The Unique Nature of the Serine Interactions for \( \alpha_1 \)-Adrenergic Receptor Agonist Binding and Activation*

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Activation of the \( \beta_2 \) and \( \alpha_2 \)-adrenergic receptors (AR) involves hydrogen bonding of serine residues in the fifth transmembrane segment (TMV) to the catechol hydroxyls of the endogenous agonists, epinephrine and norepinephrine. With the \( \beta_2 \)-AR both Ser204 and Ser207 but not a third TMV serine (Ser203) are required for binding and full agonist activity. However, with the \( \alpha_2 \)-AR only one of two TMV serines (Ser204, equivalent to Ser207 in the \( \beta_2 \)-AR) appears to contribute partially to agonist-binding and activation. Because the \( \alpha_2 \)-AR uniquely contains only two TMV serines, this subtype was used to systematically evaluate the role of hydrogen bonding in \( \alpha_2 \)-AR activation. Binding of epinephrine or its monohydroxyl congeners, phenylephrine and synephrine, was not decreased when tested with alanine-substitution mutants that lacked either Ser204 (Ser204 → Ala) or Ser207 (Ser207 → Ala). With the substitution of both serines in the double mutant, Ser204/207 → Ala, binding of all three ligands was significantly reduced (10-100-fold) consistent with a single hydrogen bond interaction. However, receptor-mediated inositol phosphate production was markedly attenuated only with the Ser204 → Ala mutation and not with Ser207 → Ala. In support of the importance of Ser204 binding of phenylephrine (meta-hydroxyl only) by Ser207 → Ala increased 7-fold over that observed with either the wild type receptor or the Ser204 → Ala mutation. Binding of synephrine (para-hydroxyl only) was unchanged with the Ser207 → Ala mutation. In addition, when combined with a recently described constitutively active \( \alpha_2 \)-AR mutation (Met207 → Leu), only the Ser204 → Ala mutation and not Ser207 → Ala relieved the high affinity binding and increased agonist potency observed with the Met207 → Leu mutation. A simple interpretation of these findings is that the meta-hydroxyl of the endogenous agonists preferentially binds to Ser204, and it is this hydrogen bond interaction, and not that between the para-hydroxyl and Ser207, that allows receptor activation. Furthermore, since Ser204 and Ser207 are separated by three residues on the TMV \( \alpha \)-helix, whereas Ser204 and Ser207 of the \( \beta_2 \)-AR are separated by only two residues, the orientation of the catechol ring in the \( \alpha_2 \)-AR binding pocket appears to be unique and rotated approximately 120° to that in the \( \beta_2 \)-AR.

Adrenergic receptors (ARs) are members of the superfamily of receptors that exert their physiological effects through coupling to guanine nucleotide-binding proteins (G-proteins). Strictly conserved among this superfamily is the presence of seven transmembrane-spanning domains connected by hydrophilic loops alternately exposed to the extracellular and intracellular environment. The intracellular loops bind and activate G-proteins (1, 2). The AR family (\( \alpha_1 \), \( \alpha_2 \), \( \alpha_3 \), \( \alpha_4 \), \( \alpha_5 \), \( \alpha_6 \), \( \beta_1 \), \( \beta_2 \), \( \beta_3 \)) mediate the effects of the sympathetic nervous system through the actions of epinephrine and norepinephrine and control the homeostasis of the cardiovascular system. However, the ligand binding pockets are distinct between the receptor subtypes, as they can discriminate a wide variety of synthetic agonists and antagonists (3).

