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Site-Directed Mutagenesis of α_{2A}-Adrenergic Receptors: Identification of Amino Acids Involved in Ligand Binding and Receptor Activation by Agonists

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SUMMARY

Molecular cloning of the α_{2A}-adrenergic receptor has shown that this receptor is a member of the gene superfamily of guanine nucleotide-binding protein (G protein)-coupled receptors. The α_{2A}-adrenergic receptor expressed in Chinese hamster ovary (CHO) cells attenuates and potentiates forskolin-stimulated cAMP production through independent signaling pathways. To examine the role of three conserved aspartic acid and two conserved serine residues in α_{2A}-adrenergic receptor function, we substituted asparagine for aspartic acid or alanine for serine and characterized the mutant receptors in stably transfected CHO cells. Asn^{113} mutant α_2-adrenergic receptors display no [^{3}H] yohimbine specific binding, at concentrations up to 1000 nM. In transfected cells expressing the Asn^{113} mutant receptor, agonists, at concentrations up to 0.1 mM, produce small decreases (~10% of wild-type values) in forskolin-stimulated cAMP and potentiate forskolin-stimulated cAMP concentrations in a dose-dependent manner, with EC_{50} values ~500-fold higher than those for the wild-type receptor. These findings suggest that Asp^{113} may be involved in high affinity binding of agonists and antagonists, as has been previously reported for β_2-adrenergic and m1 muscarinic acetylcholine receptors. Asn^{79} mutant α_2-adrenergic receptors display high affinity agonist binding that is insensitive to guanine nucleotides, suggesting an altered receptor-G protein coupling. Furthermore, agonist binding to Asn^{79} mutant receptors elicits no change in forskolin-stimulated cAMP concentrations, similar to earlier findings that the corresponding residue in β_2-adrenergic and muscarinic receptors is required for effector stimulation. Asp^{130} appears to influence receptor-G protein coupling. Mutation of this residue eliminates high affinity, guanine nucleotide-sensitive, agonist binding and produces a rightward shift in the dose-response curves for agonist-mediated inhibition of forskolin-stimulated cAMP production, compared with the wild-type receptor. Moreover, agonist potentiation of forskolin-stimulated cAMP levels is abolished if Asp^{130} is replaced by Asn, supporting the hypothesis that inhibition and potentiation of forskolin-stimulated cAMP production in CHO cells proceed through distinct signaling pathways. Characterization of Ala^{204} mutant α_{2A}-adrenergic receptors suggests a possible role for Ser^{204} in hydrogen bond interactions with the *para*-hydroxyl group of the phenyl ring of the catecholamines, as has been previously described for the corresponding serine in β_2-adrenergic receptors. Analysis of Ala^{200} mutant α_{2A} receptors suggests that this residue does not directly participate in receptor-agonist interactions, in contrast to the corresponding serine residue in the β_2-adrenergic receptor, which has been postulated to interact with the *meta*-hydroxyl group of catecholamines. Data in this study, together with earlier reports on the mutagenesis of the β_2-adrenergic receptor, suggest that the molecular interactions between catecholamines and α-adrenergic and β-adrenergic receptors may be subtly different.

α_2-Adrenergic receptors modulate a number of physiological processes peripherally, including aggregation of platelets, release of insulin from the pancreas, and inhibition of lipolysis, and they are implicated in the control of noradrenergic activity in the brain (Ref. 1 and references therein). Pharmacological data have suggested that α_2-adrenergic receptors are not a homogeneous class of receptors and can be subclassified based on differences in ligand-binding properties. At least three distinct genes encoding α_2-adrenergic receptor subtypes have been isolated and characterized in heterologous expression systems (2–5). These studies have validated the hypothesis of multiple α_2 receptor subtypes that has been derived from pharmacological data.

Molecular cloning experiments have demonstrated that α_2-adrenergic receptors are members of a large gene superfamily that includes other receptors known to mediate the signal of hormone binding to intracellular effectors through G proteins (6–9). The hallmark of G protein-coupled receptors is the presence of seven stretches of hydrophobic amino acids that are presumed to form transmembrane helices, analogous to the

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ABBREVIATIONS: G protein, guanine nucleotide-binding protein; PCR, polymerase chain reaction; CHO, Chinese hamster ovary; bp, base pair(s).

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structure of bacteriorhodopsin (10). The primary sequences of the hydrophobic domains of G protein-coupled receptors display marked homology, whereas the sequences of the hydrophilic domains are more divergent.

Much information on the molecular properties of the ligand binding sites of adrenergic receptor subtypes has been inferred based on structure-activity relationships of adrenergic ligands (11). The Easson-Stedman hypothesis for phenethylamine binding to adrenergic receptors predicts that the optically active enantiomer will have three important functional groups in the correct configuration for interaction with adrenergic receptors (Ref. 11 and references therein). These include the aromatic ring, the nitrogen atom, and the β-hydroxyl group. Site-directed mutagenesis experiments with β-adrenergic receptors have identified an aspartic acid in transmembrane domain III and two serines in transmembrane domain V as potential sites of interaction with the nitrogen moiety and the catechol hydroxyl groups of the phenethylamine ligands, respectively (12, 13). Two other conserved aspartic acid residues in the second and third transmembrane domains have been shown to influence agonist binding and be essential for normal β_2-adrenergic and m1 muscarinic receptor-mediated activation of G proteins (14, 15).

Because catecholamines serve as endogenous ligands for both α-adrenergic and β-adrenergic receptors, it was of interest to examine whether amino acids implicated in β receptor mechanisms also play a role in α receptor function. In this report, we utilize site-directed mutagenesis of human α_{2A}-adrenergic receptors to investigate the role of three conserved aspartic acid and two conserved serine residues in receptor function. These data were compared with results previously reported on analysis of β-adrenergic receptor function using mutagenesis techniques (12–14, 16).

Experimental Procedures

Materials. Tissue culture reagents were from GIBCO Laboratories. CHO-K1 cells were from the American Type Culture Collection. [^{3}H] Yohimbine (70–90 Ci/mmol) and cAMP radioimmunoassay kits were from New England Nuclear/Dupont. Restriction endonucleases were from Bethesda Research Laboratories or New England Biolabs. pSVL was from Pharmacia. Phentolamine (Regitine) was a generous gift from CIBA-GEIGY; prazosin and UK-14304 were generously provided by Pfizer. All other reagents were from Sigma or as previously described (14, 15, 17).

Site-directed mutagenesis and continuous expression of receptors in CHO cells. For construction of site-directed mutant receptors, a 1.5-kilobase *Nco*I-*Hind*III fragment of a genomic clone containing the entire coding region of the human α_{2A}-adrenergic receptor (4) was inserted into the *Sma*I-*Hind*III site of M13mp19. The single base mutation that converts Asp (GAC) to Asn (AAC) or Ser (TCC) to Ala (GCC) was introduced into the human α_2-adrenergic receptor gene at base 235, 337, 388, 598, or 610 by oligonucleotide-directed mutagenesis, according to the method of Kunkel (18). Authenticity of each mutation was confirmed by single-stranded dideoxy sequencing, using a sequencing kit from Promega. For gene expression, a 1.5-kilobase *Hind*III-*Kpn*I fragment from M13mp19 was blunt-ended and inserted into the *Sma*I site of the plasmid expression vector pSVL (Pharmacia, LKB Biotechnology, Inc.). CHO-K1 cells (American Type Culture Collection, Rockville, MD), which do not express α_2-adrenergic receptors, were co-transfected with pSVL containing the wild-type or mutant α_2-adrenergic receptor gene and pMSVneo (14) containing the dominant selectable marker aminoglycoside phosphotransferase, using the $CaPO_4$ precipitation method (19). Two days after transfection, cells were placed in selective medium containing Geneticin (G-418, 500 μg/ml). Colonies were isolated and expanded; membranes were prepared from transfected cells and assayed for the presence of α_2-adrenergic receptors using [^{3}H]yohimbine.

Radioligand binding assays. Membrane-associated α_2-adrenergic receptors (25 μg of membrane protein) were labeled with increasing concentrations of [^{3}H]yohimbine, in the presence or absence of 5 μM phentolamine, by incubation at 37° for 30 min in Tris/saline buffer (150 mM NaCl, 20 mM EDTA, 50 mM Tris, pH 7.4), in a final incubation volume of 250 μl. Incubations were terminated by filtration over Whatman GF/C glass fiber filters using a Brandel cell harvester, and each filter was washed with 10 ml of 20 mM $NaPO_4$ buffer, pH 7.4. Scatchard analysis of saturation isotherms was performed to yield estimates of K_D (equilibrium dissociation of [^{3}H]yohimbine) and B_{max} (total number of binding sites). The K_D value determined for each receptor was utilized in computer analysis of competition displacement curves.

For competition displacement studies, membranes (containing 30–40 fmol of [^{3}H]yohimbine binding sites) were incubated with [^{3}H] yohimbine (0.75–1.25 × the K_D concentration) plus the indicated concentrations of competing ligands. In some agonist competition displacement experiments, GTP (100 μM final concentration) was included in the assay buffer. Incubations were performed and filtered as described above.

Analysis of data. Competition displacement curves were analyzed according to a mass action model for receptor-ligand interactions, using a computerized iterative nonlinear least-squares curve-fitting program (GraphPAD INPLOT). Competition displacement experiments were performed three to six times in triplicate. Triplicate values from each experiment were averaged, and nonlinear regression was performed on averaged data from all competition displacement curves for a single ligand. A two-site model for ligand binding was evaluated for its statistical superiority over a single-site model using an F test based on the residual variance between the actual and predicted data points. If p values were <0.05, the two-site model was chosen as the better fit of the data.

