some but not all antagonists, mutation of Phe311 produces a global change in receptor conformation that impairs not only ligand binding, but also protein folding and membrane expression.

To further characterize the effects of alanine or leucine substitution at the Phe310 and Phe311 positions, the binding of group-specific agonists was evaluated. The agonists evaluated included the natural catecholamines, (−)-norepinephrine, and (−)-epinephrine, as well as (−)-phenylephrine, which is also a full agonist but lacks the para-hydroxy at the 4-position of the catechol ring, and a group of partial agonists: (−)-sympathomimetic 

[1] S. Chen, M. Xu, F. Lin, and R. M. Graham, unpublished data.

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As shown in Table III, the free energy change (∆ΔG) observed for the full agonists, with substitution of Phe310 with alanine, was close to 4 kcal/mol, but only about 2 kcal/mol for the partial agonists. For the leucine substitution the free energy change in agonist binding was approximately 2 kcal/mol for the full agonists, and <1 kcal/mol for the partial agonists. Given that the theoretical bond energy of an aromatic-aromatic interaction is approximately 2 kcal/mol (23), one interpretation of these findings is that the leucine substitution, because of the hydrophobic character of its side chain, but not the alanine substitution, which lacks both hydrophobicity and aromaticity, partially compensates for the wild-type phenylalanine. The greater free energy loss observed for full agonists with the alanine substitution suggests that the aromatic component of the Phe310 interaction with the catechol ring is not only essential for ligand binding, but for critical positioning of the catechol ring to allow optimal interaction of other ligand moieties with the receptor, such as the catecholamine meta-hydroxyl and Ser207. With partial agonists, which lack at least one of the critical moieties of full agonists and, thus, most likely have a less constrained binding geometry, positioning of the catechol ring by an aromatic interaction with the Phe310 side chain may be less critical. As a result, the free energy losses for partial agonists are less than those for full agonists, and almost exactly those anticipated (2 kcal/mol) for the loss of a single aromatic-aromatic interaction.

Like the Phe310 mutants, the Phe311 mutants also displayed decreased affinity for both full and partial agonists (Fig. 4). However, unlike the Phe310 mutants, the loss of agonist affinity with leucine substitution of Phe311 was greater than with ala-
FIG. 2. Model and secondary structure of the hamster α1b-AR. A, a model of the α1b-AR showing epinephrine docked into the ligand-binding pocket. The receptor is modeled as it would appear looking down onto the membrane from the extracellular side. White circles with Roman numerals indicate the respective transmembrane helices. The projection of the helices from the extracellular surface of the membrane is indicated by the yellow cylinders. Epinephrine is shown interacting via its protonated amine with Asp125 in TMIII, via the catechol ring meta-hydroxyl with Ser207 in TMV, and via the catechol ring with Phe310 in TMVI. Previously identified interactions between Asp125 in TMIII and Lys313 in TMVII (3), and Ala204 in TMV and Leu314 in TMVI (10, 19), as well as the orientation of Phe311 are shown. B, secondary structure of the hamster α1b-AR indicating the location of the native cysteine residues, including the putative, solvent inaccessible disulfide-linked extracellular pair, Cys119 and Cys195 (38), and the residues (Phe303, Phe312, Phe311, and Tyr348) evaluated in this study.
Phe\textsuperscript{310} in the α\textsubscript{1B}-AR and Catecholamine Bonding

Table I

|                 | WT | F310A | F310L | F311A | F311L | F303A | F303L | Y348L |
|----------------|-----|-------|-------|-------|-------|-------|-------|-------|
| Amount of DNA transfected (μg/100-mm dish) | 2   | 2     | 2     | 25    | 20    | 2     | 2     | 2     |
| Receptor density \(B_{max}\) (pmol/mg)   | 8.12 ± 0.77 | 8.21 ± 1.53 | 8.09 ± 1.66 | 0.95 ± 0.05\textsuperscript{a} | 1.70 ± 0.34\textsuperscript{b} | 5.88 ± 0.72 | 4.80 ± 0.18\textsuperscript{c} | 6.18 ± 0.51 |
| \(K_d\) (μM) | 66.5 ± 9.4 | 66.2 ± 15.2 | 84.3 ± 30 | 454 ± 10\textsuperscript{a} | 60.2 ± 17 | 121 ± 32 | 116 ± 36 | 145 ± 26\textsuperscript{b} |

\(\text{a} p < 0.001\) indicates significant differences versus the wild-type α\textsubscript{1B}-AR.