Previous studies with the \( \beta_2 \)-AR have identified key residues involved in binding epinephrine and norepinephrine. These interactions likely are conserved in the other ARs since they all bind the natural hormones with similar affinity. In particular, a highly conserved aspartic acid residue (Asp113 in the \( \beta_2 \)-AR) in TMIII is involved in forming a salt bridge with the protonated amine of the catecholamine (4). There are also two serine residues in TMV (Ser203 and Ser207 in \( \beta_2 \)-AR) that are highly conserved among receptors that bind catecholamines, but not in other G-protein-coupled receptors (Fig. 1). In the \( \beta_2 \)-AR, one of these two serine residues, Ser207, as well as a non-conserved serine (Ser204), have been shown to be involved in hydrogen bond interactions with the hydroxyl groups on the catechol ring (5). Both residues are required for high affinity binding of agonists and for full agonist activity. These interactions were confirmed with the use of various agonists that lack meta- and/or para-hydroxyl groups of the catechol ring. A model was therefore proposed in which Ser204 hydrogen bonds with the meta-hydroxyl group of the catechol ring while Ser207 interacts with the para-hydroxyl group. Extrapolation of this model to the \( \alpha_2 \)-AR is unclear. Mutation of either of the equivalent serines in the \( \alpha_2 \)-AR (Ser200 and Ser204) resulted in a 10-fold decrease in affinity for epinephrine but no change in affinity for synephrine that lacks a meta-hydroxyl group (suggesting the para-hydroxyl is unimportant) (6). The Ser204 mutant attenuated the functional activity (65% active) but only with synephrine, implicating a para-hydroxyl interaction. From these studies it was concluded that the para-hydroxyl group of the catechol ring is involved in a hydrogen bonding interaction with Ser203, as has been described previously for the corresponding serine in the \( \beta_2 \)-AR. Furthermore, in contrast to the situation with the \( \beta_2 \)-AR, it was suggested that the \( \alpha_2 \)-AR Ser200 residue does not participate directly in receptor-agonist interaction.

With the recent cloning of the \( \alpha_3 \)-AR, which uniquely con-
contains only two TMV serines, we explored the serine requirements for α₁-AR agonist binding and activation. We also evaluated if the interaction proposed for the β₂-AR was conserved.

**EXPERIMENTAL PROCEDURES**

Site-directed Mutagenesis—The construct used was the rat α₁-AR (7). Site-directed mutagenesis was performed as described previously utilizing the oligonucleotide-mediated double primer method (8). Positive plaques were purified and sequenced to verify the mutation. The mutagenic construct was subcloned into the expression vector, pMT2. The full-length plasmid DNA was again sequenced to verify the mutation.

Cell Culture and Transfection—COS-1 cells (ATCC) were grown in Dulbeccos modified Eagles medium supplemented with 10% fetal bovine serum. Transient expression in COS-1 cells was accomplished by the DEAE-dextran method (8).

Radioligand Binding—COS-1 membranes were prepared as described previously (9). Competition reactions contained 200 nM HEPS, pH 7.5, 1.4 mM EGTA, 12.5 mM MgCl₂, 200 nM [¹²³I]HEAT, COS-1 membranes, and increasing amounts of unlabeled ligands. Nonspecific binding was determined in the presence of 10⁻⁴ M phentolamine. Reactions were stopped after 1 h by the addition of cold HEPS buffer and were filtered with a Brandel cell harvester. Binding data were analyzed by the iterative curve-fitting program LIGAND. Statistical testing was performed using an ANOVA and Student-Newman-Keuls multiple comparisons test to determine significant differences (p < 0.05) for both ligand binding and functional assays. Free energy calculations were based upon the equation ΔG = -RT lnKd/Ka, where Kd and Ka are the equilibrium association constants determined with two different receptor constructs. The protein concentration was measured using the method of Bradford (10).

IP Hydrolysis—IP determination was performed as described (11). Cells were labeled for 16–24 h with [³H]Inositol at 1 μCi/ml in media. The cells were washed followed by a 20-min incubation with 10 mM LiCl. Agonists were then added for 30 min, the medium was removed, and the cells lysed, neutralized, and centrifuged. The supernatant was harvested with a Brandel cell harvester. Binding data were analyzed by the iterative curve-fitting program LIGAND. Statistical testing was performed using an ANOVA and Student-Newman-Keuls multiple comparisons test to determine significant differences (p < 0.05) for both ligand binding and functional assays. Free energy calculations were based upon the equation ΔG = -RT lnKd/Ka, where Kd and Ka are the equilibrium association constants determined with two different receptor constructs. The protein concentration was measured using the method of Bradford (10).