PCR amplification of RNA. Poly(A)$^+$ RNA was isolated from control or transfected cells using a QuickPrep mRNA purification kit (Pharmacia, Milwaukee, WI). α_2-Adrenergic receptor-specific RNA sequences were amplified using cDNA and PCR methodologies. For the reverse transcription reaction, 500 ng of poly(A)$^+$ RNA, 1 mM each dATP, dCTP, dGTP, and dTTP, 1 unit/μl RNasin (Promega Corporation), 100 pmol of random hexamer oligonucleotides (Pharmacia), and 200 units of Moloney murine leukemia virus (Bethesda Research Laboratories) were combined in a final volume of 20 μl of 1 × PCR buffer (10 mM Tris·HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin; Perkin-Elmer Cetus). Samples were incubated at 23° for 10 min and then at 42° for 60 min. Reactions were heated at 95° for 10 min and quick-chilled on ice. For PCR amplification, 10 μl of the reverse transcription reaction were added to 40 μl of 1 × PCR buffer containing 25 pmol each of upstream and downstream primers and 1 unit of AmpliTaq DNA polymerase (Perkin-Elmer Cetus). The upstream primer was 5′-CACGCTCGTCATCCCTTT-3′ and the downstream primer was 5′-GACGAGCTCTCCTCCAGG-3′, which are complementary to bases 249–266 of the (−)-strand of the coding region of the α_2-adrenergic receptor and to bases 876–893 of the (+)-strand of the coding region of the α_2-adrenergic receptor, respectively. This primer pair amplifies a 644-bp target fragment of DNA from the α_2-adrenergic receptor sequence. Thermal cycling was performed for 35 cycles of 1) 94° for 1 min, 2) 56° for 2 min, and 3) 72° for 3 min. After the last cycle, the extension step at 72° was increased to 7 min. An aliquot of each PCR reaction (10 μl) was analyzed on a 1.6% agarose gel containing 0.5 μg/ml ethidium bromide.

Determination of agonist-induced changes in intracellular cAMP levels. Confluent cultures of cells expressing wild-type or mutant α_2-adrenergic receptors were incubated in Hanks' solution, containing 1 mM 3-isobutyl-1-methylxanthine, for 20 min at 37°. The indicated concentrations of agonists, with or without 10 μM forskolin,

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were added to the cultures, which were incubated for an additional 20 min at 37°. The solution containing the drugs was aspirated from the plates and saved; intracellular cAMP was extracted from cells with either 6% trichloroacetic acid or cold 0.1 N HCl, 0.1 mM $CaCl_2$ (adapted from Ref. 20). Both solutions were combined, and the total cAMP (extracellular and intracellular) concentration in each sample was determined using a cAMP radioimmunoassay kit. For certain experiments, cells were pretreated with pertussis toxin (100–200 ng/ml; List) for 18 hr at 37°. Data are expressed as percentage of forskolin-stimulated cAMP production. Forskolin produced an average increase in intracellular cAMP levels of 20.5 ± 2.9-fold basal levels.

Results

To examine the function of conserved aspartic acid residues at positions 79, 113, and 130 and serine residues at positions 200 and 204 in α_{2A}-adrenergic receptor function (Fig. 1), the amino acid sequence of the receptor was modified using site-directed mutagenesis and wild-type or mutant receptor genes were permanently expressed in CHO cells. Aspartic acid was replaced with asparagine, eliminating the negative charge on the side chain of this amino acid. Serine was replaced with alanine, removing the hydroxyl moiety of this residue.

Using established procedures, we obtained multiple colonies of cells from each transfection. Ten to 15 colonies from each experiment were isolated, expanded, and assayed for the presence of α_2-adrenergic receptors using [^{3}H]yohimbine. A majority (>75%) of colonies assayed expressed α_2 receptors at densities ranging from approximately 300 to 6000 fmol of receptor/mg of membrane protein.

We previously reported that agonist activation of α_{2A}-adrenergic receptors expressed in CHO cells produces a biphasic response associated with an inhibition of forskolin-stimulated cyclic AMP production at low epinephrine (up to 0.1 μM) concentrations that reverses at higher epinephrine (>0.1 μM) doses (4). In cells pretreated with pertussis toxin, agonists potentiate forskolin-stimulated cAMP levels through a mechanism distinct from that involved in inhibition of adenylate cyclase and independent of G_i (4). We selected for study cell lines that expressed similar receptor densities, because we had previously observed that the magnitude of α_{2A}-adrenergic re-

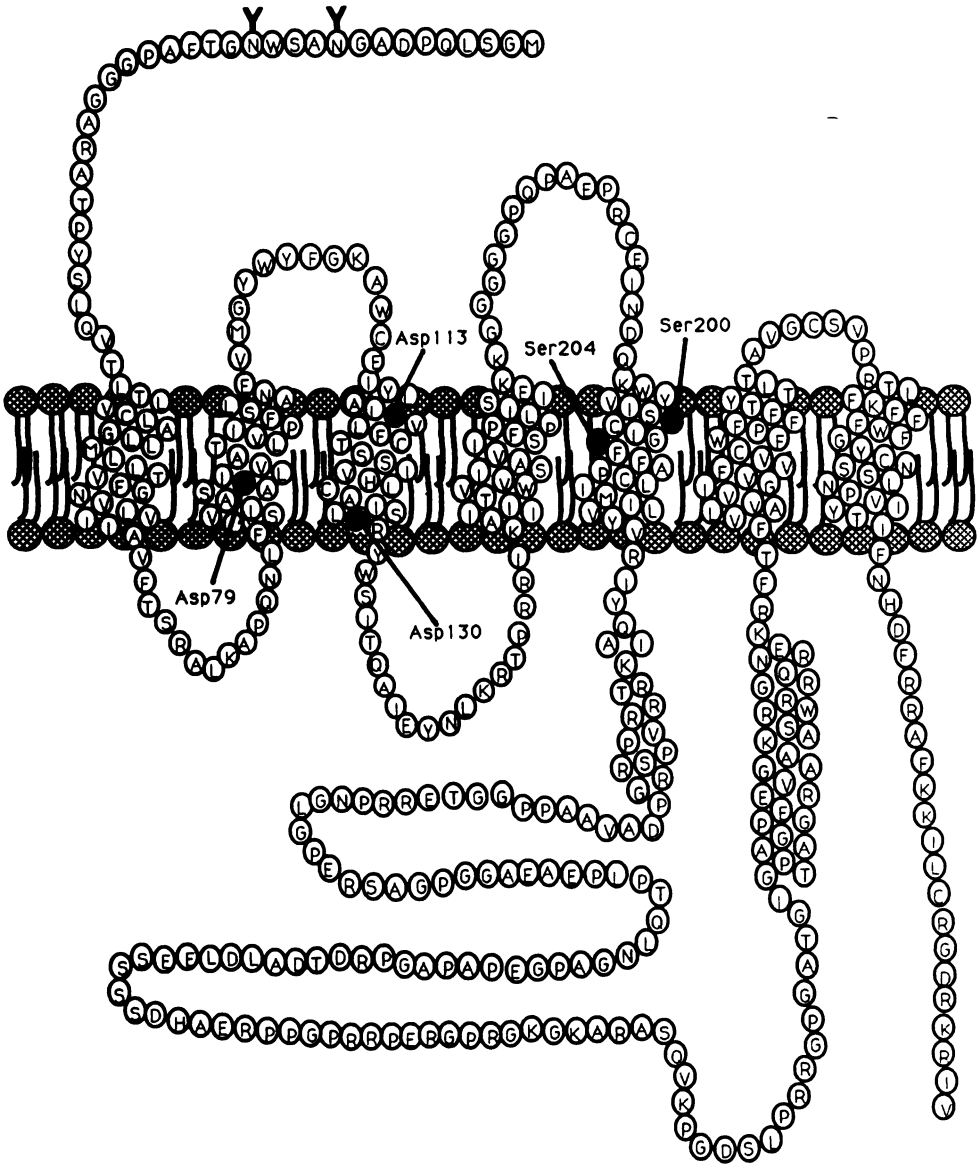

Fig. 1. Schematic diagram of the human α_{2A}-adrenergic receptor. The amino acid sequence of the receptor is indicated by the single-letter amino acid code. The conserved amino acid residues studied by site-directed mutagenesis are *highlighted*. The amino acid sequence illustrated in this figure has been reanalyzed and corrected (34), compared with that originally reported in Ref. 4.

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ceptor-mediated changes in forskolin-stimulated cAMP levels in transfected CHO cells correlates with the density of receptors. Increasing of receptor densities from 50 to 1200 fmol of receptor/mg of membrane protein increased the maximal inhibition of forskolin-stimulated cAMP production elicited by 0.1 μM epinephrine from 30 to 60% (4). We have noted in subsequent experiments that receptor densities above approximately 1 pmol of receptor/mg of membrane protein produce no significant increase in the maximal agonist-mediated inhibition or potentiation of forskolin-stimulated cAMP in transfected CHO cells[3] and, therefore, we chose cell lines expressing ~1–2 pmol of receptor/mg of membrane protein for analysis.