\(\text{b} p < 0.01\) indicates significant differences versus the wild-type α\textsubscript{1B}-AR.

To further evaluate the postulate that Phe\textsuperscript{310} forms a critical aromatic-aromatic interaction with the catechol ring, whereas Phe\textsuperscript{311} is not directly involved in ligand binding but rather in global receptor structure, additional Phe\textsuperscript{310} and Phe\textsuperscript{311} mutants were constructed and their agonist-binding properties evaluated. As seen in Fig. 5, the Phe\textsuperscript{310} mutants produced graded decreases in agonist affinity. Whereas the affinity for epinephrine, phenylephrine, and halostachine was up to 1000-fold lower with the F310A mutant than with the wild-type receptor, substitution of Phe\textsuperscript{310} with a tryptophan, which although slightly larger than the native phenylalanine, preserved both its hydrophobicity and aromaticity, resulted in a receptor protein with near wild-type agonist affinities. Substitution with a tyrosine, which also has an aromatic side chain, however, resulted in decreases in agonist affinity (25–50-fold) that were comparable to those observed with the F310L mutation. This may be due to the fact that the tyrosine ring contains an hydroxyl moiety. As a result, its side chain, unlike those of phenylalanine or tryptophan, is unlikely to be planar, and also has H-bonding potential. Thus, a tyrosine at the 310 position may perturb optimal positioning of the catechol ring due either to a steric clash or to loss of a favorable planar stacking interaction. Other substitutions at the 310 position with smaller hydrophobic or \(\beta\)-branched residues (valine and isoleucine) or polar residues (asparagine) resulted in significantly greater reductions in agonist affinity than did tyrosine, leucine, or phenylalanine. Not surprisingly, therefore, a positive and highly significant correlation was evident between the volume of the substituent side chain and agonist affinity (Fig. 5). This correlation indicates a van der Waals component to the bonding between the catechol ring and the side chain at the 310 position.
Phe\(^{310}\) in the \(\alpha_{1B}\)-AR and Catecholamine Bonding

Antagonist binding affinities (\(K_i\) values, nM) were determined in \[^{125}\text{I}]\text{HEAT}\) competition binding studies using membranes prepared from transfected COS-1 cells as described under "Experimental Procedures." Data are presented as mean ± S.E. of at least three independent experiments, each performed in duplicate. Values in parentheses are the ratio of \(K_i\) values of the mutant to the wild-type \(\alpha_{1B}\)-ARs.

| Antagonist        | WT                  | F310A | F310L | F311A | F311L |
|-------------------|---------------------|-------|-------|-------|-------|
| Phenotamine       | 31.4 ± 2.9          | 315 ± 19\(^a\) | 233 ± 37\(^b\) | 265 ± 18\(^b\) | 276 ± 18\(^b\) |
| Prazosin          | 0.77 ± 0.06         | 33.8 ± 3.2\(^c\) | 1.08 ± 0.12 | 51.7 ± 11\(^b\) | 72.2 ± 6.5\(^b\) |
| 5-Methylurapidil  | 94.7 ± 0.01         | 494 ± 0.02\(^d\) | 762 ± 15\(^e\) | 443 ± 84\(^e\) | 648 ± 61\(^e\) |

\(^{a}\) \(p < 0.001\) indicates significance differences versus the wild-type \(\alpha_{1B}\)-AR.

\(^{b}\) \(p < 0.01\) indicates significance differences versus the wild-type \(\alpha_{1B}\)-AR.

