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Site-Directed Mutagenesis of the Human A_1 Adenosine Receptor: Influences of Acidic and Hydroxy Residues in the First Four Transmembrane Domains on Ligand Binding

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SUMMARY

To provide new insights into ligand/A_1 adenosine receptor (A_1 AR) interactions, site-directed mutagenesis was used to test the role of several residues in the first four transmembrane (TM) domains of the human A_1 AR. Based on multiple sequence analysis of all known ARs, both acidic (glutamic acid and aspartic acid) and polar hydroxy (serine and threonine) amino acids were identified that could potentially play a role in binding adenosine. Glu16 (TM1), Asp55 (TM2), Ser93 and Ser94 (TM3), Ser135 (TM4), and Thr 141 (TM4) were identified in all ARs, and Ser6 and Ser23 (TM1) were identified in all A_1 ARs. To test the role of these residues, each was individually mutated to alanine. When Ala6, Ala23, Ala50, Ala93, Ala135, and Ala141 constructs were tested, affinities for [^{3}H]2-chloro-N^6-cyclopentyladenosine (CCPA) and [^{3}H]1,3-dipropyl-8-cyclopentylxanthine (DPCPX) were similar to those seen for the wild-type receptor. After conversion of Glu16 to Ala16, the affinity for [^{3}H]CCPA and other agonists fell 10–100-fold, whereas the affinity for [^{3}H]DPCPX and other antagonists was not affected. After conversion of Asp55 to Ala55, the affinity for [^{3}H]CCPA and other agonists increased ≤100-fold, whereas the affinity for [^{3}H]DPCPX and other antagonists was not affected. Studies of the Ala55 construct also revealed that Asp55 is responsible for allosteric regulation of binding by sodium because the affinity for [^{3}H]CCPA did not change over broad ranges of sodium concentrations. When Ser94 was converted to Ala94, A_1 AR immunoreactivity was present on stable cell lines; however, functional binding sites could not be detected. When Ser94 was converted to Thr94, the affinity for some xanthine antagonists fell. These data show that Glu16 in TM1 and Asp55 in TM2 play important roles in agonist/A_1 AR interactions and show that Asp55 is responsible for allosteric regulation of ligand/A_1 AR binding by sodium. We also identify Ser94 as an important site for ligand binding.

Adenosine, which contains a purine ring connected to a ribose group, acts via specific receptors that include A_1 ARs (1, 2). A_1 ARs belong to the superfamily of GPCRs, contain TM spanning domains, and couple to G_i (2). In mammals, A_1 ARs play vital roles in neural and cardiac physiology (3). Activation of A_1 ARs in the brain can mitigate seizure activity and protect against hypoxic damage (4, 5). Activation of cardiac A_1 ARs can help terminate arrhythmias and confer protection against ischemia (3, 6). Therefore, there is considerable interest in understanding how adenosine interacts with A_1 ARs.

To date, structure-function studies of A_1 ARs have largely focused on specific sites within TMs 5–7. Site-directed mutagenesis studies have suggested that His256 in TM6 plays a role in binding antagonists (7). Within TM7, the amino acid at position 270 is believed to account for species-related differences in affinity for A_1-selective drugs (8). Two separate studies have suggested that the amino acid at position 277 interacts with the 5′ position of the ribose moiety in adenosine (8, 9). A role for His278 has been suggested, although it is not clear whether receptors mutated at this site are expressed (7).