Molecular Modeling—The coordinates of the α₁-AR were determined by an overlay of the putative α₁-AR TM residues with the TM coordinates of bacteriorhodopsin (12) using data files generated using the Insight II molecular modeling software from Biosym Technologies. The boundaries of the putative TM domain were determined by an algorithm based upon the weighted pairwise comparisons of adjacent residues (13). The α₁-AR model was then minimized and conflicts adjusted as described previously (14).

Materials—Drugs were obtained from the following manufacturers: epinephrine,norepinephrine,phenylephrine,phentolamine, and phentolamine from Sigma, [¹²⁵I]HEAT,[¹³⁰I]Inositol, and the [³H]IP₃ radio-receptor kit were from DuPont NEN; AG1-X8 was from Bio-Rad. The construct used was the rat α₁-AR (7). Site-directed mutagenesis was performed as described previously utilizing the oligonucleotide-mediated double primer method (8). Positive plaques were purified and sequenced to verify the mutation. The mutagenic construct was subcloned into the expression vector, pMT2. The full-length plasmid DNA was again sequenced to verify the mutation.

RESULTS AND DISCUSSION

Using site-directed mutagenesis and pharmacophore mapping of catecholamine agonists, Strader et al. (5) proposed that hydrogen bond interactions involving the hydroxyl groups on the phenyl ring are important for ligand binding to the β₂-AR. A model was proposed in which two of three serines in the TMV are involved in these hydrogen bond interactions with the catechol hydroxyl groups of catecholamine agonists. In particular, it was proposed that Ser²₀⁴ of the β₂-AR forms a hydrogen bond with the meta-hydroxyl group of the catechol ring while Ser²₀⁷ forms a hydrogen bond with the para-hydroxyl group. In support of this model, substitution of alanines for either of these two serines resulted in mutants that had a 30-fold decrease in their affinity for all catecholamine agonists, with each serine contributing about 50% to the activation of the receptor (5). Consistent with the importance of serine residues for catecholamine binding, it is of interest that two of the three serines in TMV of the β₂-AR are highly conserved, although only one of these two conserved residues is implicated in catecholamine binding (Fig. 1). The involvement of the serine residues (Ser²₀⁴ and Ser²₀⁷) in the β₂-AR paradigm is still incomplete.

With the cloning of the α₁-AR and the finding that this subtype contains only two serine residues in TMV, we have been able to study the interaction of the catechol hydroxyls of agonists with α₁-AR serine residues. In order to assess the interactions between each of the conserved serine residues and the catechol hydroxyls, site-directed mutagenesis was used to create three different mutated α₁-ARs analogous to those created by Strader et al. (5). These receptors are denoted as Ser¹⁸⁸ → Ala, Ser¹⁹² → Ala, and Ser¹⁸⁸/¹⁹² → Ala and correspond to the substitution of serine residues by alanines at the indicated amino acid number of the α₁-AR (Fig. 1). However, in the α₁-AR there is no equivalent serine residue at position 204 as in the β₂-AR. The N-terminal TMV α₁-AR serine is located one residue higher in the helix at the analogous position 203 of the β₂-AR (Fig. 1).

The binding of epinephrine and norepinephrine to the wild type receptor is consistent with a single population of binding sites and both displayed similar Kᵢ values (Table I). There is no apparent high affinity component of agonist binding, as is typically seen with other G-protein-coupled receptors, and addition of GTP analogs does not produce a low affinity shift of epinephrine binding. With the wild type α₁-AR, binding of synephrine, a congener of epinephrine that lacks the meta-hydroxyl, was 15-fold lower than that of epinephrine. However, binding of phenylephrine, a congener of epinephrine that lacks the para-hydroxyl, was similar to that of epinephrine. This suggests that the meta-hydroxyl group contributes predominantly to determination of catecholamine affinity for α₁-ARs (Table I, Fig. 2).