Free energy change of agonist binding to wild-type and mutant \(\alpha_{1B}\)-ARs

| Agonists        | \(K_{i,mblank}^{\text{mutant}}\) | \(\Delta G^{\text{mutant}}\) | \(K_{i,WT}^{\text{mutant}}\) | \(\Delta G^{\text{WT}}\) |
|-----------------|-------------------------------|-------------------------------|-------------------------------|-------------------------------|
| \(-\) Epinephrine| 583                           | 3.76                          | 33                           | 2.07                          |
| \(-\) Norepinephrine | 395               | 4.94                          | 29                           | 1.39                          |
| \(-\) Phenylephrine | 425               | 3.57                          | 42                           | 2.22                          |
| \(-\) Synephrine  | 8                            | 1.23                          | 3                            | 0.65                          |
| \(-\) Halostachine | 34                | 2.01                          | 5                            | 0.95                          |
| \(-\) Phenylethylamine | 45              | 2.25                          | 11                           | 1.41                          |

\(^{a}\) \(\Delta G = [\Delta G_{\text{mutant}} - \Delta G_{\text{WT}}] = [-RT \ln K_{i,\text{mutant}}] - [-RT \ln K_{i,\text{WT}}]\). This is not inconsistent with an aromatic-aromatic interaction, which involves both a dipole-dipole and a van der Waals component (23). In keeping with an aromatic-aromatic interaction is the finding that the wild-type receptor and the F310W mutant, which both have a planar aromatic side chain at the 310 position, bind with considerably higher affinity than the F310L mutant. In the case of this latter mutant the 310 side chain is of similar volume to phenylalanine, but rather than being aromatic and planar, is aliphatic and bulky.

In contrast to the Phe\(^{310}\) mutations, no correlation was observed between the volume of the substituent side chain at the 311 position and agonist affinity (Fig. 5). Thus, small polar residues, such as asparagine, produced lesser decreases in affinity than larger hydrophobic residues, such as leucine or isoleucine.

If Phe\(^{310}\) indeed interacts with the catechol ring, as suggested by the above findings, it should be solvent accessible with its side chain projecting into the agonist-binding pocket. By contrast, Phe\(^{311}\), which we speculate projects toward TMV, being aromatic and planar, is aliphatic and bulky. Phe\(^{311}\) in the F311C/C128S/C129S/C137S mutant was not due to derivatization.
Moreover, in keeping with this contention is the finding that, in contrast to the effect of MTSEA on the F311C quadruple mutant, substituting Phe311 with a variety of other residues that have considerably smaller side chain volumes (\(\leq 200 \text{ Å}^3\), Fig. 5, D-F) than that of the charged MTSEA moiety, -SCH2CH2NH3\(^+\) (volume \(= 528 \text{ Å}^3\)), markedly perturbs \([^{125}\text{I}]\)HEAT binding (Fig. 5, D-F). Taken together therefore, these data indicate that the side chain of Phe310, but not Phe311, is both solvent accessible and directed toward the agonist-binding pocket.

To determine the role of Phe310 and Phe311 in receptor activation, the ability of substitution mutants to mediate agonist-stimulated PI hydrolysis was compared with that of the wild-type \(\alpha_{1B}\)-AR. With the F310L mutant, the maximal response was unaltered but the potency of the epinephrine-stimulated PI response was decreased by about 10-fold (EC\(_{50}\) = 0.57 mM versus 0.08 mM for the wild-type \(\alpha_{1B}\)-AR), which is comparable to its decrease in epinephrine binding affinity (Fig. 7 A). However, the F310A mutant showed a significant decrease not only in potency (EC\(_{50}\) = 120 mM versus 0.08 mM for the wild-type \(\alpha_{1B}\)-AR), but also in efficacy of epinephrine-stimulated PI hydrolysis (\(E_{\text{max}}\) = 60% of the wild-type response) (Fig. 7 A). This decrease in agonist efficacy was also observed with two other catecholamine analogues, halostachine and phenethylamine,
wild-type (hydroxyls and the chiral hydroxyl on the mutant C128S/C129S/C137S was determined in intact COS-1 cells stimulated with 10 \textsuperscript{-4} M epinephrine for 30 min as detailed under "Experimental Procedures.""

The binding affinity of [\textsuperscript{125}I]HEAT (K\textsubscript{d}, pM) was determined by saturation binding studies performed as described under "Experimental Procedures." Values in parentheses are the fold change in the dissociation constant compared to that of the wild-type \(a\_1B\)-AR.

| Antagonist (nM) | Prazosin | Epinephrine | Cirazoline | Function PI hydrolysis |
|----------------|----------|-------------|------------|------------------------|
| [\textsuperscript{125}I]HEAT | | | | |
| WT | 0.077 ± 0.02 | 0.48 ± 0.13 | 0.53 ± 0.03 | 1.49 ± 0.52 | 100 |
| C128S/C129S/C137S | 0.195 ± 0.06 | 0.30 ± 0.04 | 0.44 ± 0.02 | 2.72 ± 0.22 | 102 ± 8 |

\(a\) \(p < 0.05\) indicates significant differences versus the wild-type \(a\_1B\)-AR.

