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Identification of Two Serine Residues Involved in Agonist Activation of the β-Adrenergic Receptor*

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Pharmacophore mapping of the ligand binding domain of the β-adrenergic receptor has revealed specific molecular interactions which are important for agonist and antagonist binding to the receptor. Previous site-directed mutagenesis experiments have demonstrated that the binding of amine agonists and antagonists to the receptor involves an interaction between the amine group of the ligand and the carboxylate side chain of Asp^{113} in the third hydrophobic domain of the receptor (Strader, C. D., Sigal, I. S., Candelore, M. R., Rands, E., Hill, W. S., and Dixon, R. A. F. (1988) *J. Biol. Chem.* 263, 10267–10271). We have now identified 2 serine residues, at positions 204 and 207 in the fifth hydrophobic domain of the β-adrenergic receptor, which are critical for agonist binding and activation of the receptor. These serine residues are conserved with G-protein-coupled receptors which bind catecholamine agonists, but not with receptors whose endogenous ligands do not have the catechol moiety. Removal of the hydroxyl side chain from either Ser^{204} or Ser^{207} by substitution of the serine residue with an alanine attenuates the activity of catecholamine agonists at the receptor. The effects of these mutations on agonist activity are mimicked selectively by the removal of the catechol hydroxyl moieties from the aromatic ring of the agonist. The data suggest that the interaction of catecholamine agonists with the β-adrenergic receptor involves two hydrogen bonds, one between the hydroxyl side chain of Ser^{204} and the *meta*-hydroxyl group of the ligand and a second between the hydroxyl side chain of Ser^{207} and the *para*-hydroxyl group of the ligand.

Many cell-surface receptors mediate their intracellular responses through activation of one or more of a family of guanine nucleotide binding regulatory proteins (G-proteins). The recent cloning and expression of several of these G-protein coupled receptors has shown them to constitute a family of related proteins with conserved structural features which are thought to reflect their similar mechanisms of action (1–15). The common structural motif of G-protein-coupled receptors is a pattern of seven hydrophobic domains, believed to form transmembrane helices, connected by hydrophilic loops which are postulated to be exposed alternately extracellularly and cytoplasmically. The primary sequences of the hydrophobic regions of these various receptors are highly conserved, while the hydrophilic regions are more divergent. Mutagenesis studies of the β-adrenergic receptor (βAR)[1] and biophysical analysis of rhodopsin indicate that the ligand binding domains of these receptors involve residues within the conserved hydrophobic core (16–24). In contrast, coupling of the receptors to G-proteins involves residues within the divergent third intracellular hydrophilic loop (24–28).

The sequence homology among G-protein-coupled receptors and the structural similarities among ligands which bind to different receptors suggests that the amino acid residues involved in ligand binding to many of these proteins may be conserved. This sequence similarity should allow the rational development of small molecule inhibitors of these receptors. To design specific agonists and antagonists for G-protein-coupled receptors, a detailed characterization of the ligand binding pocket of the protein must be performed, with all points of contact between the ligand and the receptor identified. The identification of the molecular interactions which differentiate agonists from antagonists would also aid in understanding the mechanism of receptor activation. The βAR is proving a useful model system for the construction of such a map of the ligand binding pocket, as decades of structure-activity studies of adrenergic ligands have yielded a wealth of information about the structural properties of compounds which interact with the receptor. Pharmacaphore mapping studies have revealed specific structural requirements for ligands to bind to the βAR (29–31). The endogenous agonists for this receptor are catecholamines, with a protonated amine group separated from an aromatic catechol ring by a β-hydroxyethyl chain. Antagonists are characterized by increased hydrophobicity of the aromatic ring system and by an increased distance between the amine group and the aromatic ring, usually by substitution of a phenoxymethylene moiety for the phenyl ring of the agonists. Thus, specific molecular requirements for ligand binding to the βAR include an interaction with an amine group, hydrogen bonds with β-hydroxyl and catechol hydroxyl groups, and aromatic ring interactions.

The binding of agonists and antagonists to the βAR involves an interaction, presumably a salt bridge, between the amine group of the ligand and the carboxylate side chain of Asp^{113} in the third hydrophobic domain (17, 19). In the present report, we have further characterized the ligand binding site of the receptor. In order to identify hydrogen bonding interactions involved in agonist binding to the βAR, amino acid residues which could potentially serve as hydrogen bond donors or acceptors were substituted by alanine or leucine. The amino acid substitutions were further restricted to residues which were located within the hydrophobic ligand binding

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[1] The abbreviations used are: βAR, β-adrenergic receptor; Gpp(NH)p, guanyl-5′-yl imidodiphosphate; ^{125}I-CYP, ^{125}I-cyanopindolol.

domain of the receptor and which were present in those G-protein-coupled receptors that bind catecholamine ligands. Two serine residues, at positions 204 and 207 in the fifth hydrophobic domain of the βAR, were determined to be critical for full activation by catecholamine agonists. The conservation of these serine residues with other G-protein-coupled receptors which bind catecholamine agonists suggests that the structural parameters responsible for agonist activation are shared by these functionally related receptors.