Although the above sites in TMs 5–7 may play important roles in ligand binding, it is unlikely that they will distinguish between A_1- and A_{2a}-selective ligands. Comparison of the primary amino acid sequences between A_1 and A_{2a} ARs reveals that the corresponding amino acids at positions 256, 270, 277, and 278 of the human A_1 AR are identical (10). Furthermore, recent studies of chimeric A_1/A_{2a} ARs have

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ABBREVIATIONS: AR, adenosine receptor; TM, transmembrane; PCR, polymerase chain reaction; CPA, N^6-cyclopentyladenosine; CCPA, 2-chloro-*N*6-cyclopentyladenosine; PIA, *N*6-phenylisopropyladenosine; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; DPX, 1,3-diethyl-8-phenylxanthine; NECA, 5′-*N*-ethylcarboxamidoadenosine; GPCR, G protein-coupled receptor; CHO, Chinese hamster ovary; PBS, phosphate-buffered saline; ANOVA, analysis of variance.

shown that TMs 1–4 of A_1 ARs are sufficient to confer the ligand-binding characteristics of an A_1 AR (10).

To identify additional sites involved in ligand/A_1 AR interactions, we used site-directed mutagenesis to test the role of several amino acids within the first four TM domains. Because polar amino acids and those with hydroxyl groups can interact with heteroatoms in the ligand (11, 12), we focused on examining the role of such sites within TMs 1–4. We report that Glu16 in TM1 and Asp55 in TM2 play important roles in agonist/A_1 AR interactions and show that Asp55 is responsible for allosteric regulation of ligand/A_1 AR binding by sodium. We show that conversion of Asp55 to Ala55 results in the formation of a mutant receptor with 100-fold higher affinity for agonists compared with the wild-type A_1 AR. Ser94 is also identified as an important site for agonist and antagonist binding.

Materials and Methods

cDNAs. The cDNA encoding the full-length human A_1 AR was provided by Dr. S. M. Reppert (Massachusetts General Hospital, Boston, MA). This cDNA has been extensively characterized (13).

Generation of mutant receptors. Mutant receptors were made by the PCR overlap-extension method of Ho *et al.* (14). Primer pairs were designed to introduce mutations of wild-type amino acids, similar to a previously described procedure (10). Oligonucleotides were synthesized using an Applied Biosystems oligonucleotide synthesizer (Norwalk, CT).

To generate the front part of mutant receptors, oligonucleotide primer pairs (primers A and B) were designed to generate a 5′ fragment of the A_1 AR. Another set of oligonucleotide primer pairs (primers C and D) were designed to generate a 3′ fragment of the A_1 AR receptor. B and C primers contained sequences that encoded for the desired mutations. To introduce an alanine, a glutamine, or a threonine mutation, codon sequences were changed to GCA, CAA, or ACC, respectively.

Receptor fragments were generated using >1 μg of DNA as the substrate for PCR reactions, and PCR reactions were performed using the Gene Amp Kit reagents (Perkin-Elmer Cetus, Norwalk, CT). PCR was generally performed using 30 cycles at 94° for 1 min, 55° for 1 min, and 72° for 2 min. PCR products were then separated on a 1% agarose gel and eluted using NA45 paper (Schleicher & Schuell, Keane, NH). Receptor fragments (A–B and C–D) were then combined in a third PCR reaction to generate a full-length A_1 AR using flanking primers (A and D).

Flanking PCR primers contained *Hind*III (A primers) or *Xba*I (D primers) restriction endonuclease sites at the ends. After fusion reactions, PCR products were digested with *Hind*III and *Xba*I and were subcloned into the mammalian expression vector pcDNA$_1$ (InVitrogen, San Diego, CA). Mutant receptors were then sequenced using Sequenase Version 2 (USB/Amersham, Arlington Heights, IL).

Acute transfections. Receptor cDNA expression was characterized using COS 7 cells as described previously (10). COS cells were grown as monolayers in Dulbecco's modified Eagle's medium (GIBCO BRL, Baltimore, MD) supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 100 g/ml streptomycin in 5% CO_2 at 37°. Cells were acutely transfected using the DEAE-Dextran method (15, 16). Plates (10-cm) were individually transfected with 5–10 μg of DNA or sham-transfected. At 48–72 hr after transfection, cells were tested by radioreceptor assay.