\(b\) \(p < 0.001\) indicates significant differences versus the wild-type \(a\_1B\)-AR.

![Graphs showing agonist efficacy](image)

which lack either the catechol para-hydroxyl, or both catechol hydroxyls and the chiral hydroxyl on the \(\beta\)-carbon, respectively (Fig. 7, B and C). Like the Phe\textsuperscript{310} mutants, alanine and leucine substitutions at the 311 position also affected (\(\sim\))-epinephrine-stimulated PI hydrolysis. However, in this case the leucine substituent produced a greater perturbation of receptor signaling (decreased potency, EC\textsubscript{50} = 219 \textmu M \text{versus} 0.08 \textmu M for the wild-type \(a\_1B\)-AR, and efficacy, \(E\text{\textsubscript{max}} = 40\%\) of the wild-type response) than the alanine substituent (decreased potency only, EC\textsubscript{50} = 4.9 \textmu M). Together with the alterations in agonist binding observed above with these Phe\textsuperscript{310} and Phe\textsuperscript{311} mutants, the data can be interpreted to indicate that at the 310 position, substitution of the native phenylalanine with a hydrophobic residue, such as leucine, can partially compensate for the loss of both aromaticity and hydrophobicity associated with an alanine substitution. At the 311 position, however, the greater perturbation of receptor signaling with leucine than with alanine may be due to the bulky, non-planar leucine side chain disrupting interhelical packing. In keeping with the requirement of an aromatic side chain at the 310 position for both agonist binding and receptor activation, is the finding that a mutant in which Phe\textsuperscript{310} was replaced with another aromatic residue, tryptophan, displayed not only near wild-type agonist affinities, but also wild-type PI hydrolysis (Fig. 7, A-C).

Finally, to confirm that the effects of the F310A and F310L mutations on PI signaling are not due to a global change in receptor structure, we combined them with a previously described constitutively active mutant, A293E (25, 26), to produce the double mutants, F310A/A293E and F310L/A293E. Since Ala\textsuperscript{293} is about four helical turns below Phe\textsuperscript{310}, it is possible that a structural change caused by the alanine or leucine substitutions will be transmitted to Ala\textsuperscript{293}, thus altering its function PI hydrolysis. The expression levels of the wild-type and F310W, F310L, F310A, F311A, and F311L mutants were 5.1 ± 0.4, 6.2 ± 0.6, 4.6 ± 0.8, 4.2 ± 0.6, 1.0 ± 0.1, and 2.1 ± 0.3 pmol/mg protein, respectively.

![Graphs showing agonist efficacy](image)
**Phe<sup>310</sup> in the α<sub>1B</sub>-AR and Catecholamine Bonding**

Fig. 8. Basal PI hydrolysis. Total inositol phosphates generated in the absence of agonist were determined in intact COS-1 cells transfected with vector alone (mock), or plasmid encoding the wild-type (WT), or F310A or F310L mutant α<sub>1B</sub>-ARs. Total inositol phosphates were quantitated as described under “Experimental Procedures” in cells incubated for 30 min in the presence of 10 mM LiCl. The expression levels of the wild-type, F310A, and F310L mutants were 6.2 ± 0.2, 5.7 ± 0.1, and 5.9 ± 0.3 pmol/mg protein, respectively. *Inset*, basal PI hydrolysis determined as described above in cells transfected with various amounts of plasmid encoding the wild-type ■ or mutant □, A293E; □, F310A/A293E; ●, F310L/A293E; ○, F310L/A293E α<sub>1B</sub>-ARs, to produce the receptor expression levels (densities) shown. Receptor densities were determined from parallel saturation binding studies. Data points for each receptor construct did not differ from a linear relationship, as determined from test runs (p > 0.05). The slopes of the regression lines (0.68 ± 0.28, wild-type; 3.79 ± 1.65, A293E; 3.2 ± 1.31, F310A/A293E; 2.53 ± 0.86, F310L/A293E) provide an index of the amount of inositol phosphates generated per pmol of receptor.