EXPERIMENTAL PROCEDURES

Mutagenesis and Expression—The cloning, expression, and oligonucleotide-directed mutagenesis of the hamster βAR have been reported previously (16). Expression of the wild-type and mutant receptors in COS-7 and L cells was performed as previously described, using the SV40 early promoter-based expression cassette pSVL, and L cell lines expressing the receptors were selected by resistance to G-418 (16).

Membrane Preparation and Receptor Assays—Membranes were prepared from COS-7 cells by freeze-thaw lysis, as previously described (16), and resuspended at 1–3 mg/ml in TME buffer (75 mM Tris, pH 7.5, 12.5 mM $MgCl_2$, 1.5 mM EDTA) for use in ligand binding assays. For protein immunoblotting, membranes were further purified by centrifugation on a sucrose cushion to remove nuclei (18) and resuspended in TME buffer. Membranes were prepared from L cells by hypotonic lysis in 1 mM Tris, pH 7.4, as previously described, and resuspended at 1–2 mg/ml in TME buffer.

^{125}I-CYP binding to membranes was measured in a final volume of 250 μl of TME buffer, containing 10–20 μg of membrane protein and 225 pM ^{125}I-CYP, for 90 min at 23 °C. Bound ^{125}I-CYP was measured with a γ counter after filtration over GF/C filters. Nonspecific binding was measured in the presence of 10 μM alprenolol. Competition binding of agonists and antagonists was performed for 90 min at 23 °C in 250 μl of TME buffer, containing 35 pM ^{125}I-CYP, 10–20 μg of membrane protein (representing a final receptor concentration of 3–10 pM), and the competing ligand at concentrations of 10^{-10}–10^{-3} M. Data were analyzed using the LIGAND program of Munson and Rodbard (32).

Adenylyl cyclase activity was measured for 30 min at 30 °C in 50 μl of TME buffer containing 10–20 μg of membrane protein in the presence of [α-^{32}P]ATP and an ATP regenerating system (16). [^{32}P]cAMP was determined by the method of Saloman *et al.* (33). Data were analyzed by nonlinear regression analysis, according to the equation:

$$V = V_{max}[A]/(K_{act} + [A])$$

where V is the level of cyclase stimulation (in picomoles/mg/min) measured at a given agonist concentration $[A]$, and V_{max} is the calculated maximal stimulation at infinite agonist concentration (34).

Protein immunoblotting was performed on membrane samples separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, using an antibody raised to a synthetic peptide corresponding to the C-terminal region of the hamster βAR, exactly as described previously (18). Blots were incubated with antibody at a final dilution of 1:1000, followed by ^{125}I-protein A at a final concentration of 10^6 cpm/ml.

RESULTS

For analysis of hydrogen bonding interactions between the βAR and adrenergic ligands, amino acid substitutions removing potential hydrogen bonding side chains were introduced into the *βAR* gene by site-directed mutagenesis. The resulting mutant receptors were expressed in COS-7 cells and the effects of the substitutions on the binding of agonists and antagonists determined. The results of nine such mutations, all involving serine or tyrosine residues within the hydrophobic domain of the receptor, are summarized in Table I. As previously reported, the wild-type βAR appeared by protein immunoblotting to be a glycosylated protein, migrating on sodium dodecyl sulfate gels with an apparent molecular mass of 67 kDa (18, Table I). Attempts to generate mutant receptors having substitutions for the serine residues at positions 120, 165, and 203 failed to produce immunoreactive protein, while a mutant receptor having a substitution for Tyr^{316} displayed anomalous electrophoretic mobility (Table I). Of those mutant receptors which appeared to be folded correctly, substitution for Ser^{161} or Tyr^{219} did not appear to affect the ligand binding properties of the βAR (Table I). In contrast, substitution of an alanine residue for Ser^{204}, Ser^{207}, or Ser^{319} resulted in a 10–25-fold decrease in the affinity of the receptor for the agonist isoproterenol without substantially affecting the binding of the antagonist alprenolol (Table I). This decreased affinity for agonists reflects a specific disruption of the receptor-ligand interaction rather than an effect on G-protein coupling, since G-protein coupling of the βAR is not observed in COS-7 cell membranes (16). Ser^{319} is conserved among the adrenergic, dopamine, serotonin, muscarinic, and substance K receptors, whereas the serine residues at positions 204 and 207 appear to be more specific for receptors which bind catecholamine ligands (1–15). Therefore, the effects of substitution of the latter 2 serine residues on the functional properties of the βAR were characterized further.