In three separate transfections, we were only able to isolate cells expressing the Ala^{204} mutant α_2-adrenergic receptor at the lowest (100–300 fmol/mg of membrane protein) or highest (>5 pmol of receptor/mg of membrane protein) receptor densities that are routinely observed with this expression system. We chose a cell line expressing a higher density of the Ala^{204} mutant receptor for further characterization. We reasoned that any decrease in the ability of this receptor to modulate cAMP levels would be better quantitated in a cell line providing a larger biochemical signal and that any loss in receptor activity, compared with the wild-type receptor expressed at a lower density, would be significant.

Characterization of the Asn^{113} mutant α_2-adrenergic receptor. Analysis of [^{3}H]yohimbine binding to 15 colonies of cells transfected with the Asn^{113} mutant α_2-adrenergic receptor revealed that none of the colonies display specific radioligand binding at ligand concentrations up to 1000 nM (200 × K_D for [^{3}H]yohimbine binding to the wild-type receptor). These results suggest that Asp^{113} may be required for high affinity ligand binding to α_2-adrenergic receptors or that this mutation interferes with normal receptor processing and/or membrane insertion. Our screening protocols could not distinguish between altered receptor processing or a direct effect of the mutation on ligand binding.

The lack of specific radioligand binding to the Asn^{113} mutant receptor precluded its pharmacological characterization. However, we utilized cDNA and PCR methodologies to amplify mRNA sequences from control and transfected CHO cells, to identify cell lines that contained detectable levels of mutant receptor-specific mRNA and had, therefore, been stably transfected with the mutant receptor gene. As illustrated in Fig. 2, receptor-specific mRNA was detected in three of five lines of transfected cells examined. The size of the amplified region in each cell line was 644 bp, identical to that observed with the wild-type receptor. Control CHO cells contained no detectable levels of α_{2A}-adrenergic receptor mRNA. We chose one cell line (see Fig. 2, *lane i*) for additional biochemical analysis.

In cells containing Asn^{113} mutant receptor-specific mRNA, and, therefore, presumably expressing the mutant receptor, agonists, at a concentration of 0.1 mM, produce small but detectable decreases (~10%) in forskolin-stimulated cAMP accumulation (Fig. 3C). Agonists produce a larger, dose-dependent potentiation of forskolin-stimulated cAMP production in cells stably transfected with the Asn^{113} mutant receptor gene. Both epinephrine and UK-14304, at a concentration of 10^{-4} M, elicit increases in intracellular cAMP that represent 35% and 60%, respectively, of those observed with the wild-type receptor

[3]C. M. Fraser, unpublished observations.

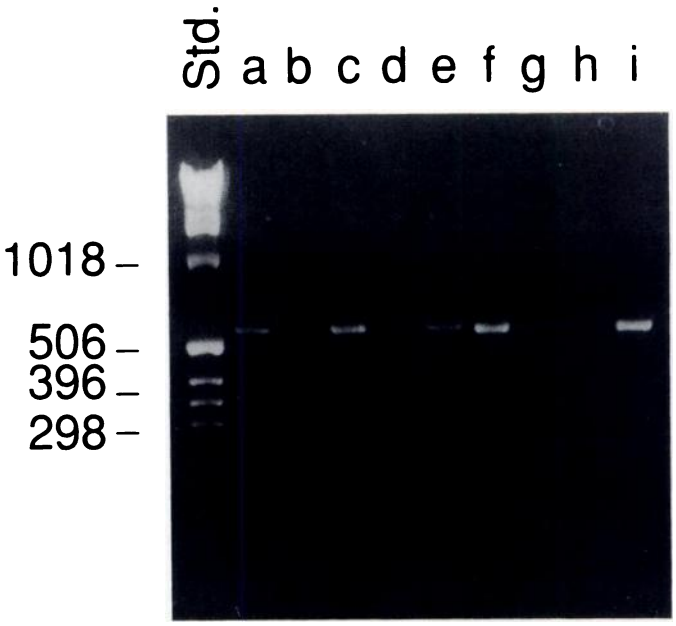

Fig. 2. Amplification of α_{2A}-adrenergic receptor target sequences from control and transfected CHO cells. Poly(A)$^+$ RNA was isolated from one confluent 150-mm plate of control or transfected CHO cells, and cDNA was synthesized as described in Experimental Procedures. A 644-bp DNA sequence from the coding region of the α_{2A}-adrenergic receptor was amplified using PCR. One fifth of the reaction was examined by ethidium bromide/agarose gel electrophoresis. *Lane a*, product amplified from 1 ng of the expression vector pSVL containing the coding region of the α_{2A}-adrenergic receptor (positive control); *lane b*, product amplified from 1 ng of pSVL alone (negative control). *Lanes c–i*, product from reverse transcription reactions with mRNA isolated from CHO cells expressing wild-type receptor (840 fmol of receptor/mg of membrane protein) (*lane c*), control CHO cells (*lane d*), and five cell lines isolated from a transfection with the Asn^{113} mutant α_2-adrenergic receptor (*lanes e–i*). *Numbers on the left*, bp standards, as determined from the migration of DNA size markers.

(Table 1). The EC_{50} values for agonist-mediated increases in forskolin-stimulated cAMP production in cells expressing the Asn^{113} mutant receptor are 3.2×10^{-5} and 2.8×10^{-6} M, respectively, for epinephrine and UK-14304 (Table 1).

Antagonist binding properties of mutant α_{2A}-adrenergic receptors. The affinities of Asn^{79}, Asn^{130}, Ala^{200}, and Ala^{204} mutant α_2 receptors for α-adrenergic antagonists were examined in competition displacement studies with [^{3}H]yohimbine and were compared with those of the wild-type receptor (Table 2). Each mutant receptor displays a rank order of antagonist potency of yohimbine > phentolamine > prazosin, consistent with an α_{2A}-adrenergic receptor subtype (21). The affinities of the Asn^{79}, Asn^{130}, Ala^{200}, and Ala^{204} mutant α_2 receptors for yohimbine, phentolamine, and prazosin are similar to those of the wild-type receptor (K_i = 5.1 M, 120 M, and 3.8 M, respectively) (Fig. 4; Table 2). The affinities of the Asn^{79} and Asn^{130} mutant receptors for antagonists are similar to those reported by Horstmann *et al.* (22) for the same mutations in the porcine α_{2A}-adrenergic receptor.

Agonist binding properties and biochemical characterization of the wild-type α_2-adrenergic receptor. Agonist binding properties of mutant α_2-adrenergic receptors were assessed using phenethylamine-type (epinephrine and norepinephrine) and imidazoline-type (oxymetazoline, *p*-aminoclonidine, and UK-14304) agonists and were compared with those of the wild-type receptor. As summarized in Table 2 and illus-

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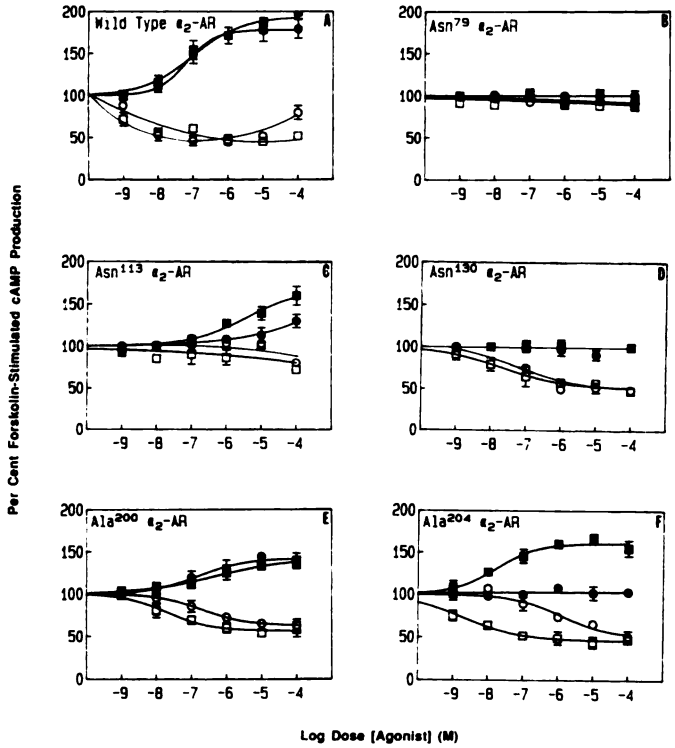

Fig. 3. Measurement of agonist-induced changes in forskolin-stimulated cAMP production in CHO cells transfected with wild-type or mutant α_{2A}-adrenergic receptors. CHO cells expressing wild-type (A) or Asn[79] (B), Asn[113] (C), Asn[130] (D), Ala[200] (E), or Ala[204] (F) mutant α_2-adrenergic receptors were assayed for (−)-epinephrine- or UK-14304-mediated changes in forskolin-stimulated cAMP levels, as described in Experimental Procedures. Data are expressed as percentage of forskolin-stimulated cAMP. Values for basal and forskolin-stimulated cAMP accumulation, respectively, for each cell line were as follows: wild-type, 15.3 ± 11.2 and 368 ± 120 pmol/min (13 experiments); Asn[79], 13.0 ± 6.4 and 303 ± 126 pmol/min (10 experiments); Asn[113], 29 ± 15 and 545 ± 136 pmol/min (nine experiments); Asn[130], 18 ± 11 and 400 ± 110 pmol/min (seven experiments); Ala[200], 12 ± 8 and 235 ± 140 pmol/min (14 experiments); and Ala[204], 26 ± 13 and 406 ± 148 pmol/min (14 experiments). *Open and solid circles*, data with epinephrine in untreated and pertussis toxin-treated cells, respectively. *Open and solid squares*, data with UK-14304 in untreated and pertussis toxin-treated cells, respectively.

trated in Fig. 4, computer analysis of agonist binding to the wild-type α_2-adrenergic receptor reveals the presence of two agonist affinity states for epinephrine, norepinephrine, and imidazolines. The high affinity component of agonist binding presumably reflects the formation of a ternary receptor-G protein complex, because inclusion of guanine nucleotides in competition displacement experiments eliminates all high affinity agonist binding (Fig. 5, A and B; Table 2).