Radioreceptor assays. Radioligand-binding studies were performed using intact cells as described previously (10, 16). The radioligands used were [^{3}H]CCPA (DuPont-New England Nuclear, Boston, MA; specific activity, 33 Ci/mmol) and [^{3}H]DPCPX (DuPont-New England Nuclear; specific activity, 100 Ci/mmol). All determinations were done in quadruplicate. When constructs with different levels of expression were compared, the amount of tissue per tube was adjusted so that amounts of specific binding per tube were similar among the different constructs.

Generation of stable cell lines. Stable cell lines were generated as described previously (13). cDNAs encoding mutant A_1 ARs were subcloned into the mammalian expression vector pcDNA$_1$-NEO (InVitrogen) and were used to transfect CHO cells. Transfections were performed using Lipofectin (GIBCO BRL), and transfected cells were selected in the presence of geneticin (GIBCO BRL; 600–800 g/liter). Cell lines were then screened for receptor mRNA expression by dot-blotting and tested for ligand binding as described previously (13). Cell lines expressing mutant receptors were tested in parallel with a previously characterized CHO cell line (H6) that stably expresses the wild-type human A_1 AR (13). cAMP studies using stable cell lines were performed similarly to as described previously (13). Different stable cell lines with comparable levels of receptor expression were directly compared in functional studies.

Immunocytochemistry. Immunocytochemistry was performed on stable CHO cell lines as described previously (17) using validated antisera (17, 18). Cells were cultured in individual wells on the same slide using Lab-Tek tissue culture chambers (Nunc, Naperville, IL) that had been previously coated with poly-L-lysine (Sigma Chemical, St. Louis, MO). When cells were ~50% confluent, they were fixed for 15 min in 4% paraformaldehyde (21°) in PBS and processed for labeling in small, plastic slide mailers as reaction vessels. Endogenous peroxidase activity was blocked by incubating sections in chilled 3% H_2O_2 (Sigma Chemical) in PBS (10 min). The tissue sections were then washed in ice-cold PBS (three times for 10 min). Sections were next incubated with the A_1 AR antisera for 24 hr on an orbital shaker at room temperature (0.6 rpm; Lab-Line, Melrose Park, IL). The primary antisera dilution was 1:1000 in PBS containing 1.3% goat serum (Kirkegaard and Perry, Gaithersburg, MD) and 0.02% Triton X-100 (Sigma Chemical). Specimens were then washed in ice-cold PBS (three times for 5 min). Sections were incubated with a biotinylated secondary, goat anti-rabbit antisera (Kirkegaard and Perry) for 30 min at 21° with shaking. The slides were then washed in ice-cold PBS (twice for 5 min). Next, the slides were incubated with streptavidin-peroxidase for 30 min at 21° and washed in PBS (twice for 5 min), and the reaction product was generated using HistoMark BLACK (Kirkegaard and Perry). Slides were then counterstained with 0.5% methyl green and examined by light microscopy.

Statistical analysis. Saturation and competition binding data were analyzed by computer using an iterative nonlinear regression program (19). Comparisons among multiple groups were performed by one-way ANOVA with post-test comparisons among specific treatment groups performed by the Bonferroni method. Comparisons between paired groups were performed by the paired *t* test. The InStat statistics program (GraphPAD, San Diego, CA) was used for statistical computations.

Drugs. All adenosinergic compounds tested were obtained from Research Biochemicals (Natick, MA).

Results

Based on what is known about how other small molecules bind to GPCRs, we used computer analysis to identify receptor sites in TMs 1–4 that could potentially interact with ligands. For other receptors, charged amino acids and those with hydroxyl groups within TM regions can interact with ligand nitrogen and oxygen atoms (11, 12). Because adenosine contains several nitrogens within the adenine ring system and hydroxy groups in the ribose moiety (1), we focused on identifying charged or hydroxy amino acids within the first four TM domains of the human A_1 AR. To identify such sites, we compared primary sequence information of cloned ARs present in GenBank and >50 small molecule GPCRs (adrenergic, serotonergic, and muscarinic) using the SeqApp 1.9 sequence analysis software (D. Gilbert, Indiana University, Indianapolis, IN).