If the decrease in agonist affinity observed upon substitution of an alanine residue for Ser^{204} or Ser^{207} reflects the disruption of a hydrogen bond between the ligand and the mutant receptor, then the effect of the mutation on the binding properties of the receptor should be mimicked by structural alterations of the ligands which remove potential hydrogen bonding moieties. The binding of variously substituted ligands to the wild-type and mutant receptors is summarized in Table II, with specific examples shown in Fig. 1. The catechol agonists epinephrine and isoproterenol (compounds 1 and 2, respectively, in Table II) bound [Ala^{204}]βAR and [Ala^{207}]βAR with affinities 25–35-fold lower than for the wild-type receptor. For compounds 1 and 2, the affinities of these two mutant receptors were reduced by approximately the same amount, corresponding to a relative affinity for catecholamine ligands of βAR > [Ala^{204}]βAR = [Ala^{207}]βAR. When the β-hydroxyl group of the ligand was substituted with a ketone moiety (compound 3), the affinity of the wild-type

TABLE I

Characterization of mutant βAR

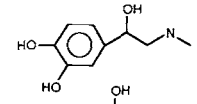
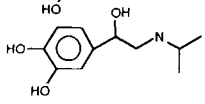
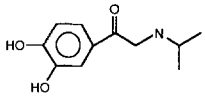
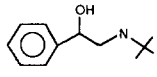
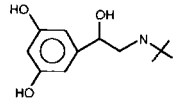
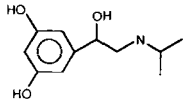
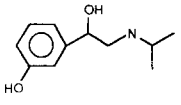
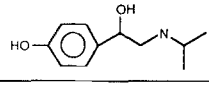
All determinations were on membranes prepared from COS-7 cells transfected with DNA encoding the indicated mutant or wild-type βAR, as described under "Experimental Procedures." PSVL membranes were prepared from control cells transfected with the expression vector alone. The apparent molecular mass of the receptor was determined by protein immunoblotting. NP signifies that no protein could be detected by this technique. The receptor concentration in the membrane preparations was determined by ^{125}I-CYP binding and is expressed as femtomoles/mg protein. K_d values for isoproterenol and alprenolol were determined by competition binding, as described under "Experimental Procedures." Values given are the means of two to five separate experiments. ND signifies that the value was not determined.

Mutant	Wild-type residue	Receptor molecular mass	fmol/mg	K_d	
				Isoproterenol	Alprenolol
		kDa		*M*	
pSVL			50		
βAR		67	500	2×10^{-7}	9×10^{-10}
[Ala^{120}]βAR	Ser	NP	25	ND	ND
[Ala^{161}]βAR	Ser	67	725	1×10^{-7}	ND
[Ala^{165}]βAR	Ser	NP	75	ND	ND
[Ala^{203}]βAR	Ser	NP	60	ND	ND
[Ala^{204}]βAR	Ser	67	650	5×10^{-6}	7×10^{-10}
[Ala^{207}]βAR	Ser	67	490	5×10^{-6}	1×10^{-9}
[Leu^{219}]βAR	Tyr	67	315	2×10^{-7}	6×10^{-10}
[Ala^{316}]βAR	Tyr	55	95	ND	ND
[Ala^{319}]βAR	Ser	67	450	2×10^{-6}	9×10^{-10}

TABLE II

Ligand binding characteristics of mutant βAR

The binding of ligands 1–8 (structures as indicated) to wild-type βAR, [Ala^{204}]βAR, and [Ala^{207}]βAR was measured by competition with 35 pM ^{125}I-CYP, as described under "Experimental Procedures," using 10^{-10}–10^{-3} M competing ligands.

	Ligand	Kd (M) βAR	Kd (M) [Ala^{204}]βAR	Kd (M) [Ala^{207}]βAR
(1)		6.1×10^{-7}	1.8×10^{-5}	2.2×10^{-5}
(2)		2.0×10^{-7}	5.2×10^{-6}	5.1×10^{-6}
(3)		9.0×10^{-4}	$>5 \times 10^{-3}$	$>5 \times 10^{-3}$
(4)		4.1×10^{-6}	7.1×10^{-6}	2.4×10^{-6}
(5)		7.4×10^{-6}	4.6×10^{-5}	1.2×10^{-5}
(6)		1.3×10^{-5}	1.3×10^{-4}	2.5×10^{-5}
(7)		3.0×10^{-5}	1.1×10^{-4}	1.5×10^{-5}
(8)		5.2×10^{-6}	6.7×10^{-6}	6.7×10^{-6}