Replacement of either Ser¹⁸⁸ or Ser¹⁹² in TMV of the α₁-AR with an alanine did not significantly reduce the binding affinity for any of the agonists tested compared to the wild type receptor (Table I, Fig. 2). In fact, the binding affinity for phenyleph-
rine was significantly increased (7-fold) with the Ser\textsuperscript{192} \rightarrow Ala mutant (Table I, Fig. 2). There also was a noticeable higher affinity for the other monohydroxyl agonist, synephrine with the Ser\textsuperscript{192} \rightarrow Ala mutant, but this increase in affinity was not significant. These results are quite distinct from the \(\beta\)-\(2\)-and \(\alpha\)-\(2\)-\textsuperscript{a}-\textsuperscript{b}-ARs paradigms, where either serine mutation was able to reduce agonist-binding affinity. To confirm a hydrogen bond interaction, the double mutant, Ser\textsuperscript{188}/192 \rightarrow Ala, was created and found to decrease the binding affinity by 25–120-fold for epinephrine, norepinephrine and phenylephrine, consistent with a decrease in binding energy upon substitution of \(\Delta G = 3–5\) kcal/mol. The decrease in synephrine binding affinity by this mutant was only 6-fold. The free energy values are consistent with the disruption of a single hydrogen bond (\(\Delta G = 3–7\) kcal/mol). Since either serine residue is sufficient in itself in maintaining the wild type binding affinity but the free energy

The table below shows the agonist binding profiles of wild type \(\alpha\)-\textsuperscript{a}-\textsuperscript{AR} and serine mutations. Competition binding studies were used to determine \(K_i\) values (\(\mu M \pm SE\)) of membranes prepared from COS-1 cells expressing the wild type \(\alpha\)-\textsuperscript{a}-\textsuperscript{AR}, Ser\textsuperscript{188} Ala (S188A), Ser\textsuperscript{192} Ala (S192A), and Ser\textsuperscript{188} Ala/Ser\textsuperscript{192} Ala (S188A/S192A). All competition binding isotherms were best fit to a single-site model. Receptor densities (\(B_{\text{max}}\)), and the \(K_i\) (\(\mu M\)) for [\(\text{I}^{125}\text{I}\)]HEAT were determined on the same membranes from equilibrium binding studies. An ANOVA was performed followed by a Student-Newman-Keuls post-test analysis to determine significant differences. ***indicates significant differences from the wild type receptor (***, \(p < 0.001\)).

### Table I

| Agonist            | \(\text{S188A}\) | \(\text{S192A}\) | \(\text{S188A/S192A}\) |
|--------------------|-----------------|-----------------|-------------------|
| \((-\)Epinephrine   | 3.3 ± 0.4       | 2.6 ± 0.2       | 3.2 ± 0.7         |
| \((-\)Phenylephrine| 6.2 ± 1.5       | 12.6 ± 1.6      | 0.9 ± 0.1***      |
| \((\pm)\)Symphephrine| 52.5 ± 2.4      | 104.7 ± 11.4    | 28.2 ± 3.1       |
| \((-\)Norepinephrine| 4.6 ± 0.3       | 9.8 ± 1.3       | 7.4 ± 1.1        |
| \(K_i\) [\(\text{I}^{125}\text{I}\)]HEAT (\(\mu M\)) | 81              | 50              | 282              |
| \(B_{\text{max}}\) (pmol/mg protein) | 0.30            | 0.31            | 0.61             |

**Fig. 2.** Competition binding and IP stimulation with substituted catechol hydroxyls to wild type, Ser\textsuperscript{188} \rightarrow Ala, and Ser\textsuperscript{192} \rightarrow Ala mutant \(\alpha\)-\textsuperscript{a}-\textsuperscript{ARs}. Competition binding and IP stimulation was performed on cells prepared from transfected COS-1 cells with the wild type \(\alpha\)-\textsuperscript{a}-\textsuperscript{AR} receptor (\(\bullet\)), Ser\textsuperscript{188} Ala (\(\bigcirc\)), or Ser\textsuperscript{192} Ala (\(\bigtriangleup\)) as described under “Experimental Procedures,” in the presence of epinephrine (panel A), phenylephrine (panel B), and synephrine (panel C). The experiment shown is the mean curve (\(\pm\) S.E.) generated from at least three separate experiments.
The binding of phenylephrine (6.2 μM) to the wild type receptor is essentially the same as that of epinephrine (Table I, Fig. 2). The meta-hydroxyl group is closer to Ser188 than Ser192, and the remaining serine residue remains capable of activating the receptor. This also supports the hypothesis of promiscuity of ligand docking, since the para-hydroxyl group is closer to Ser192. Therefore, it appears that α1-ARs maintain their catechol hydroxyls not equal distant from the two serine residues but in a parallel position relative to the surface of the receptor (Fig. 4B) with the meta-hydroxyl and Ser188 forming the strongest interaction. This model is not only valid for the mutated receptors but also for the wild type receptors since the binding of phenylephrine (6.2 μM) is similar to epinephrine (3.3 μM), while synephrine binding decreased by 10-fold for the wild type receptor. These results demonstrate a strong binding interaction and, therefore, a closer distance of the meta-hydroxyl position to a serine residue and a weaker and more distant interaction of the para-hydroxyl. Also in support of this model for the wild type receptor, synephrine's affinity is minimally changed (6-fold) for the wild type receptor (52.5 μM) as compared to the double serine mutant (324 μM), while all other agonists tested displayed 25-120-fold affinity differences (Table I). This also suggests that the para-hydroxyl is only weakly binding with a serine residue.

