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Amino Acid Substitutions at Position 312 in the Seventh Hydrophobic Segment of the β_2-Adrenergic Receptor Modify Ligand-Binding Specificity

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SUMMARY

We previously reported that Asn^{312} of the β_2-adrenergic receptor and Asn^{385} in the homologous position in the 5-hydroxytryptamine$_{1A}$ receptor are important for binding to a class of β-adrenergic receptor antagonists including propranolol and alprenolol. We proposed that the asparagine may be forming a hydrogen bond with the phenoxy oxygen common to these ligands. To further test this hypothesis we made alanine, threonine, phenylalanine, and glutamine substitutions at position 312 in the β_2-adrenergic receptor. We observed that substitution with amino acids that permit formation of hydrogen bonds (threonine and glutamine) supported binding to aryloxyalkylamines, whereas substitution with amino acids that cannot form hydrogen bonds (alanine and phenylalanine) did not permit binding to these compounds. We were surprised to find that two of these substitutions led to an increase in affinity for α-adrenergic ligands. Substitution with glutamine and threonine at position 312 led to a 11–15-fold increase in affinity for yohimbine and enabled *p*-aminoclonidine to act as an agonist. These results further emphasize the role of position 312 in the formation of the ligand binding site for multiple ligands.

The primary structures of all the guanine nucleotide-binding protein-coupled receptors cloned to date contain seven stretches of hydrophobic amino acids (reviewed in Refs. 1 and 2). Each of these hydrophobic segments is believed to span the plasma membrane as an α-helix. Mutagenesis, proteolysis, and biophysical studies showed that the ligand binding site of adrenergic receptors is formed by the seven helices (2–5). Asp^{113} of the β_2-adrenergic receptor is believed to form an ionic interaction with the amino group of both agonists and antagonists (6), and Ser^{204} and Ser^{207} may hydrogen bond with the *meta*- and *para*-hydroxyls of the catachol moiety of agonists (7, 8). Similar conclusions have been drawn from mutagenesis studies on the α_2C10-adrenergic receptor (9). Studies on chimeric α_2/β_2-adrenergic receptors revealed that the seventh hydrophobic segment is mainly responsible for subtype-specific binding of antagonists (10). To define the specificity further, Phe^{412} in the seventh hydrophobic segment of human α_2C10 was mutated to asparagine, the residue conserved in the homologous position in all cloned β-adrenergic receptors. This mutant receptor (α_2F→N) expressed in COS-7 cells or Raji cells showed a 300–3000-fold increase in affinity for aryloxyalkylamines such as alprenolol, pindolol, and propranolol and an approximately 370-fold decrease in affinity for the α_2 antagonists yohimbine, rauwolscine and atipamezole (11).[1] However, this mutant receptor showed only a 3.5-fold increase in affinity for sotalol, a β-adrenergic receptor antagonist that does not contain an ether oxygen atom linking the amino side chain with the aromatic ring.

5-HT$_{1A}$ serotonin receptors show high affinity binding to a class of β-adrenergic antagonists. Mutation of Asn^{385} (homologous to position 312 in the β_2-adrenergic receptor) in the seventh hydrophobic segment of the human 5-HT$_{1A}$ receptor to valine resulted in a selective decrease in affinity for aryloxyalkylamine-type β-adrenergic antagonists (about 100-fold), while producing only minor changes in the binding of other 5-HT agonists and antagonists (12). The affinity of labetalol, a β-adrenergic anatgonist containing no ether oxygen, was not reduced by this mutation in the 5-HT$_{1A}$ receptor. The change in free energy [$\Delta(\Delta G) = RT\ln[K_d(\alpha_2)/K_d(\alpha_2\text{F}\rightarrow\text{N})] = 2.8–4.7$ kcal/mol] associated with the increased affinity of the α_2F→N mutant receptor and the decreased affinity of the 5-HT$_{1A}$ mutant receptor for aryloxyalkylamines is consistent with formation of a hydrogen bond between asparagine in the seventh hydrophobic segment and the ligand. Based on these observations it was proposed that Asn^{312} of the β_2-adrenergic receptor is involved in forming a hydrogen bond with the ether oxygen atom of the aryloxyalkylamines (11). In an attempt to test this

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[1] S. Suryanarayana and B. K. Kobilka, unpublished observations.

ABBREVIATION: 5-HT, 5-hydroxytryptamine.

hypothesis directly, Asn^{312} of the human β_2-adrenergic receptor was substituted with glutamine, threonine, phenylalanine, or alanine and the pharmacological properties of the mutant receptors expressed in COS-7 cells or Raji cells were studied. The data presented in this report show that only the amino acids capable of forming a hydrogen bond through their side chain (glutamine and threonine but not phenylalanine or alanine) supported the binding of aryloxyalkylamines. Furthermore, substitutions at this position enhanced affinity for α receptor ligands.