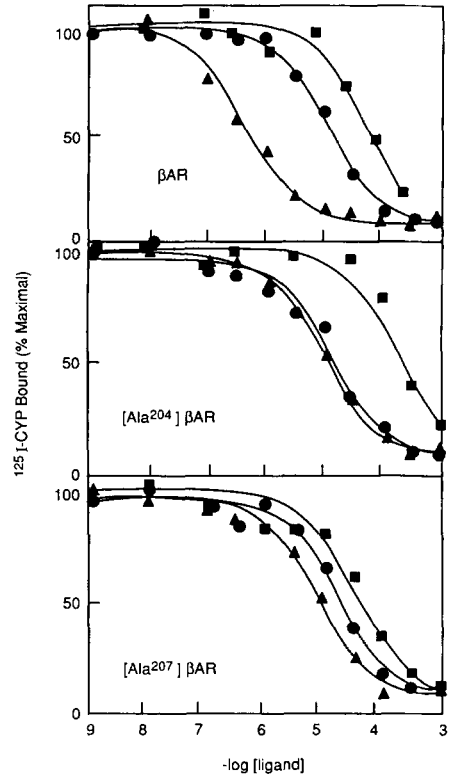


FIG. 1. **Competition binding of ligands with substituted catechol hydroxyls to wild-type and mutant βAR.** Competition binding was performed on membranes prepared from transfected COS-7 cells as described under "Experimental Procedures," using 35 pM ^{125}I-CYP in the presence of isoproterenol (compound 2 in Table II) (▲), ligand 8 (●), or ligand 7 (■). The structures of the ligands are given in Table II. The results for the wild-type βAR are shown in the *top panel*, [Ala^{204}]βAR in the *middle panel*, and [Ala^{207}]βAR in the *bottom panel*. Maximal ^{125}I-CYP binding was 0.5–1.5 fmol/tube in 250 μl. The experiment shown is representative of two similar experiments.

receptor was decreased by 1500-fold, whereas the affinities of [Ala^{204}]βAR and [Ala^{207}]βAR were reduced by >8000-fold, suggesting that the effects of the serine substitutions in the receptor were additive with the effects of the β-hydroxyl modification in the ligand.

The *meta-* or the *para*-catechol hydroxyl groups of the ligand were replaced, either separately or together, in the series of compounds shown as ligands 4–8 in Table II. Except for the *tert*-butyl-substituted amine group in compounds 4 and 5, the only difference among these ligands was in the positions of the hydroxyl groups on the aromatic ring. Removal of both of these hydroxyl groups to give the unsubstituted phenyl compound 4 resulted in a 15-fold decrease in the affinity of the ligand for the wild-type βAR, compared to isoproterenol. The affinity of compound 4 was not further decreased by the substitution of an alanine residue for Ser^{204} or Ser^{207}, so that βAR = [Ala^{204}]βAR = [Ala^{207}]βAR for binding to the unsubstituted phenyl compound (Table II). In compounds 5–7, the *para*-hydroxyl group of the agonist was replaced by a hydrogen, either alone (compound 7) or with the addition of a second hydroxyl at the other meta position on the ring (compounds 5 and 6). These ligands showed a relative affinity for the three mutant receptors of βAR = [Ala^{207}]βAR > [Ala^{204}]βAR (Table II). Replacement of the *meta*-hydroxyl group of isoproterenol with a hydrogen (compound 8) caused a 10-fold reduction in affinity for the wild-type βAR. Interestingly, this compound showed the same affinity for the two mutant receptors in which the serine residues at positions 204 or 207 were replaced, such that βAR = [Ala^{204}]βAR = [Ala^{207}]βAR (Table II).

The individual contributions of the *meta-* and *para*-hydroxyl groups to the binding interactions with the wild-type and mutant βARs are compared in Fig. 1. Removal of either hydroxyl group from the ligand caused a decrease in affinity for the wild-type βAR (Fig. 1, *top*). With [Ala^{204}]βAR, removal of the *meta*-hydroxyl group from the ligand did not alter its binding affinity, whereas substitution of the *para*-hydroxyl moiety dramatically reduced the affinity of the ligand for this mutant receptor (Fig. 1, *middle*). In contrast, the binding of [Ala^{207}]βAR was less sensitive to the removal of the hydroxyl groups from the ligand (Fig. 1, *bottom*).

In order to examine the agonist activation of the wild-type and mutant receptors by various ligands, stable clonal L cell lines expressing the βAR, [Ala^{204}]βAR, or [Ala^{207}]βAR were established, and the coupling of the receptors to the G_s-adenylyl cyclase effector system was measured. Receptor-G_s coupling was determined by the ability of the nonhydrolyzable GTP analog Gpp(NH)p to decrease the affinity of the receptor for isoproterenol. As seen in Fig. 2, isoproterenol binding to the wild-type and both mutant βARs was responsive to Gpp(NH)p, indicating that all three of these receptors were coupled to a G-protein in these cells. The ability of the modified ligands to function as agonists to promote the activation of the wild-type and mutant receptors was determined by measuring stimulation of adenylyl cyclase activity, with

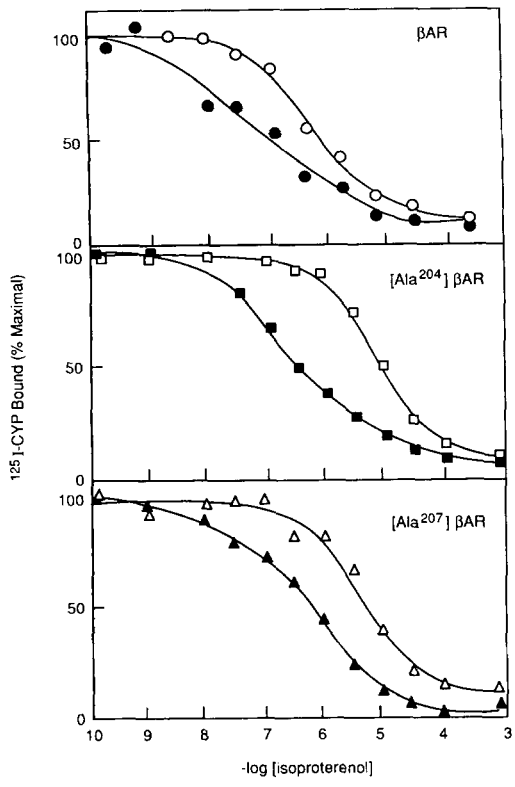


FIG. 2. **Effects of Gpp(NH)p on agonist binding to the wild-type and mutant βAR.** Isoproterenol binding to membranes prepared from L cells expressing the βAR (*top*), [Ala^{204}]βAR (*middle*), or [Ala^{207}]βAR (*bottom*) was performed as described under "Experimental Procedures" in the absence (*closed symbols*) or the presence (*open symbols*) of 100 μM Gpp(NH)p.