To compare agonist activation of wild-type versus mutant α_2-adrenergic receptors, we measured changes in forskolin-stimulated cyclic AMP concentrations in stably transfected CHO cells. As illustrated in Fig. 3A and summarized in Table 1, epinephrine and UK-14304 produce dose-dependent decreases in forskolin-stimulated cAMP concentrations in cells expressing the wild-type receptor, with EC_{50} values of 4 and 0.5 M, respectively. Maximal inhibition of forskolin-stimulated cAMP production elicited by each agonist is ~55%. In cells pretreated with pertussis toxin, agonists produce a dose-dependent potentiation of forskolin-stimulated cAMP accumulation, with EC_{50} values of 64 and 96 nM, respectively, for epinephrine and UK-14304. Maximal potentiation of forskolin-stimulated cAMP production is ~180% of control values (Fig. 3A). Addition of epinephrine or UK-14304 to untransfected CHO cells has no effect on forskolin-stimulated cAMP concentrations (data not shown).

TABLE 1

Functional characterization of mutant α_2-adrenergic receptors

Data were analyzed using a nonlinear regression program of GraphPAD INPLOT software. Data were fit to the equation $Y = A + \frac{B - A}{1 + (10^C/10^X)^D}$, *where X* represents the logarithm of ligand concentration, *A* and *B* represent the bottom and top plateaus of the curve, respectively, *C* is the *X* value at the middle of the curve [or the log(EC_{50})], and *D* is the slope factor. Data represent the average of three to five separate experiments performed in duplicate. Forskolin produced an average increase in intracellular cAMP levels of 20.5 ± 2.9-fold (range, 15.6–24-fold) basal levels, which was defined as 100%. Values in parentheses represent the maximal agonist-induced changes in intracellular cAMP levels relative to the wild-type receptor (defined as 100%) in each cell line studied.

Receptor	Agonist	Forskolin-stimulated cAMP EC_{50}	
		Inhibition	Potentiation[a]
		M	
Wild-type	Epinephrine	4.0×10^{-9} (100%)	6.4×10^{-8} (100%)
	UK-14304	5.1×10^{-10} (100%)	9.6×10^{-8} (100%)
Asn[79]	Epinephrine	ND[b]	ND
	UK-14304	ND	ND
Asn[113]	Epinephrine	$\geq 10^{-4}$ (10%)	3.2×10^{-5} (35%)
	UK-14304	$\geq 10^{-4}$ (13%)	2.8×10^{-6} (60%)
Asn[130]	Epinephrine	1.2×10^{-7} (100%)	ND
	UK-14304	4.0×10^{-8} (100%)	ND
Ala[200]	Epinephrine	1.9×10^{-7} (90%)	1.6×10^{-7} (50%)
	UK-14304	1.9×10^{-8} (90%)	5.3×10^{-7} (50%)
Ala[204]	Epinephrine	1.3×10^{-6} (100%)	ND
	UK-14304	2.0×10^{-9} (100%)	1.6×10^{-8} (75%)

[a] These experiments were performed with cells that had been preincubated with pertussis toxin before assay, as described in Experimental Procedures.
[b] ND, value could not be determined.

Agonist binding properties and biochemical characterization of the Asn[79] mutant α_2-adrenergic receptor. The binding of epinephrine and norepinephrine to the Asn[79] mutant receptor is consistent with a single population of binding sites (Table 2; Fig. 4) that display K_i values similar to those for high affinity agonist binding to the wild-type receptor. When competition displacement experiments were performed in the presence of guanine nucleotides, there was no significant change in the affinity of the Asn[79] mutant receptor for epinephrine (Fig. 5D). Because the receptor-G protein complex has a higher affinity for agonists than does the free receptor, one possible interpretation of these data is that the Asn[79] mutant receptor forms an unusually stable complex with a G protein, such that only the high affinity state is observed in both the absence and presence of guanine nucleotides. Alternatively, it is possible that this mutation stabilizes the conformation of the receptor when occupied by agonist and eliminates the driving force for receptor-G protein coupling. The high affinity of the Asn[79] mutant α_2-receptor for catecholamines is in contrast to data previously described by Chung *et al.* (14) and Strader *et al.* (23) for Asn[79] mutant human and hamster β_2-adrenergic receptors that display a 10–100-fold decreased affinity for agonists. However, this same mutation in both α_2-adrenergic and β_2-adrenergic receptors is similar, in that it eliminates guanine nucleotide-sensitive agonist binding.

Imidazoline binding to the Asn[79] mutant adrenergic receptor in the absence of exogenous guanine nucleotides is similar to that observed with the wild-type receptor and is consistent with two classes of binding sites (Table 2; Fig. 4). As illustrated in Fig. 5C, however, the high affinity component of imidazoline

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TABLE 2

Ligand-binding properties of mutant α_2-adrenergic receptors

K_i values for competing ligands were calculated using the GraphPAD INPLOT curve-fitting program as described in Experimental Procedures. Values represent averages from three to six competition displacement experiments performed in triplicate. Values in parentheses show percentage of receptors in each affinity state.

	K_i				
	Wild-type	Asn[79]	Asn[130]	Ala[200]	Ala[204]
			M		
B_{max} (fmol/mg)[a]	840	960	1400	2000	6000
Antagonists					
Yohimbine[b]	5.1×10^{-9}	1.0×10^{-8}	9.6×10^{-9}	4.5×10^{-9}	1.2×10^{-8}
Phentolamine	1.2×10^{-7}	2.9×10^{-8}	2.1×10^{-7}	2.6×10^{-7}	4.8×10^{-8}
Prazosin	3.8×10^{-6}	8.0×10^{-6}	5.8×10^{-6}	1.2×10^{-5}	1.4×10^{-5}
Agonists					
(−)-Epinephrine	3.5×10^{-6} (61%) 5.3×10^{-8} (39%)	1.5×10^{-7}	4.0×10^{-6}	4.5×10^{-5}	2.1×10^{-5}
+100 μM GTP	3.3×10^{-6}	2.1×10^{-7}	6.8×10^{-6}	2.6×10^{-5}	2.3×10^{-5}
(+)-Epinephrine	2.7×10^{-5}	1.9×10^{-5}	1.8×10^{-4}	5.3×10^{-4}	4.8×10^{-4}
(−)-Norepinephrine	3.1×10^{-5} (48%) 1.4×10^{-6} (52%)	9.0×10^{-7}	2.7×10^{-5}	2.3×10^{-4}	1.1×10^{-4}
Oxymetazoline	8.3×10^{-8} (22%) 7.8×10^{-9} (78%)	4.2×10^{-7} (23%) 1.3×10^{-8} (77%)	2.7×10^{-8}	1.3×10^{-8}	4.4×10^{-9}
p-Aminoclonidine	2.2×10^{-7} (62%) 1.0×10^{-8} (38%)	2.9×10^{-7} (51%) 2.6×10^{-8} (49%)	2.1×10^{-7}	1.2×10^{-6}	1.5×10^{-7}
UK-14304	2.4×10^{-7} (58%) 3.1×10^{-9} (42%)	3.6×10^{-7} (68%) 8.1×10^{-9} (32%)	3.3×10^{-7}	7.8×10^{-7}	8.2×10^{-8}
+100 μM GTP	2.5×10^{-7}	1.9×10^{-7} (61%) 6.3×10^{-9} (39%)	2.8×10^{-7}	1.8×10^{-6}	2.9×10^{-7}

[a] B_{max} = maximum number of binding sites determined by Scatchard analysis of saturation isotherms.
[b] Affinity constant for the radioligand [^{3}H]yohimbine was determined directly in equilibrium binding studies.