The activation requirements for α1-ARs appear distinct from its binding interactions (Fig. 2). At equal receptor numbers of 0.3 pmol/mg protein, only Ser188 plays a major role in receptor activation, contributing 70–90% of the wild type response. On the other hand, the effect of Ser192 on receptor activation is minimal. Both full agonists, epinephrine and phenylephrine, produced similar effects on activation with either serine mutant, consistent with the meta-hydroxyl of epinephrine being nearest to Ser188 (Fig. 2, A and B). Synephrine produced effects similar to those of epinephrine and phenylephrine on receptor activation (Fig. 2C) with either serine mutation (but with less efficacy). Only Ser192 → Ala (Ser188 intact) allowed full receptor activation. Since synephrine can activate the receptor, this indicates that either hydroxyl group on the catechol ring is capable of activating the receptor but only an interaction with Ser188 will produce a wild type response. This also supports the hypothesis of promiscuity of ligand docking, since the para-hydroxyl would need to move to allow interaction with Ser188. Since this would not be an optimal interaction, it accounts for the partial agonist properties of synephrine. Likewise, the full agonism of phenylephrine is due to its close contact with Ser188, as has been postulated in earlier studies (15) in which the greatest activity among monophenolic analogs of phenethylamines always resides in the meta-substituted derivative, with the para-hydroxylated phenethylamines being significantly weaker.

We recently described a chimeric point mutation in the α1b-AR, Met292 → Leu, that was created to explore the agonist binding pocket differences between α1a- and α1b-ARs. This mutant is constitutively active as evidenced by increased basal signaling and increased agonist binding and potency (16). The mechanism by which this mutant imparted constitutive activity appears to be by preventing normal packing of an adjacent valine residue in TMV (Val185), which, in turn, perturbs the