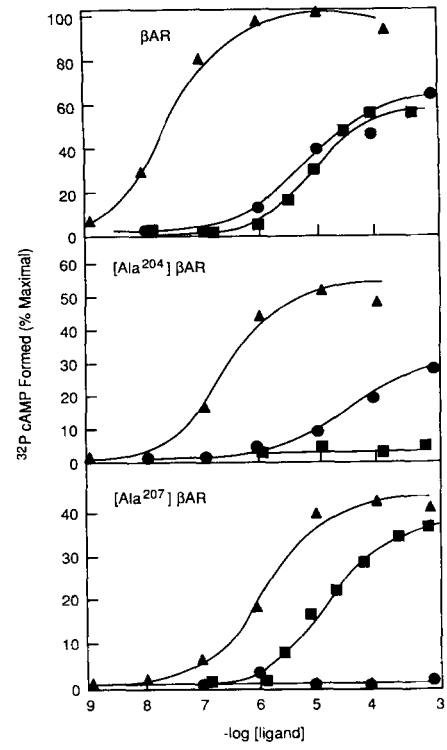


FIG. 3. **Adenylyl cyclase stimulation by wild-type and mutant βAR.** Adenylyl cyclase stimulation was measured on membranes prepared from L cells expressing βAR (*top panel*), [Ala^{204}]βAR (*middle panel*), and [Ala^{207}]βAR (*bottom panel*), as described under "Experimental Procedures." The data are presented as percentage of the maximal stimulation (picomoles of ^{32}P-cAMP/mg of protein/min) obtained with 10 mM NaF. Stimulating ligands were isoproterenol (compound 2 in Table II) (▲), ligand 8 (●), and ligand 7 (■). The experiment shown is representative of two similar experiments.

TABLE III

Adenylyl cyclase stimulation by mutant βAR

The wild-type and mutant receptors, as indicated, were expressed in L cells, and adenylyl cyclase stimulation by the ligands listed in column 1 was measured on membranes prepared from the cells. The structures of the ligands are given in Table II. K_{act} and V_{max} were calculated as described under "Experimental Procedures." V_{max} is expressed as percentage of the maximal stimulation with 10 mM NaF. Results are the means of two to four separate experiments.

Ligand	βAR		[Ala^{204}]βAR		[Ala^{207}]βAR	
	K_{act}	V_{max}	K_{act}	V_{max}	K_{act}	V_{max}
	M	%	*M*	%	*M*	%
2	4.3×10^{-8}	100	4.3×10^{-7}	54	5.8×10^{-6}	40
4	3.0×10^{-5}	16	1.6×10^{-5}	14	2.5×10^{-6}	36
5	3.1×10^{-6}	91	2.3×10^{-5}	37	2.5×10^{-6}	53
6	4.5×10^{-6}	98	4.6×10^{-5}	43	6.3×10^{-6}	45
7	9.2×10^{-6}	67		0	1.0×10^{-5}	37
8	3.1×10^{-6}	64	2.8×10^{-5}	27		0

the results summarized in Table III and Fig. 3. Whereas isoproterenol functioned as a full agonist at the wild-type βAR, this ligand was only a partial agonist with reduced affinity at [Ala^{204}]βAR or [Ala^{207}]βAR. Removal of both of the catechol hydroxyl groups (compound 4) resulted in a large decrease in the efficacy of the ligand at the mutant and wild-type receptors (Table III). Simple replacement of the *para*-hydroxyl substituent of isoproterenol with a hydrogen (compound 7) caused a decrease in adenylyl cyclase stimulation by the wild-type and [Ala^{207}]βAR. In addition, this ligand failed to activate [Ala^{204}]βAR at concentrations up to 10^{-3} M (Table III and Fig. 3). A similar pattern of activities was observed with the full agonist metaproterenol (compound 6). Replacement of the *meta*-hydroxyl of isoproterenol with a hydrogen (compound 8) resulted in partial agonist activity at the wild-type βAR. The efficacy of this compound was also reduced at [Ala^{204}]βAR, whereas the compound failed to stimulate [Ala^{207}]βAR at all (Table III and Fig. 3).