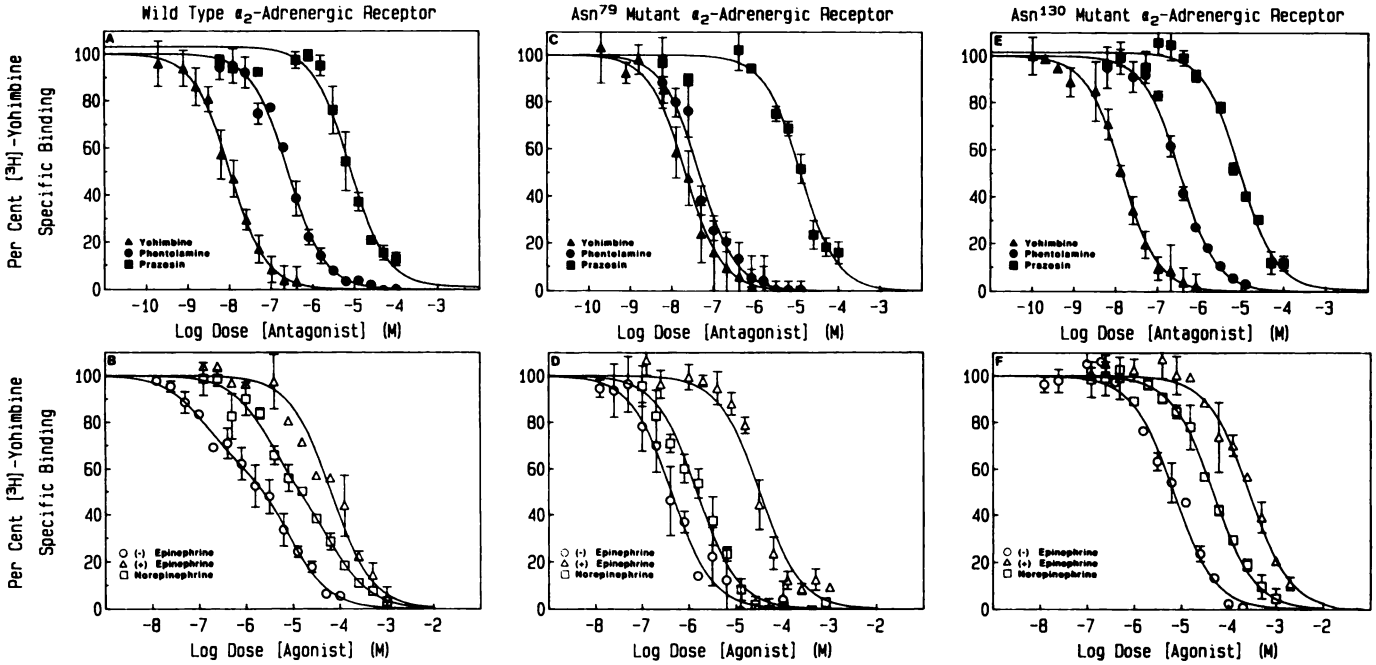

Fig. 4. Competition binding of antagonists and agonists to wild-type and mutant α_{2A}-adrenergic receptors. Competition displacement experiments were performed on membranes prepared from transfected CHO cells expressing wild-type or the indicated mutant α_2-adrenergic receptors. Best-fit curves were obtained as described in Experimental Procedures. Data represent averages of three to six experiments performed in triplicate.

binding to the Asn[79] mutant receptor is unaltered by inclusion of 100 μM GTP in the reaction mixtures, suggesting an altered receptor-G protein coupling.

Addition of increasing concentrations of either epinephrine or UK-14304 to control or toxin-treated cells expressing the Asn[79] mutant receptor has no effect on forskolin-stimulated cAMP levels (Fig. 3B) and is consistent with the apparent loss of normal receptor-G protein interactions inferred from agonist binding studies.

Agonist binding properties and biochemical characterization of the Asn[130] mutant α_2-adrenergic receptor. The Asn[130] mutant α_2-adrenergic receptor displays a single class of low affinity agonist binding sites for phenethylamine and imidazoline agonists (Table 2; Fig. 4). The affinity of this class of binding sites is similar to the low affinity agonist binding site of the wild-type receptor (4 μM for epinephrine and 0.33 μM for UK-14304), suggesting that this mutation may interfere with the ability of the α_2-adrenergic receptor to interact with

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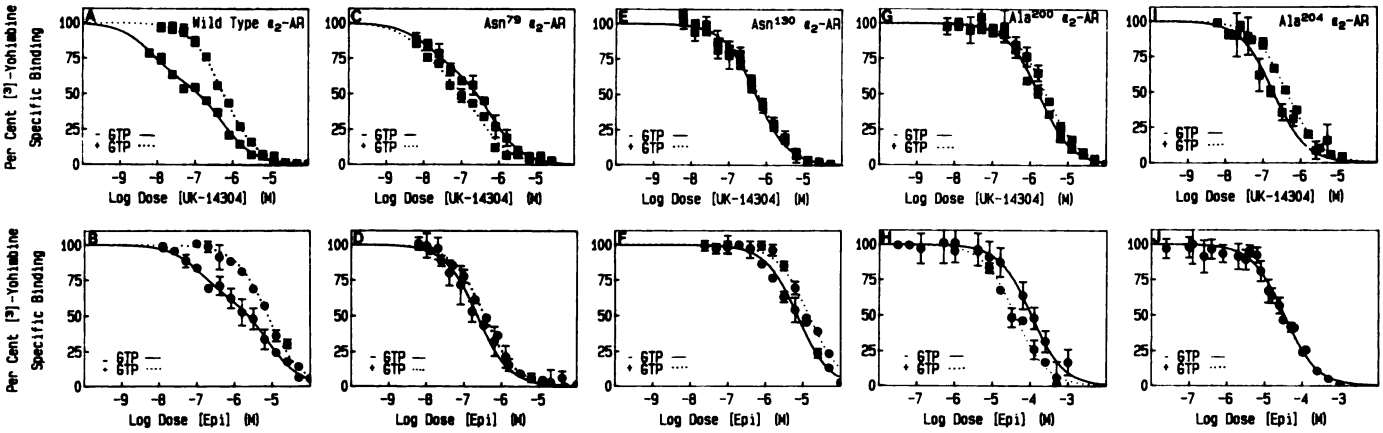

Fig. 5. Guanine nucleotide effects on agonist binding to wild-type and mutant α_{2A}-adrenergic receptors. Competition binding experiments with either epinephrine (*Epi*) or UK-14304 were performed as described in the legend to Fig. 4, in the absence or presence of 100 μM GTP.

G proteins to form a ternary complex associated with high affinity agonist binding. This idea is also supported by the finding that inclusion of 100 μM GTP in agonist competition displacement experiments produces only a 1–1.7-fold decrease in agonist affinity (Fig. 5, E and F). These results are in contrast to an earlier report from our laboratory, which demonstrated that the same mutation at the corresponding locus in the β_2-adrenergic receptor produces a mutant receptor with agonist binding affinities approximately 10-fold higher than those of the wild-type β_2-receptor (16). The opposing changes in agonist affinities observed between the Asn^{79} and Asn^{130} mutant α- and β-adrenergic receptors suggest that these aspartic acid residues may play different roles in maintaining the conformation of the agonist-occupied wild-type receptor and suggest that catecholamines may differentially interact with these two classes of receptors.

Addition of epinephrine or UK-14304 to cells expressing the Asn^{130} mutant α_2-adrenergic receptor produces a maximal decrease in forskolin-stimulated cAMP levels comparable to that seen with the wild-type α_2-adrenergic receptor (Fig. 3D), with EC_{50} values of 120 and 40 nM, respectively (Table 1). These values are 30-fold and 78-fold greater, respectively, than those for the wild-type receptor. The shifts to the right in the agonist dose-response curves for inhibition of forskolin-stimulated cAMP production are consistent with the lower affinity of the Asn^{130} mutant receptor for agonists. Our data are similar to those previously reported with the m1 muscarinic acetylcholine receptor containing the same mutation at the corresponding position in the third transmembrane helix, where agonists display an impaired potency to elicit increases in phosphoinositide hydrolysis in transfected cells (15). In contrast, removal of the negative charge at position 130 in the human β_2-adrenergic receptor eliminates agonist-mediated increases in adenylate cyclase activity (16).

The Asn^{130} mutant α_2-adrenergic receptor is unable to mediate agonist-induced potentiation of forskolin-stimulated cAMP production in pertussis toxin-treated cells (Fig. 3D). Neither epinephrine nor UK-14304, at concentrations from 10^{-9} to 10^{-4} M, produces any increase in forskolin-stimulated cAMP levels. These data support our earlier hypothesis that α_2 receptor-mediated inhibition and potentiation of forskolin-stimulated cAMP levels in transfected CHO cells represent two distinct signaling pathways (4), because we can differentially affect these responses with a single mutation. It is not clear whether potentiation of forskolin-stimulated cAMP accumulation is mediated by a G protein(s) distinct from G_i in CHO cells, because this response is insensitive to pertussis toxin. A mutation in rhodopsin at this locus generates a protein that does not bind transducin (G_t) or stimulate GTPase activity, suggesting that a direct interaction may occur between rhodopsin and G_t at this site (24). It is possible that the α_{2A}-adrenergic receptor may interact with a second G protein in CHO cells to potentiate forskolin-stimulated cAMP accumulation and that Asp^{130} is required to form the binding site for such a G protein.

Agonist-binding properties and biochemical characterization of the Ala^{200} mutant α_2-adrenergic receptor. Substitution of Ser^{200} with Ala produces a mutant receptor that displays a single class of agonist binding sites with affinities equal to or less than those of the low affinity agonist binding sites of the wild-type receptor (Table 2; Fig. 4). Agonist binding to the Ala^{200} mutant α_2 receptor is essentially unchanged by addition of exogenous guanine nucleotides (Fig. 5, G and H; Table 2).

Addition of epinephrine or UK-14304 to cells expressing the Ala^{200} mutant receptor produces dose-dependent changes in forskolin-stimulated cAMP levels in control and toxin-treated cells (Fig. 3E). The maximal decrease in forskolin-stimulated cAMP levels observed in cells expressing the Ala^{200} mutant receptor is similar to that seen with the wild-type receptor; however, EC_{50} values are shifted ~40-fold to the right, compared with the wild-type receptor (Fig. 3; Table 1), consistent with a reduction in receptor affinity for both agonists. Epinephrine and UK-14304 potentiate forskolin-stimulated cAMP levels in toxin-treated cells expressing the Ala^{200} mutant receptor; however, the potencies of epinephrine and UK-14304 are decreased 2.5-fold and 5.5-fold, respectively, and the maximal response observed is only 50% of that seen with the wild-type receptor (Table 1).