Experimental Procedures

The details of the experimental procedures used for the construction and expression of mutant and chimeric receptors are described elsewhere (11, 13). In brief, the mutant and chimeric receptors were constructed by polymerase chain reaction techniques using human α_2C10 and β_2-adrenergic receptors cloned into the pGEM-3Z vector (Promega Biotec) as templates. After the authenticity of the DNA sequence was confirmed by sequence analysis, the wild-type, mutant, and chimeric receptor constructs were transferred from pGEM-3Z to eukaryotic expression vectors pBC12MI and pWS290 and were transfected into COS-7 cells and Raji cells by a DEAE-dextran technique and electroporation, respectively.

Ligand binding and agonist-stimulated adenylyl cyclase assays with membranes prepared from Raji cells 9–12 days after transfection were performed as described (13). Binding assays with membranes prepared from transfected COS-7 cells were performed 3 days after transfection. Nonspecific binding was determined by including 1 μM (−)-alprenolol in [^{3}H]dihydroalprenolol binding studies. Equilibrium dissociation constants were determined from saturation isotherms and competition curves. Saturation isotherm data were analyzed by a nonlinear, least squares, curve-fitting technique, and the competition data were analyzed according to a four-parameter logistic equation to determine EC_{50} values, using GraphPAD software (GraphPAD Software Inc., San Diego, CA).

Results and Discussion

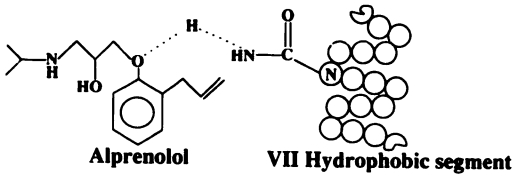
Evidence that high affinity binding of aryloxyalkylamines to the β_2-adrenergic receptor involves a hydrogen bond between Asn^{312} and the ligand. Previous mutagenesis studies on the human α_2C10-adrenergic receptor and the human 5-HT_{1A} serotonin receptor suggested that Asn^{312} in the seventh hydrophobic segment of the β_2-adrenergic receptor may be involved in forming a hydrogen bond with the ether oxygen of aryloxyalkylamines (Fig. 1A) (11, 12). Additional support for this hypothesis comes from the observation that changing the residue at position 412 in the α_2 receptor from phenylalanine to asparagine has little effect on the binding affinity for sotalol (11) or labetolol (Fig. 1B), two β receptor antagonists lacking the ether oxygen found in the aryloxyalkylamines. To further test this hypothesis, Asn^{312} of the human β_2-adrenergic receptor was replaced by glutamine and threonine, which have side chains capable of forming hydrogen bonds, or by alanine and phenylalanine, which have side chains that cannot form hydrogen bonds (Fig. 1, B and C). Only substitutions capable of forming a hydrogen bond (glutamine and threonine but not phenylalanine or alanine) resulted in a mutant receptor capable of binding to the β-antagonists alprenolol, pindolol, and propranolol.

Although the failure of the alanine and phenylalanine mutants to bind the aryloxyalkylamines could be due to the inability of these amino acids to form hydrogen bonds with the ligand, our results also suggest that substitutions at position 312 result in more general changes in the structure of the receptors that could influence binding to all ligands. The latter appears to be the case for Phe^{312}, because we previously showed that this mutation results in retention of the receptor in the endoplasmic reticulum (11). Furthermore, the affinities of the glutamine and threonine mutants for the agonists isoproterenol and epinephrine and for the nonaryloxyalkyamine antagonists sotolol and labetolol are reduced. Nevertheless, a comparison of the Ala^{312} mutant and the Thr^{312} mutant may provide support for the model shown in Fig. 1A. Although K_d values for the Ala^{312} mutant could not be determined (no binding of [^{3}H] dihydroalprenolol or [^{3}H]yohimbine could be detected), this mutant can mediate catecholamine stimulation of adenylyl cyclase in Raji cells (Fig. 2), and the EC_{50} values of isoproterenol and epinephrine for this mutant are essentially the same as those for the Thr^{312} mutant (Table 1). Thus, if agonist affinity can be used as an index of the influence of the amino acid substitution on the general structure of the receptor, the threonine and alanine substitutions have similar effects. Nevertheless, the threonine mutant is capable of binding alprenolol, whereas the alanine mutatnt is not.