DISCUSSION

Pharmacaphore mapping of adrenergic agonists has suggested that hydrogen bonding interactions with the catechol hydroxyl groups on the aromatic ring of the ligand are essential for agonist binding and activation of the βAR (29–31). In the present study, we have used site-directed mutagenesis of the βAR to investigate the molecular basis for these putative hydrogen bonding interactions. Molecular replacement of conserved amino acids which are potential hydrogen bond donors or acceptors revealed that four of these residues, located in hydrophobic domains 3, 4, 5, and 7, are required for proper folding and membrane insertion of the receptor. Thus, hydrogen bonding interactions involving hydroxyl amino acid side chains may be important in maintaining the secondary or tertiary structure of the βAR. It is interesting that substitution of alanine for Ser^{203} resulted in the absence of normally processed receptor protein, whereas substitution of the adjacent Ser^{204} residue did not appear to affect the folding of the receptor. Thus, the side chain of Ser^{203} may be important for structural interactions essential for receptor stability, while Ser^{204} is not.

Replacement of either Ser^{204} or Ser^{207} in the fifth hydrophobic domain of the receptor with an alanine residue removing the hydroxyl group from the amino acid side chain,

reduced the affinity and efficacy of the receptor for catecholamine agonists but not for antagonists. The normal antagonist binding properties which were observed for these mutant receptors argue for the disruption of a specific receptor-agonist interaction by these mutations, rather than general disruption of protein structure. The decrease in binding energy upon substitution of either Ser^{204} or Ser^{207} ($\Delta\Delta G$ = +1.9 kcal/mol) is consistent with their involvement in hydrogen bonding interactions with the ligand. Hydrogen bonds between these serine residues and the β-hydroxyl group of the ligand seem unlikely, since the β-hydroxyl moiety is common to both agonists and antagonists. The absence of a direct interaction between the serine side chains and the β-hydroxyl substituent is further supported by the additivity of the decreases in binding affinity measured upon substitution of either serine residue and the β-hydroxyl group. In contrast, the decreased agonist activity seen at the wild-type βAR for the ligand in which both of the catechol hydroxyl groups were removed (compound 4) was not additive with the decreases observed upon substitution of Ser^{204} or Ser^{207}. These data are consistent with the existence of agonist-specific hydrogen bonds linking the catechol hydroxyl groups of isoproterenol to the hydroxyl side chains of Ser^{204} and Ser^{207} in the wild-type receptor. Furthermore, as discussed below and outlined schematically in Fig. 4, the data suggest that the *meta*-hydroxyl group of the ligand interacts preferentially with the side chain of Ser^{204}, whereas the hydroxyl group at the *para* position of the ligand interacts with the Ser^{207} side chain in the receptor.

Substitution of the *para*-hydroxyl group of isoproterenol with a hydrogen generates compound 7, which has a single hydroxyl substituent at the *meta* position of the phenyl ring. This compound showed decreased affinity and efficacy at the wild-type receptor, reflecting the loss of the hydrogen bond to the absent *para*-hydroxyl group of the ligand (Fig. 4*c*). The affinity of compound 7 was further decreased and its partial agonist activity abolished by the substitution of alanine for Ser^{204}, suggesting the removal of a second receptor-ligand interaction (Fig. 4*i*). These data are consistent with the existence of a hydrogen bond between the *meta*-hydroxyl group of the catecholamine and Ser^{204} in the wild-type receptor. In contrast, the replacement of Ser^{207} and the substitution of the *para*-hydroxyl group of the ligand had similar, nonadditive effects on receptor-agonist interactions. The lack of additivity of these effects suggests that the same hydrogen bond is removed by both substitutions (Fig. 4*f*), consistent with the existence of a hydrogen bond between Ser^{207} in the wild-type βAR and the *para*-hydroxyl group of isoproterenol. Further support for these hydrogen bond assignments arises from an examination of the binding and activation properties of compound 8, in which the *meta*-hydroxyl group has been removed, leaving a single hydroxyl substituent at the *para* position of the phenyl ring. This ligand exhibited decreased agonist activity at the wild-type receptor, consistent with the loss of a hydrogen bond between the receptor and the absent *meta*-hydroxyl substituent of the ligand (Fig. 4*b*). Neither the affinity nor the efficacy of this ligand was further reduced by the substitution of alanine for Ser^{204}, further supporting the interaction of the *meta*-hydroxyl group with Ser^{204} in the wild-type βAR (Fig. 4*h*). The effects of the substitution of Ser^{207} in the receptor and the substitution of the *meta*-hydroxyl group of the ligand were additive with respect to agonist efficacy, but not with respect to ligand binding. This apparent contradiction might be explained if hydrogen bonding between the *para*-hydroxyl group of the ligand and the hydroxyl side chain of Ser^{204} could occur in the absence of both Ser^{207} and the *meta*-hydroxyl substituent (Fig. 4*e*). This hydrogen bond could serve to increase the affinity of [Ala^{207}]βAR for compound 8, but would presumably not allow the receptor to assume its active conformation, resulting in a lack of agonist activity.