Agonist binding properties and biochemical characterization of the Ala^{204} mutant α_2-adrenergic receptor. Computer analysis of agonist binding to the Ala^{204} mutant receptor reveals the presence of a single class of binding sites for the phenethylamines and imdazolines, with affinities equal to or less than the low affinity agonist binding sites of the wild-type receptor (Fig. 4; Table 2). Addition of 100 μM GTP to

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agonist competition displacement experiments has little effect on agonist affinity (Fig. 5, I and J; Table 2).

At the Ala^{204} mutant receptor, UK-14304 elicits a similar maximal inhibition of forskolin-stimulated cAMP levels as the wild-type receptor; the EC_{50} value for this response is increased 4-fold to 2 nM (Table 1). UK-14304 also potentiates forskolin-stimulated cAMP accumulation in these cells. The maximal response is 75% of that observed with the wild-type receptor (Fig. 3F), with a similar EC_{50} value (Table 1).

In contrast, epinephrine displays a markedly reduced potency to inhibit forskolin-stimulated cAMP accumulation (EC_{50} = 1.3 μM versus 4 nM for the wild-type receptor) and has no effect on cAMP potentiation in toxin-treated cells (Table 1). The decrease in epinephrine potency may, in part, be explained by the loss in receptor affinity for this ligand.

Do Ser^{200} and Ser^{204} in the α_{2A}-adrenergic receptor participate in hydrogen bond interactions with the catechol hydroxyl groups? Strader *et al.* (13) reported that mutation of Ser^{204} or Ser^{207} in the β_2-adrenergic receptor results in a 25-fold reduction in receptor affinity for isoproterenol and an attenuation of the activity of catecholamine agonists. Based on structure-activity studies, it was suggested that these serine residues in the β_2-adrenergic receptor are involved in hydrogen bond interactions with the catechol hydroxyl groups of the catecholamines, 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. Consistent with this hypothesis is the observation that serine residues are conserved in the fifth transmembrane domain of all receptors that bind catecholamine ligands but are not present in other receptors in this family whose ligands lack the catechol hydroxyl groups, such as muscarinic and serotonin receptors (13).

When Ser^{200} and Ser^{204} in the α_{2A}-adrenergic receptor are replaced with Ala, we observe decreases in the affinity of each mutant receptor for epinephrine and norepinephrine (Fig. 4; Table 2), suggesting that these residues may play similar roles in ligand-binding interactions in α- and β-adrenergic receptors. In order to examine in more detail whether Ser^{200} or Ser^{204} participates in hydrogen bonding with the *meta*- and *para*-hydroxyl groups of the catecholamines, we first analyzed the binding of synephrine and phenylephrine to wild-type and Ala^{200} and Ala^{204} mutant α_2-adrenergic receptors. Synephrine and phenylephrine are analogs of epinephrine that lack a catechol hydroxyl group at the *meta*- and *para*-positions, respectively. As suggested by Strader *et al.* (13), if the decrease in agonist affinity seen after substitution of either Ser^{200} or Ser^{204} with Ala reflects the loss of a hydrogen bond interaction between a catechol hydroxyl group and the mutant receptor, then a similar reduction in agonist binding affinity for the wild-type receptor should be observed if either of the catechol hydroxyl groups is removed from the parent compound epinephrine. Elimination of both potential sites of ligand-receptor interactions would be predicted to have a greater effect on receptor affinity than that observed after disruption of a single hydrogen bond.

Removal of either hydroxyl group from epinephrine reduces ligand affinity for the wild-type α_2-receptor. The rank order of agonist potency is epinephrine (average $K_i = 1.6 \times 10^{-6}$ M) > phenylephrine ($K_i = 4.7 \times 10^{-6}$ M) > synephrine ($K_i = 2.8 \times 10^{-5}$ M) (Table 3), indicating that the *para*-hydroxyl group contributes more to determination of catecholamine affinity than does the *meta*-hydroxyl group. As compared with the wild-type receptor, the affinity of the Ala^{200} mutant receptor is 28-fold lower for epinephrine, 10-fold lower for phenylephrine, and essentially unchanged for synephrine (Table 3). The Ala^{204} α_2-adrenergic receptor displays a 13-fold and a 4.3-fold decrease, respectively, in affinity for epinephrine and phenylephrine, with no change in affinity for synephrine, compared with the wild-type receptor (Table 3). The changes in ligand-binding affinities of the Ala^{200} and Ala^{204} mutant α_2-adrenergic receptors are qualitatively similar to those of the Ala^{204} and Ala^{207} mutant β_2-adrenergic receptors (13).

To further analyze the effects of the serine to alanine mutations on α_2-adrenergic receptor function, agonist activation of wild-type and mutant receptors by epinephrine, phenylephrine, and synephrine was assessed by measuring inhibition of forskolin-stimulated cAMP production in transfected cells. As illustrated in Fig. 6 and summarized in Table 3, phenylephrine and synephrine exhibit decreased potencies ($EC_{50} = 1.4 \times 10^{-6}$ and 3.6×10^{-6} M, respectively) at the wild-type receptor, compared with epinephrine ($EC_{50} = 1.0 \times 10^{-8}$ M), but elicit the same maximal decrease in forskolin-stimulated cAMP levels. The rank order of agonist potency at the wild-type receptor is epinephrine > phenylephrine ≥ synephrine, consistent with the binding affinities for these ligands. At the Ala^{200} mutant α_2-adrenergic receptor, all three agonists elicit a maximal response, with a rank order of agonist potency of epinephrine ($EC_{50} = 1.7 \times 10^{-7}$ M) ≥ synephrine ($EC_{50} = 4.6 \times 10^{-7}$ M) ≥ phenylephrine ($EC_{50} = 7.4 \times 10^{-7}$ M), consistent with the ligand affinities. At the Ala^{204} mutant α_2-adrenergic receptor, epinephrine ($EC_{50} = 8.4 \times 10^{-7}$ M) and phenylephrine ($EC_{50} = 1.4 \times 10^{-7}$ M) elicit similar maximal decreases in intracellular cAMP levels; however, synephrine displays decreased potency ($EC_{50} = 1.3 \times 10^{-5}$ M) and partial agonist activity (65%), compared with the wild-type receptor (Fig. 6; Table 3).

Discussion

Using site-directed mutagenesis and permanent expression of wild-type and mutant receptors in cell lines lacking endogenous receptors, we previously examined the role of conserved aspartic acid and cysteine residues in β_2-adrenergic and m1 muscarinic acetylcholine receptor function (14–16, 25). The results of the present study extend our findings to another receptor in this gene superfamily, the α_{2A}-adrenergic receptor, and allow for a comparison with earlier work on the β-adrenergic receptor. This was of interest because α- and β-adrenergic receptors bind the same endogenous ligands but differ in their pharmacological and biochemical properties.

Three conserved aspartic acids contained within the putative second and third transmembrane helices of the α_{2A}-adrenergic receptor (Fig. 1) appear to play critical roles in receptor function. Substitution of Asp^{113} with Asn eliminated the ability of the receptor to bind [^{3}H]yohimbine with high affinity. We measured agonist-mediated changes in forskolin-stimulated cAMP levels over a range of agonist concentrations, from 10^{-9} to 10^{-4} M, in cells shown to be stably transfected with the mutant receptor gene. This assay presumably would allow for detection of lower affinity receptor-ligand interactions than could be detected in radioligand binding assays. Over this concentration range, agonists elicited small but detectable decreases in forskolin-stimulated cAMP accumulation. Our data

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TABLE 3

Properties of Ser → Ala mutant α_{2A}-adrenergic receptors

K_i values were determined in competition displacement experiments, as described in Experimental Procedures (value for epinephrine is average K_i value). EC_{50} values were determined using a nonlinear regression program of GraphPAD INPLOT software and represent the average of three experiments performed in duplicate. Activity values were determined using data from Fig. 6. One hundred percent activity is defined as the percentage of inhibition of forskolin-stimulated cAMP production elicited by 10^{-4} M epinephrine in cells expressing the wild-type α_2-adrenergic receptor.