F310L, the F310L/A293E double mutant displayed a decreased potency for epinephrine-stimulated PI hydrolysis (EC<sub>50</sub> = 113 nM versus 0.81 nM for A293E) without a change in efficacy (E<sub>max</sub>). Similarly, like the F310A single mutant, the F310A/A293E double mutant displayed a greater decrease in agonist potency (EC<sub>50</sub> = 8020 nM) than F310L/A293E, as well as a decrease in efficacy (Fig. 9A).

Consistent with these changes in PI signaling, the increased epinephrine affinity observed with the A293E mutant (K<sub>i</sub> = 0.017 μM) compared with the wild-type α<sub>1B</sub>-AR (K<sub>i</sub> = 0.75 μM), was perturbed by additional substitution of Phe<sup>310</sup> with leucine in the F310L/A293E double mutant (K<sub>i</sub> = 1.84 μM), and further decreased in the F310A/A293E double mutant (K<sub>i</sub> = 64 μM) (Fig. 9B). Thus, the effects of alanine or leucine substitution of Phe<sup>310</sup> on PI signaling do not suggest distortion of global receptor structure, but are entirely consistent with the involvement of Phe<sup>310</sup> as a key switch residue involved in ligand binding and receptor activation.

Pharmacophore mapping of adrenergic agonists suggests that the catechol ring of ligands is important both for binding and receptor activity (27). In this study, we provide several lines of evidence to support the contention that Phe<sup>310</sup> in TMVI of the α<sub>1B</sub>-AR mediates such effects through the formation of an aromatic-aromatic interaction between its side chain and the catechol ring. First, computerized modeling of the α<sub>1B</sub>-AR structure, and docking of catecholamines into the ligand-binding pocket, indicate that the Phe<sup>310</sup> side chain is well positioned to interact with the catechol ring. Second, as required for a residue directly involved in ligand binding, SCAM studies reveal that the Phe<sup>310</sup> side chain is not only solvent accessible but is directed into the agonist-binding pocket. Third, since substitution of Phe<sup>310</sup> with a variety of other amino acids does not perturb membrane expression or post-translational processing (glycosylation) of the receptor, its effects on ligand binding and receptor activation are due to local interactions, and not to alterations in global receptor structure. In addition, even loss of all bonding potential with alanine substitution of Phe<sup>310</sup> does not impair spontaneous isomerization of the receptor to a partially activated conformation, as is evident with the F310A/A293E double mutant. Fourth, and most importantly, the aromatic character of the Phe<sup>310</sup> side chain is essential for wild-type agonist binding and, in terms of receptor signaling, for both wild-type agonist potency and efficacy. Thus, although substitution of Phe<sup>310</sup> with a residue that has a hydrophobic side chain, such as leucine, partially compensates for the loss of phenylalanine at the 310 position, only an aromatic residue, e.g. tryptophan, can fully restore ligand-binding and-receptor activation.

Our results do not support the direct involvement of Phe<sup>311</sup> in agonist binding as proposed by Strader et al. (9) for the equivalent residue (Phe<sup>290</sup>) in the β<sub>2</sub>-AR, or alternatively, indicate that the residues interacting with the catecholamine ring in the α<sub>1B</sub> and β<sub>2</sub>-AR, differ. Thus, substitution of Phe<sup>310</sup> with a variety of different residues markedly impaired receptor expression and post-translation processing, indicating that phenylalanine at the 311 position is essential for proper folding of the receptor protein. In addition, neither the volume nor the hydrophobicity of Phe<sup>311</sup> substituent side chains could be directly related to agonist binding or receptor activation. Finally, and most importantly, SCAM studies indicated that the Phe<sup>311</sup> side chain is neither solvent accessible nor directed into the ligand-binding pocket. Thus, effects of Phe<sup>311</sup> substitutions on ligand binding and receptor activation are likely secondary to global changes in receptor structure, rather than to loss of an interaction directly involved in ligand binding.