![Fig. 3. Basal IP₃ release (A), epinephrine binding affinity (B), and IP dose response by epinephrine (C) for wild type, Met³⁹² → Leu, and mutant α₁ARs. IP₃ production in the absence of agonists (panel A), competition binding (panel B), and IP stimulation (panel C) was measured in COS-1 cells expressing the constitutively active α₁AR mutation, Met³⁹² → Leu alone (●), or in combination with either Ser¹⁸⁸ → Ala (▲) or Ser¹⁸⁸ → Ala (▲) relative to the wild type (○) or to mock transfected cells (vector alone). Expression levels for each receptor were adjusted to similar values (0.3 pmol/mg membrane protein) by titrating the amount of DNA used in the transfection. The results are the mean ± S.E. of at least three separate experiments. ** indicates significant differences from the wild type (**, p < 0.01; ***, p < 0.001).](http://www.jbc.org/Content/dam/journals/jbc/Full/40153816/Fig3B.png)
helix resulting in constitutive activity (16). Since this \( \alpha_{1b} \) mutation, Met292 \( \rightarrow \) Leu, appeared to be operating through a conformational change of TMV, we explored the possibility that the serine residues on TMV might also be involved in manifesting its constitutive activity. Therefore, we combined the constitutively active mutation, Met292 \( \rightarrow \) Leu, with either serine mutation, Ser253 \( \rightarrow \) Ala or Ser192 \( \rightarrow \) Ala, in a single receptor and evaluated the resulting double mutant for changes in potency, basal IP3 release, and agonist binding affinity. The Met292 \( \rightarrow \) Leu mutation alone was constitutively active, as evidenced by its higher IP3 basal activity (Fig. 3A), higher binding affinity (Fig. 3B), and increased potency (Fig. 3C) compared to the wild type receptor. However, basal IP3 signal transduction remained higher when combined with either serine mutation (Met292 \( \rightarrow \) Leu/Ser253 \( \rightarrow \) Ala or Met292 \( \rightarrow \) Leu/Ser192 \( \rightarrow \) Ala), indicating an agonist-independent property of this mutant. In contrast, the high binding affinity for epinephrine as seen in Met292 \( \rightarrow \) Leu alone was abolished by combination with either serine mutation. This is in contrast to the single serine mutations and most likely is due to both serines in the Met292 \( \rightarrow \) Leu mutant being moved closer to the agonist binding pocket. However, the exact nature of the conformational change and whether it truly mimics the native activated state are unknown. Nevertheless, these results are still consistent with both serines participating in binding affinity. Likewise, in dose-response studies with epinephrine, the Met292 \( \rightarrow \) Leu/Ser188 \( \rightarrow \) Ala combination virtually abolished the signal transduction and lowered the EC50 back to wild type values, essentially reversing the agonist-dependent manifestations of constitutive activity. These results are also consistent with the proposed \( \alpha_{1a} \)-AR paradigm that both serines contribute to binding affinity but only Ser188 is critical in receptor activation.

The data presented here are consistent with the model of the \( \alpha_{1a} \)-AR ligand binding site presented in Fig. 4. Two serine residues in TMV of the \( \alpha_{1b} \)-AR, Ser188 and Ser192, are responsible for part of the agonist binding affinity. Each catechol hydroxyl is not located equal distant from its respective serine as the \( \beta_2 \)-AR. Both hydroxyls appear closer to Ser188 with the meta-hydroxyl forming the closest interaction. For receptor activation, however, only Ser188 is necessary for full agonism. This \( \alpha_{1a} \)-AR paradigm is likely conserved to the other two \( \alpha_{1a} \)-AR subtypes, the \( \alpha_{1b} \) and \( \alpha_{1d} \)-ARs. Although both these \( \alpha_{1a} \)-receptor subtypes have an extra serine located at the analogous position of 189 in the \( \alpha_{1b} \)-AR (Ser253) facing away from the ligand binding pocket. As shown in Fig. 4B, the meta-hydroxyl serine residue (Ser188) of the \( \alpha_{1b} \)-AR would be closer to TMV and the para-hydroxyl serine residue (Ser192) closer to TMVI. Therefore, the two serine residues in the \( \alpha_{1b} \)-ARs are located three residues apart in the helix while the \( \beta_2 \)-AR serines are located two residues apart. Due to the helical nature of the TM domains, this displacement by one residue can be predicted to result in a total rearrangement of the \( \alpha_{1a} \)-AR catechol hydroxyls in the ligand binding pocket compared to the \( \beta_2 \)-AR. As shown in Fig. 4B, the meta-hydroxyl serine residue (Ser188) of the \( \alpha_{1b} \)-AR would be closer to TMV and the para-hydroxyl serine residue (Ser192) closer to TMVI with the extra serine residue in the \( \alpha_{1b} \)-AR facing away from the ligand binding pocket. This arrangement of the hydroxyls in the \( \alpha_{1a} \)-AR would be opposite to the alignment in the \( \beta_2 \)-AR where the meta-hydroxyl serine residue (Ser253) would be closer to TMVI and the para-hydroxyl serine residue (Ser264) would be closer to TMV. Therefore, the orientation of the catechol ring in the \( \alpha_{1a} \)-AR binding pocket appears to be unique and rotated approximately 120° to that in the \( \beta_2 \)-AR. This resulting difference between the \( \alpha_{1a} \) and \( \beta_2 \)-ARs has major implications for drug design and possible mechanistic differences in receptor activation.

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