	Wild-type	Ala^{200}	Ala^{204}
(−)-Epinephrine	$K_i = 1.6 \times 10^{-6}$ M $EC_{50} = 1.0 \times 10^{-8}$ M 100% activity	$K_i = 4.5 \times 10^{-5}$ M $EC_{50} = 1.7 \times 10^{-7}$ M 100% activity	$K_i = 2.1 \times 10^{-5}$ M $EC_{50} = 8.4 \times 10^{-7}$ M 100% activity
(−)-Phenylephrine	$K_i = 4.7 \times 10^{-6}$ M $EC_{50} = 1.4 \times 10^{-6}$ M 100% activity	$K_i = 4.9 \times 10^{-5}$ M $EC_{50} = 7.4 \times 10^{-7}$ M 100% activity	$K_i = 2.0 \times 10^{-5}$ M $EC_{50} = 1.4 \times 10^{-7}$ M 100% activity
(±)-Synephrine	$K_i = 2.8 \times 10^{-5}$ M $EC_{50} = 3.6 \times 10^{-6}$ M 100% activity	$K_i = 4.0 \times 10^{-5}$ M $EC_{50} = 4.6 \times 10^{-7}$ M 100% activity	$K_i = 3.0 \times 10^{-5}$ M $EC_{50} = 1.3 \times 10^{-5}$ M 65% activity

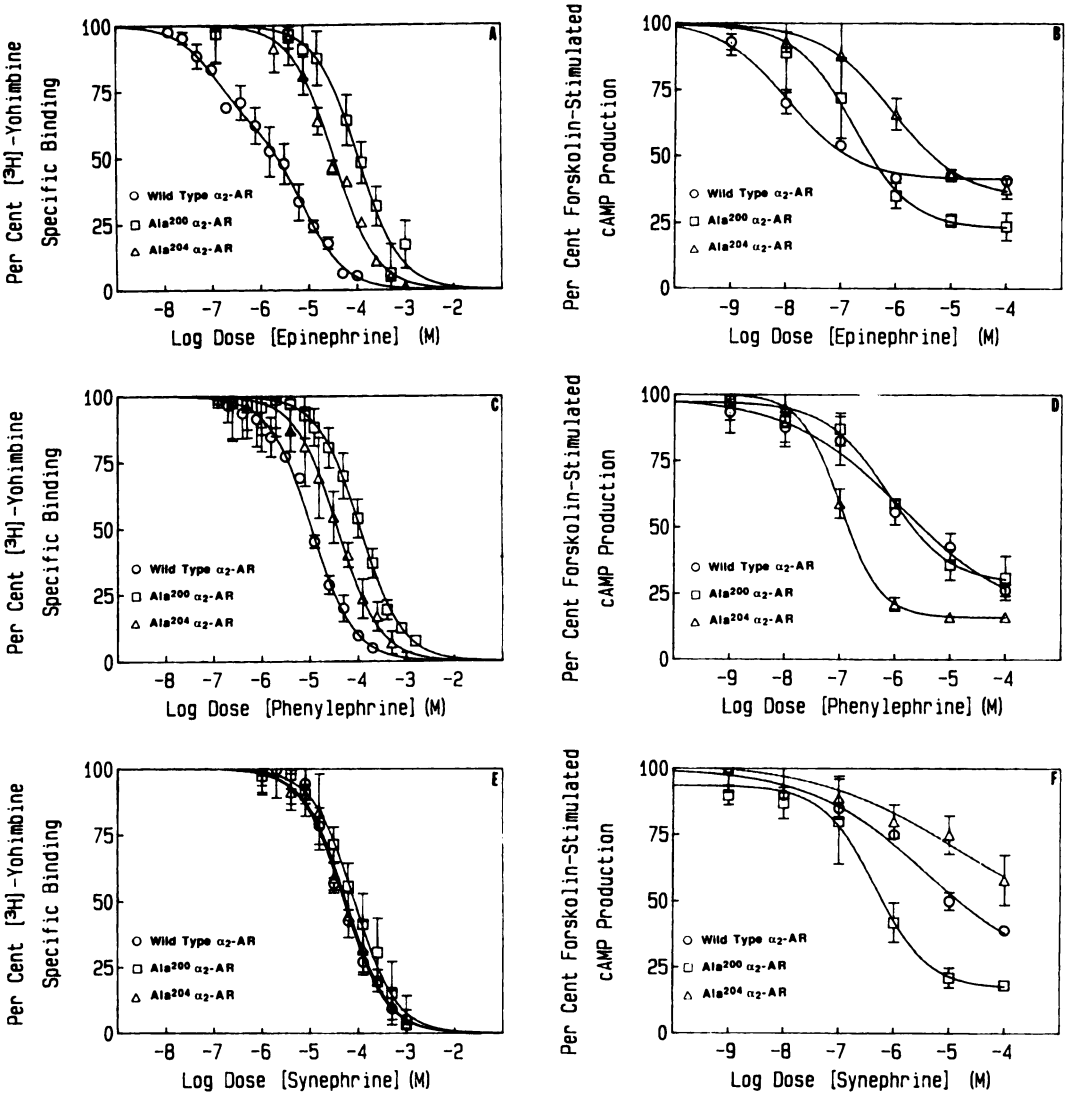

Fig. 6. Effects of catechol hydroxyl substitution on ligand binding affinities and agonist activation of wild-type and mutant α_{2A}-adrenergic receptors. Competition binding of epinephrine, phenylephrine, or synephrine to wild-type or Ala^{200} or Ala^{204} mutant α_{2A}-adrenergic receptors was determined as described in the legend to Fig. 4 (A, C, and E). Agonist-mediated inhibition of forskolin-stimulated cAMP production was also assessed, as described in the legend to Fig. 3 (B, D, and F).

are similar to those reported by Strader *et al.* (12) on the ability of the Asn^{113} mutant β_2-adrenergic receptor to partially stimulate adenylate cyclase and of Fraser *et al.* (15) on the ability of the Asn^{105} m1 muscarinic receptor to marginally increase inositol phosphate production in the presence of high agonist concentrations. The finding that the Asn^{113} mutant α_2-adrenergic receptor elicited a small decrease in intracellular cAMP concentrations at high agonist concentrations suggests that agonists do bind to the Asn^{113} mutant receptor, but with markedly lower affinities. Consistent with this idea is the observation that the Asn^{113} mutant α_2-adrenergic receptor was able to potentiate forskolin-stimulated cAMP accumulation in transfected cells, albeit with reduced potency and efficacy, compared with the wild-type receptor.

Our data are not inconsistent with the hypothesis that this aspartic acid, which is conserved in all G protein-linked recep-

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tors known to bind amine ligands, is critical for high affinity ligand binding and is involved in an electrostatic interaction with the positively charged nitrogen of phenethylamine and imidazoline ligands (12, 15). However, an alternative explanation for our findings is that mutation of Asp^{113} to Asn alters normal receptor processing and insertion into the membrane. The small agonist-mediated changes in intracellular cAMP concentrations that were observed may relate to a low density of Asn^{113} mutant receptors on the cell surface. Without independent evidence regarding expression of the Asn^{113} mutant receptor, we cannot at present make a more definitive conclusion regarding the role of this amino acid in receptor function.

The predicted amino acid sequences of all G protein-coupled receptors contain an aspartic acid at the locus corresponding to Asp^{79} in the second transmembrane domain of the human α_{2A}-adrenergic receptor. Mutation of this residue in β_2-adrenergic (14) and muscarinic acetylcholine receptors (15) markedly attenuates agonist efficacy for stimulating adenylate cyclase and phospholipase C activity, respectively, similar to the findings of the present study on the effect of this mutation on α_{2A}-adrenergic receptor function. Photoaffinity labeling of the porcine Asn^{79} mutant α_2-adrenergic receptor has demonstrated that this protein undergoes normal processing and membrane insertion (22), suggesting that the altered properties of the Asn^{79} mutant α_2-adrenergic receptor do not reflect changes in the overall structural integrity of the receptor. Rather, it seems more likely that the effect of this mutation on receptor function reflects a change in receptor conformation.

Because this aspartic acid is so highly conserved among all G protein-coupled receptors and because elimination of the negative charge at this position has been shown to abolish the ability of three different classes of receptors to stimulate three different intracellular signaling pathways (14, 15) (this study), it is possible that this residue may be critical for agonist-induced conformational change(s) that catalyze G protein activation. We previously speculated that cation transfer between negatively charged amino acid side chains may play a role in receptor activation of G proteins and suggested that this process may involve the invariant aspartic acid in the second transmembrane helix of this family of proteins (8). In part, this hypothesis was based on the known function of corresponding conserved aspartic acid residues in bacteriorhodopsin, which form a charge network for the translocation of protons across the cell membrane (26, 27). This idea is further supported by recent data from Horstman *et al.* (22), suggesting that cations such as Na^+ directly bind to the carboxylate form of Asp^{79} in the porcine α_2-adrenergic receptor.

Substitution of Asp^{130} with Asn in the porcine α_2-adrenergic receptor has been shown to have no deleterious effect on receptor processing (22). As demonstrated in the present study, this mutation abolishes high affinity, guanine nucleotide-sensitive, agonist binding, reduces agonist potency to inhibit forskolin-stimulated cAMP production, and eliminates agonist-mediated potentiation of forskolin-stimulated cAMP levels, suggesting a possible role for this residue in receptor-G protein interactions. The low affinity of the Asn^{130} mutant receptor for agonists that is unchanged by guanine nucleotides is consistent with a lack of receptor-G protein coupling.

Evidence from earlier kinetic studies suggested that the high affinity conformation of the α_2-adrenergic receptor is functionally relevant in terms of G protein activation (28). However, the Asn^{130} mutant α_2-adrenergic receptor, which displays a low affinity for agonists, is able to mediate inhibition of forskolin-stimulated cAMP production. It is possible that a small population of Asn^{130} mutant α_2-adrenergic receptors may form a ternary complex with G_i that is associated with high affinity agonist binding. This subpopulation of receptors, which may be too small to be identified in computer analysis of ligand binding, may be responsible for receptor-mediated inhibition of forskolin-stimulated cAMP accumulation.

Alternatively, our findings argue that the ability of the receptor to form a high affinity agonist binding state, as measured in competition displacement studies with agonists, may not be necessary for receptor-G_i interactions and inhibition of adenylate cyclase. Recent data from Fraser *et al.* (16) and Hausdorff *et al.* (29) have demonstrated that the molecular determinants of the β_2-adrenergic receptor that are involved in the formation of the ternary receptor-G protein complex are not identical to those that transmit the agonist-induced stimulatory signal to G_s. Thus, whereas Asp^{130} may be necessary for high affinity agonist binding to the α_{2A}-adrenergic receptor, other domains may be involved in agonist-mediated activation of G_i. These observations support the hypothesis that receptor-mediated signal transduction is a complex process that may involve multiple conformational changes.