The data presented here are consistent with the model of the ligand binding site for the βAR presented in Fig. 5*A*. Previous mutagenesis studies suggested that the binding of agonists and antagonists to the receptor involves ionic interactions between the amine group of the ligand and the carboxylate side chain of Asp^{113} in the third hydrophobic domain of the receptor (16, 19). Structure-activity analysis of adrenergic ligands has shown the amine moiety of the ligand to be essential for the interaction of both agonists and antagonists with the βAR (29–31), aiding in the assignment of this specific molecular interaction. In the present study, we pre-

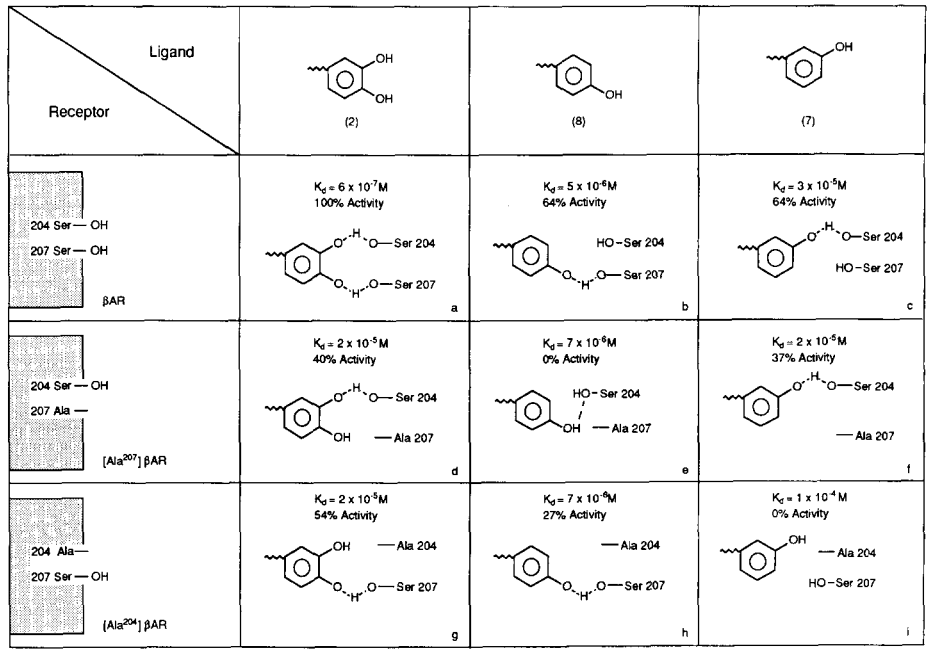
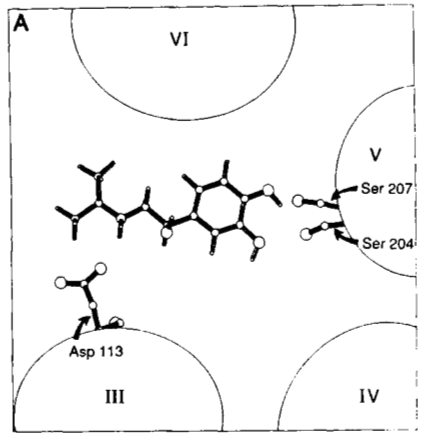


FIG. 4. **Schematic diagram of hydrogen bonding interactions postulated for agonist binding to the βAR.** The residues at positions 204 and 207 of the wild-type βAR, [Ala^{207}]βAR, and [Ala^{204}]βAR are shown along the *left* side. The substituted phenyl rings of compounds 2, 8, and 7 are shown along the *top*. The *intersecting squares* (labeled *a–i*) show the hydrogen bonding interactions which are postulated to occur for each receptor-ligand combination. The K_d values in each *square* are from Table II, and the activity values are the V_{max} values from Table III.



B

	198																			220
ham β_2	A	Y	A	I	A	S	S	I	V	S	F	Y	V	P	L	V	V	M	V	F	V	Y	S
hum β_1	A	Y	A	I	A	S	S	V	V	S	F	Y	V	P	L	C	I	M	A	F	V	Y	L
hum α_{2B}	W	Y	V	I	S	S	C	I	G	S	F	F	A	P	C	L	I	M	I	L	V	Y	V
hum α_{2A}	W	Y	I	L	S	S	C	I	G	S	F	F	A	P	C	L	I	M	G	L	V	Y	A
ham α_1	F	Y	A	L	F	S	S	L	G	S	F	Y	I	P	L	A	V	I	L	V	M	Y	C
rat D_2	A	F	V	V	Y	S	S	I	V	S	F	Y	V	P	F	I	V	T	L	L	V	Y	I
rat 5HT-1c	N	F	V	L	I	G	S	F	V	A	F	F	I	P	L	T	I	M	V	I	T	Y	F
hum 5HT-1a	G	Y	T	I	Y	S	T	F	G	A	F	Y	I	P	L	L	L	M	L	V	L	Y	G
rat 5HT-2	N	F	V	L	I	G	S	F	V	A	F	F	I	P	L	T	I	M	V	I	T	Y	F
hum M1	I	I	T	F	G	T	A	M	A	A	F	Y	L	P	V	T	V	M	C	T	L	Y	W
hum M2	A	V	T	F	G	T	A	I	A	A	F	Y	L	P	V	I	I	M	T	V	L	Y	W
hum M3	A	V	T	F	G	T	A	I	A	A	F	Y	L	P	V	V	I	M	T	V	L	Y	I
hum M4	T	I	T	F	G	T	A	I	A	A	F	Y	M	P	V	T	I	M	T	I	L	Y	W
hum ops	S	F	V	I	Y	M	F	V	V	H	F	I	I	P	L	I	V	I	F	F	C	Y	G
bov sub k	Y	H	L	I	V	I	A	L	I	Y	F	L	-	P	L	V	V	M	F	V	A	Y	S