Based on SCAM studies of the dopamine D2 receptor, it has been suggested recently that all of the aromatic residues in TMVI of biogenic amine receptors are solvent accessible (28). Furthermore, it was postulated that these aromatic residues relayed information from the site of agonist-binding in the extracellular half of the TMVI helix to produce a conformational change at the intracellular end of the helix, resulting in receptor activation. While an attractive hypothesis, the SCAM conclusions were based entirely on antagonist-inactivation data, and were not confirmed by evaluating an effect on agonist binding.

Apart from defining a critical residue (Phe<sup>310</sup>) involved in ligand binding and signaling by the α<sub>1B</sub>-AR, the findings of this study have potentially important ramifications for our understanding of the mechanisms of GPCR activation. For rhodopsin, activation involves initial disruption of an ionic bond between the protonated Schiff base formed by the interaction of 11-cis-retinal with Lys<sup>296</sup> in TMVII and Gln<sup>113</sup> in TMIII (5). Similarly, activation of the α<sub>1B</sub>-AR involves disruption of an ionic bond linking Asp<sup>125</sup> in TMVII and Lys<sup>131</sup> in TMVIII (9), and partial receptor activation can be induced by a moiety mimic (triethylamine) of the catecholamine protonated amine (29). For rhodopsin, activation has been shown to result in rigid body movement of the TMVI helix (30), and movement of this helix is also a feature of β<sub>2</sub>-AR (31) and α<sub>1C</sub>-AR activation. Thus, it is likely that movement of TMIII, due to disruption of a constraining interaction with TMVII, and movement of TMVI, are common features of GPCR receptor activation. Movement of these helices, which are contiguous with the second and third intracellular loops, is also consistent with the known involvement of these loops in G-protein activation (12).

Given these considerations, and based on the findings of this study that implicate Phe<sup>310</sup> in TMVI as a key switch residue in α<sub>1B</sub>-AR activation, it seems reasonable to postulate that activation of adrenergic receptors involves initial disruption of the
Asp<sup>125</sup>Lys<sup>331</sup> ionic interaction followed by Phe<sup>310</sup>/catechol ring-induced movement of TMVI. Indeed, the findings of our studies with the F310A or LA293E double mutants suggest this model based on the following considerations: (i) central to the recently revised ternary complex model of GPCR activation is the finding that mutant receptors can exist in a constitutively active state that allows signaling in the absence of agonist; (ii) in support of this model is the finding that overexpression of wild-type receptors can also initiate biochemical responses in the absence of agonist; (iii) accordingly, it has been proposed that receptors spontaneously resonate between a basal state, R<sub>b</sub>, and an active state, R<sup>a</sup>, with only the latter being able to productively interact with G-protein, and (iv) as a corollary, constitutively active mutants, which partially mimic the active state, represent an intermediate receptor conformation, R<sup>′</sup> (26). Based on these considerations and on the finding that agonists bind to constitutively active receptors with higher affinity than to wild-type receptors, it has been suggested that rather than inducing the active conformation upon binding, agonists merely select or “trap” the active conformation that results from spontaneous isomerization of R to R<sup>a</sup> (32). Nevertheless, recent structure-function studies of the angiotensin II (AT<sub>1</sub>) receptor provide compelling evidence for an R<sup>′</sup> conformation that is a distinct intermediate between R and R<sup>a</sup>, and that isomerization from the R<sup>′</sup> conformation to the active state involves an inductive step that requires agonist binding (33).

In the present study we demonstrate that little, if any, PI hydrolysis is observed with the wild-type α<sub>1B</sub>-AR in the absence of agonist. Moreover, signaling in the absence of agonist was similar with the F310A and F310L mutants, and, importantly, was not reduced below that observed with the unliganded wild-type α<sub>1B</sub>-AR, even though these mutants markedly impaired agonist-induced signaling. Thus the unliganded wild-type α<sub>1B</sub>-AR receptor likely represents the true basal or R conformation, whereas the receptor maximally activated by high agonist concentrations represents the R<sup>a</sup> state. By contrast, and consistent with an R<sup>′</sup> conformation, basal signaling was readily apparent with the unliganded A293E mutant. Since the double mutants F310A/A293E and F310L/A293E also showed similar basal signaling in the absence of agonist, but impaired agonist-induced signaling, the Phe<sup>310</sup>/catechol ring interaction is likely not required for isomerization between R and R<sup>a</sup>, but is critical for full receptor activation. Consistent with the above

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