Alignment of the sequences of the fifth transmembrane helix of G protein-coupled receptors illustrates the presence of serine residues analogous to Ser^{204} and Ser^{207} in the β_2-adrenergic receptor in those receptors that bind catecholamines (Fig. 7). In a recent mutagenesis study of the hamster β_2-adrenergic receptor, Strader *et al.* (13) implicated Ser^{204} and Ser^{207} in hydrogen bond interactions with the hydroxyl groups of the catechol ring. Comparison of ligand binding data from characterization of the Ala^{200} and Ala^{204} mutant α_2-adrenergic receptors with those previously reported for the corresponding mutant β_2-adrenergic receptors (13) revealed similar qualitative changes in affinities for the dihydroxy-substituted and monohydroxy-substituted phenethylamines, compared with the respective wild-type receptors. However, mutation of the conserved serine residues had markedly different effects on receptor activation by phenethylamines in α_2- and β_2-adrenergic receptors.

At the Ala^{204} mutant β_2-adrenergic receptor, isoproterenol and its *para*-substituted analog possess only partial agonist activity, whereas the *meta*-substituted analog has no intrinsic agonist activity. These data suggest a role for Ser^{204} in the β_2-adrenergic receptor in hydrogen bond interactions with the *meta*-hydroxyl group of the catecholamines. In contrast, at the Ala^{200} mutant α_2-adrenergic receptor, epinephrine, phenylephrine, and synephrine elicit maximal responses. The difference between α- and β-adrenergic receptors may be due to the fact that, in the β_2-adrenergic receptor, Ser^{204} and Ser^{207} are located three positions apart in a region of the receptor presumed to form a transmembrane α-helix, compared with a distance of four residues apart for Ser^{200} and Ser^{204} in the α_{2A}-adrenergic receptor (Fig. 7). Because one turn of an α-helix contains 3.6 amino acid residues (30), the side chains of Ser^{200} in the α_2 receptor and Ser^{204} in the β_2 receptor would assume different orientations in the transmembrane helix, which could affect their reactivity with the *meta*-hydroxyl group of the catechol ring. It is possible that the *meta*-hydroxyl group of the catecholamines may interact with the sulfhydryl side chain of Cys^{201}

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Human β_1-AR	A Y A I A S S V V S F Y V P L C I M A F V Y L
Human β_2-AR	A Y A I A S S I V S F Y V P L V I M V F V Y S
Human α_{2A}-AR	W Y I L S S C I G S F F A P C L I M G L V Y A
Human α_2 (C-4)	W Y I L S S C I G S F F A P C L I M G L V Y L
Rat α_{2B}-AR	W Y I L A S S I G S F F A P C L I M I L V Y R
Hamster α_1-AR	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-DR	A F V V Y S S I V S F Y V P F I V T L L V Y I
Dros Octop	G Y V I Y S S L G S F F I P I A I M T I V Y I
Rat 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-1C	N F V L I G S F V A F F I P L T I M V I T Y F
Human M1	I I T F G T A M A A F Y L P V T V M C T L Y N
Human M2	A V T F G T A I A A F Y L P V I I M T V L Y W
Human opsin	S F V I Y M F V V H F I I P L I V I F F C Y G

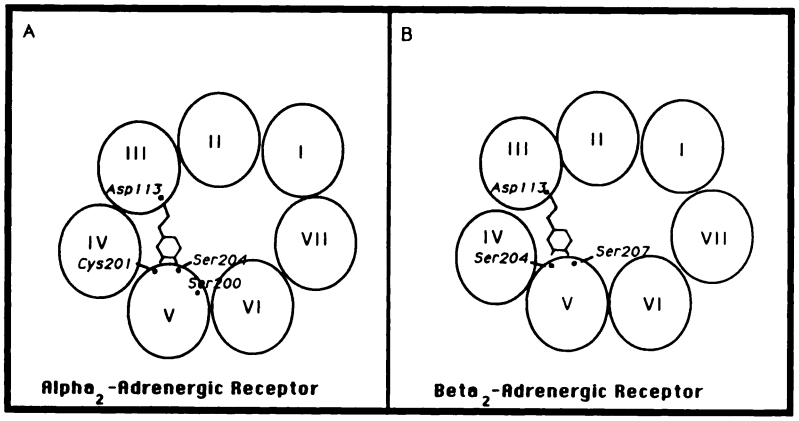

Fig. 7. *Top*, alignment of the deduced amino acid sequences from the fifth transmembrane domain of selected G protein-coupled receptors. Amino acid sequences were aligned to maximize homologies in this region. *, The conserved serine residues. Sequences are from Refs. 3–5 and 35–43. *Bottom*, model comparing putative interactions of epinephrine with α_{2A}-adrenergic (A) and β_2-adrenergic (B) receptors. The model depicts the presumed tertiary structure of the α_{2A}-adrenergic and β_2-adrenergic receptors in the plasma membrane. The seven helices are numbered (*I–VII*). The location of Asp[113] and the conserved serines implicated in ligand binding are indicated. Epinephrine has been inserted into the possible ligand binding sites of these receptors. The model for ligand binding to the β_2-adrenergic receptor has been adapted from the work of Strader *et al.* (13).

in the α_2 receptor, which is located in the same relative position in the fifth transmembrane helix as Ser[204] in the β_2-adrenergic receptor (Fig. 7).

At the Ala[207] β_2-adrenergic receptor, isoproterenol and its *meta*-substituted derivative elicit 40% of the maximal agonist activity, whereas the *para*-substituted derivative has no intrinsic activity (13). In a somewhat analogous manner, epinephrine and phenylephrine (*meta*-substituted) elicit 100% maximal agonist activity at the Ala[204] mutant α_2-adrenergic receptor, whereas synephrine (*para*-substituted) displays ~65% of the activity. Inasmuch as both the Ala[207] β_2-adrenergic receptor and the Ala[204] mutant α_2-adrenergic receptor exhibit qualitatively similar pharmacological and biochemical properties, one interpretation of the data from this study is that Ser[204] in the α_2-adrenergic receptor participates in binding the *para*-hydroxyl group of the catecholamines.

Ligand binding data for the Ala[204] mutant α_2-adrenergic receptor do not, however, unequivocally support a direct role for this residue in the wild-type receptor in binding the *para*-hydroxyl group of the catecholamines. Based on the hypothesis of Strader *et al.* (13), one would predict that the affinity of the Ala[204] mutant α_2-adrenergic receptor for epinephrine and phenylephrine should be similar and greater than that for synephrine. However, the affinity of the Ala[204] α_2 receptor is identical for all three compounds. Photoaffinity labeling of platelet α_{2A}-adrenergic receptors has implicated an amino acid contained within the fourth transmembrane helix of the receptor as the site at which the *para* substituent on the phenyl ring of clonidine binds (31). Because the imidazolines can assume a conformation that mimics a critical distance of the phenethylamines, in which the distance from the center of the phenyl ring to the positively charged nitrogen is 5.1 Å (1), it is possible that the *para*-hydroxyl group of the catechol ring binds to the same residue as the *para* substituent of clonidine. It should be possible to evaluate this hypothesis with additional biochemical and molecular genetic approaches.

UK-14304 elicits inhibition and potentiation of forskolin-stimulated cAMP accumulation at the Ala[204] mutant α_2-adrenergic receptor, as observed with the wild-type receptor. In contrast, epinephrine (at concentrations up to 10^{-4} M) has no effect on potentiation of forskolin-stimulated cAMP production. These findings support data from functional analysis of other mutant α_2-adrenergic receptors (see discussion of Asn[113] receptor and Asn[130] receptor, above), which suggest that receptor-mediated inhibition and potentiation of forskolin-stimulated cAMP production represent distinct intracellular signaling pathways. The differential ability of UK-14304 and epinephrine to potentiate cAMP production at the Ala[204] mutant α_2-adrenergic receptor also suggests that the mechanism of receptor activation by these classes of agonists may differ. This observation supports an earlier hypothesis by Ruffolo and colleagues (11, 32, 33), which proposed that phenethylamines and imidazolines differentially interact with α_2 receptors. Our data suggest that, although there may be common sites in the α_2 receptor for the binding of phenethylamine and imidazoline ligands (Asp[113], for example), the molecular mechanisms of activation by these two classes of agonists may not be identical.

In conclusion, we have utilized site-directed mutagenesis to probe the relationship between receptor structure and function and have examined the role of five conserved amino acids in

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the transmembrane helices of the human α_{2A}-adrenergic receptor. As previously demonstrated with other members of the G protein-coupled family of receptors, these amino acids influence ligand binding and receptor activation by agonists. Data from this study, together with earlier work, suggest that the molecular interactions between α- and β-adrenergic receptors and catecholamine ligands may be subtly different. Furthermore, our results suggest that the receptor-ligand interactions may be different for phenethylamine and imidazoline compounds. One of the challenges in this area of study will be to identify the domains within adrenergic receptors that confer ligand-binding specificities. The continued application of genetic techniques to the study of α_2-adrenergic receptors, in combination with the large number of compounds available for structure-activity studies, has the potential to provide a more detailed understanding of the molecular basis of receptor action and allow for the design of more specific therapeutic agents.

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