Evidence that position 312 in the seventh hydrophobic segment influences β receptor affinity for α receptor ligands. As we demonstrated previously, substituting a phenylalanine at position 312 in the β_2 receptor (β_2N→F) led to a nonfunctional receptor due to an incompatibility between Phe^{312} and residues within the first or first and second hydrophobic segments of the β_2 receptor (14). This incompatibility could be overcome if the first two hydrophobic segments of β_2N→F were exchanged with the homologous sequence from the α_2 receptor. The resulting receptor, CRS11N→F, was correctly processed and bound yohimbine with high affinity, whereas CR1 (CRS11N→F without the N→F substitution) bound alprenolol with high affinity. These results suggested that Phe^{412} in the α_2 receptor, at a position homologous to Asn^{312} in the β receptor, is involved in forming the binding site for yohimbine. In the current studies, we observed that the threonine and glutamine substitutions at position 312 in the β receptor enhanced affinity for yohimbine (Table 1). The increased affinity of glutamine and threonine mutant receptors for the hydrophobic compound yohimbine could be due either to the greater hydrophobicity of these residues, compared with asparagine, or to general changes in the structure of the receptor.

The threonine mutant is of particular interest in the context of serotonin receptors. Human and rat 5-HT_{1A} and rat and mouse 5-HT_{1B} serotonin subtypes contain an asparagine at a position homologous to Asn^{312} of the β_2-adrenergic receptor. These serotonin subtypes bind aryloxyalkylamine-type β antagonists such as alprenolol, pindolol, and propranolol with affinities (10–30 nM) close to that of the β_2-adrenergic receptor and bind α_2 antagonists such as yohimbine and rauwolscine with low affinity (>4500 nM) (12, 15–18). In contrast, human 5-$HT_{1D\beta}$ and 5-$HT_{1D\alpha}$, canine 5-HT_{1D}, and *Drosophila* octopamine receptors contain a threonine residue in the homologous position and these receptors bind yohimbine and rauwolscine with affinities (10–40 nM) considerably higher than those for aryloxyalkylamines (>5000 nM) (19–26). Because asparagine in the seventh hydrophobic segment of human 5-HT_{1A} has already been shown to be responsible for high affinity binding of aryloxyalkylamines (12), it is tempting to speculate that the homologous threonine residue might be responsible for binding of α_2 antagonists. A recent report on site-directed mutagenesis of the human 5-$HT_{1D\beta}$ receptor supports this speculation. Substitution of threonine in the seventh hydrophobic segment of this receptor with an asparagine resulted in a considerable

(A)



(B)

	Amino acid residue at position 312 of β_2 adrenergic receptor: Asn	Phe	Gln	Thr	Ala	α_2	α_2F→N
(-)Alprenolol (nM)	0.29	(-)*	2.8	28.6	(-)*	6600	2.1
Pindolol (nM)	1.5	ND	0.9	79	ND	11070	25
(-)Propranolol (nM)	0.43	ND	7.7	854	ND	12000	40
Yohimbine (μM)	81	(-)*	7.3	5.4	(-)*	0.0025	0.92

(C)

	Amino acid residue at position 312 of β_2 adrenergic receptor: Asn	Phe	Gln	Thr	Ala
(-)Isoproterenol (μM)	0.47	ND	7.6	58	ND
(-)Epinephrine (μM)	1.9	ND	56	100	ND
p-Aminoclonidine (μM)	>1000	ND	326	>1000	ND

Fig. 1. A, Proposed model for the interaction of aryloxyalkylamines, represented by alprenolol, with the seventh hydrophobic segment of the β_2-adrenergic receptor. Our previously published results suggested that Asn^{312} in the seventh hydrophobic segment is involved in forming a hydrogen bond with the ether oxygen of the aryloxyalkylamines (11, 12). B and C, The effect of the substitution of Asn^{312} in the human β_2-adrenergic receptor with phenylalanine, glutamine, threonine, or alanine on the equilibrium dissociation constants (K_d or K_i) for some α and β antagonists (B) and agonists (C). The values are representative of two or three independent experiments. Methods used for the construction and expression of the mutants in Raji cells or COS-7 cells and the determination of the equilibrium dissociation constants were described in Experimental Procedures and in Ref. 13. *, No specific binding of [^{3}H]dihydroalprenolol or [^{3}H]yohimbine was observed at concentrations of up to 80 nM. *ND*, not determined.