FIG. 5. *A*, model of the agonist isoproterenol in the ligand binding site of the βAR, viewed from the surface of the plasma membrane. The circles at the edge of the figure represent the proposed arrangement of four of the seven transmembrane helices of the βAR (numbered *III–VI*), based on the published model of the structure of rhodopsin (24). The carboxyl side chain of Asp^{113} in helix III is shown interacting with the amino group of the ligand. The hydroxyl side chains of Ser^{204} and Ser^{207} in helix V are shown interacting with the *meta*- and *para*-catechol hydroxyl groups, respectively, of the ligand. *B*, sequences of the fifth hydrophobic domains of G-protein-coupled receptors. Receptor sequences are from Refs. 1–15. The single letter amino acid code is used. The receptor subtype and the species from which it was cloned are listed to the left of the amino acid sequences (*ham* = hamster; *hum* = human; *bov* = bovine). Sequences were aligned to maximize homologies within this region, which was identified by hydropathy analysis of the amino acid sequences, as described (1–15). The numbers at the top signify the positions of the corresponding amino acids in the primary sequence of the hamster β_2AR. *Arrows* designate Ser^{204} and Ser^{207}.

sent evidence for an agonist-specific interaction between the catechol hydroxyl groups of the ligand and the hydroxyl side chains of Ser^{204} and Ser^{207} in the fifth transmembrane helix of the receptor. Further, the data suggest that Ser^{204} recognizes the *meta*-hydroxyl group of the ligand and Ser^{207} interacts with the *para*-hydroxyl group. These 2 serine residues are located three amino acid positions apart in a region of the receptor which is predicted to form a transmembrane α-helix, suggesting that these residues are positioned a single helical turn apart on the same face of the helix. Hydrogen bonds between the catechol hydroxyl groups of the agonist and the 2 serine residues could exist simultaneously by constraining the rotation of the ligand. The exact conformation of the catechol ring of the ligand in the binding pocket of the receptor is probably determined by other interactions in addition to these hydrogen bonds and awaits further biophysical and genetic analysis of the βAR.

Further evidence in support of the model for the agonist binding site of the receptor presented here arises from analysis of the primary sequences of the fifth hydrophobic domains of other G-protein-coupled receptors. As shown in Fig. 5*B*, when the sequences of G-protein-linked receptors are aligned for maximum homology, serine residues analogous to Ser^{204} and Ser^{207} are present in the α_1-, α_{2A}-, α_{2B}-, β_1-, and β_2-adrenergic and D_2-dopamine receptors, all of which bind catecholamine ligands. These serine residues are not conserved with other receptors of this class whose ligands lack the catechol hydroxyl groups (1–15). In both the α_{2A} and α_{2B} receptor subtypes, the Ser residues which appear to be analogous to Ser^{204} and Ser^{207} in the βAR are separated from each other by the insertion of an additional cysteine residue immediately C-terminal to the Ser^{204} homolog (see Fig. 5*B*). This would dictate a slightly different orientation of the agonist aromatic ring in the binding pocket of the α_2-receptor. Alternatively, the hydrogen bond to the *meta*-hydroxyl group of the agonist could involve the sulfhydryl side chain of the cysteine residue in the α_2-receptor. Interestingly, the three subtypes of serotonin receptors whose sequences are known all have serine or threonine residues at a position equivalent to that of Ser^{204} in the βAR (9, 10, 14). The side chains of these amino acids may be involved in the binding of the 5-hydroxyl group of serotonin to the receptor.

The proposed localization of the agonist-specific hydrogen bonding interactions in the fifth transmembrane helix of the βAR suggests a mechanism for agonist activation of the receptor. The interaction of receptors with G-proteins has been postulated to involve residues within the third intracellular loop of the receptor, which connects the fifth and sixth transmembrane helices (24–28). Specifically, the interaction of the βAR with G_s requires a stretch of residues at the N-terminal portion of this loop, predicted to form a cytoplasmically exposed amphipathic α-helix located at the bottom of the fifth transmembrane helix (25, 26). If agonist interactions with the receptor involve the formation of specific hydrogen bonds with serine residues in helix 5, then the binding might cause conformational changes in this helix which could be transmitted to the residues at the bottom of the helix, catalyzing the interaction of this region with G_s. Antagonists, which do not appear to interact with Ser^{204} and Ser^{207}, would not be expected to promote this conformational change. Further biophysical analysis of the βAR will be necessary to define the molecular basis for the active conformation of the receptor.

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