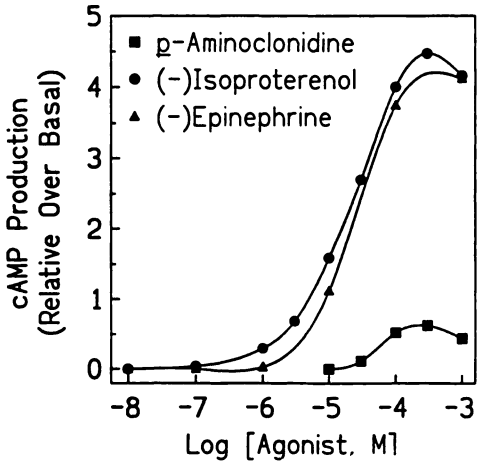


Fig. 2. Agonist-promoted stimulation of adenylyl cyclase in Raji cells by a β_2-adrenergic receptor with alanine substituted for Asn^{312}. The amount of cAMP produced by stimulation with varying concentrations of agonists was estimated as described before (13). The cyclase activity data were expressed as a fraction of the basal activity, that is, the difference between the agonist-stimulated value (X) and the basal value (Y) divided by the basal value [($X - Y$)/Y]. The *curves* are representative of two independent experiments.

TABLE 1

EC_{50} values of agonists for stimulation of adenylyl cyclase by wild-type and mutant receptors expressed in Raji cells

Agonist	EC_{50}: β_2	β_2N→F	β_2N→Q	β_2N→T	β_2N→A
			μM		
(−)-Isoproterenol (nM)	0.003	–[a]	0.3	15	20
(−)-Epinephrine (nM)	0.04	–	1.7	15	20
p-Aminoclonidine (nM)	NS	–	~170	50	55
Maximal stimulation[b] (%)	73		110	107	30

[a] –, No stimulation of adenylyl cyclase was observed at agonist concentrations of up to 1 mM. NS, no stimulation of adenylyl cyclase.

[b] % Maximal fluoride stimualtion: Maximal values were estimated by measuring adenylyl cyclase activity with 100 μM (−)-epinephrine and were normalized to stimulation with 10 mM NaF.

decrease in affinity for rauwolscine (about 46-fold) and an increase in affinity for aryloxyalkylamine β antagonists (27).

In addition to altering antagonist binding specficity, threonine, glutamine, and alanine substitutions resulted in mutant receptors capable of stimulating adenylyl cyclase in the presence of *p*-aminoclonidine, an α_2-adrenergic agonist that does not activate the wild-type β_2 receptor (Fig. 3). This result was surprising, because these substitutions did not markedly increase the receptor binding affinity for *p*-aminoclonidine (Table 1). Wild-type α_2 and β_2 receptors contain two different residues (phenylalanine and asparagine) at this position and yet they bind to epinephrine with similar affinities (14), suggesting that neither of these residues is involved in a direct interaction with epinephrine. Of interest, the glutamine mutant is most responsive to *p*-aminoclonidine, followed by the threonine and alanine mutants. This is not what one would expect because, of these three substitutions, glutamine is structurally most similar to asparagine and the glutamine substitution mutant would be expected to behave more like the wild-type β_2 receptor (as we observed for binding to aryloxyalkylamines). This suggests that the increased efficacy of *p*-aminoclonidine as an agonist for the

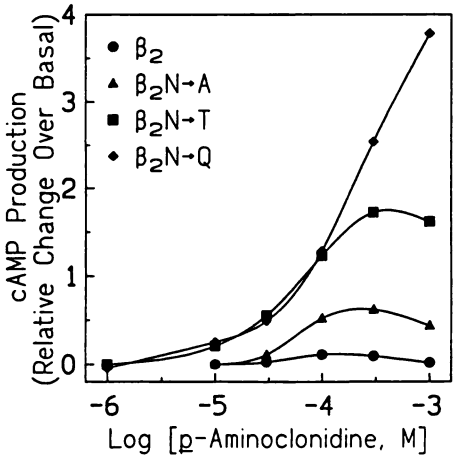


Fig. 3. *p*-Aminoclonidine-promoted stimulation of adenylate cyclase in Raji cells by the wild-type β_2-adrenergic receptor and the glutamine, threonine, and alanine mutant receptors. Details of the methods were described in Ref. 13 and in the legend to Fig. 2.

glutamine, threonine, and alanine mutant receptors may be due to global changes in receptor structure associated with the substitutions, rather than to specific interactions between the amino acid side chains and *p*-aminoclonidine.

In conclusion, the results presented above, together with our previously published studies, suggest that the side chain of the amino acid at position 312 in the β_2 receptor and position 412 in the α_2 receptor is involved in direct interactions with antagonists and may also be involved in intramolecular interactions that influence the tertiary structure of the receptor. High affinity binding of aryloxyalkylamines to the β_2-adrenergic receptor appears to involve a hydrogen bond between Asn^{312} and the ether oxygen of the ligand. Substitution with threonine or glutamine at this position of the β_2-adrenergic receptor enhances affinity for the α_2 antagonist yohimbine. We also observed that glutamine, threonine, and alanine substitutions led to the activation of adenylyl cyclase by *p*-aminoclonidine. The observation that mutations at this position alter both agonist activation and antagonist binding suggests that this position may be involved in the conformational changes associated with agonist activation.

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