Using this approach, we identified several TM domain amino acids present in all cloned ARs that could potentially interact with adenosine: Glu16 (TM1), Ser93 and Ser94 (TM3), Ser135 (TM4), and Thr141 (TM4). In addition, we identified amino acids that were present only in A_1 ARs: Ser6 (amino terminus) and Ser23 (TM1). We also identified an aspartate conserved in TM2 of several GPCRs (20–23) that also was present in the human A_1 AR (Asp55) (Fig. 1).

To test the role of the above sites, each was individually changed to alanine, which is a strategy that has been successfully applied to other receptors (21, 24, 25). In addition, Glu16 was changed to glutamine. To determine whether the mutations influenced the affinity for agonists and antagonists (Fig. 2), saturation studies were performed using [^{3}H]CCPA and [^{3}H]DPCPX, respectively. Wild-type and mutant A_1 ARs were always studied at the same time. For the mutant receptors with K_d values that differed from K_d values of the wild-type A_1 AR by more than 3-fold, competition studies were also performed.

When Ala6, Ala23, Ala50, Ala93, Ala135, and Ala 141 constructs were tested, binding was readily detectable after expression in COS cells. When directly compared with the wild-type A_1 AR, affinities for [^{3}H]CCPA and [^{3}H]DPCPX were similar (Table 1), suggesting that Ser6, Ser23, Ser50, Ser93, Ser135, and Thr141 are not essential for conferring normal affinity for the ligands tested.

Although mutation of the above tested serine or threonine residues did not alter receptor affinity, mutation of Glu16 altered ligand-binding properties. After conversion of Glu16 to Ala16, we found that the affinity for [^{3}H]CCPA fell 10-fold, whereas the affinity for [^{3}H]DPCPX was not affected (Table 1). Competition studies performed using agonists [2-chloroadenosine, NECA, CPA, (*R*)-PIA] consistently revealed that affinity was reduced ≤10-fold (Table 2). In contrast, reduced affinity for antagonists was not observed, with the exception of N 0840 (*N*-cyclopentyl-9-methyl-9*H*-purin-6-amine), which in comparison to the other antagonists tested contains an adenine rather than a xanthine core (Table 2). To examine further the role of Glu16 on ligand binding, we also examined a construct in which Glu16 was converted to Gln16. Like with the Ala16 construct, competition studies revealed reduced affinity for agonists for the Gln16 construct (Tables 1 and 2; see Fig. 3 for CPA competition curve).

Because it was suggested that Glu16 may play a role in receptor activation (26, 27), we next tested whether the Gln16 construct could functionally regulate adenylate cyclase. This construct was chosen for these studies because it generally had lower affinity for agonists than did the Ala16 construct. CHO cells that functionally expressed the Gln16 construct were studied. Similar to CHO cells that express the wild-type A_1 AR (13), we found that CPA (100 nM) inhibited forskolin-stimulated cAMP accumulation in CHO cells expressing the Ala16 construct (256 ± 23 fmol/mg of protein; 1 nM [^{3}H]DPCPX). When dose-response curves for inhibition of cAMP accumulation by CPA were examined in tandem with stable cell lines expressing the wild-type A_1 AR, the dose-response curves were shifted to the right by ~10-fold (IC_{50} = 8.5 ± 1.0 E-8 for Gln16 versus 9.9 ± 0.3 E-10 M for wild-type; n = 3; Fig. 3). Thus, although the Gln16 construct functionally regulates cellular cAMP levels, it is less potent than the wild-type A_1 AR. When CPA dose-response curves for functional and drug competition studies were compared among the Gln16 construct and the wild-type A_1 AR (Fig. 3), the curves were comparably shifted to the right for the Gln16 construct by similar magnitudes.

Studies of Asp55 in TM2 also were very revealing. When Asp55 was changed to Ala55, the affinity of [^{3}H]CCPA was significantly greater than for the wild-type A_1 AR, whereas

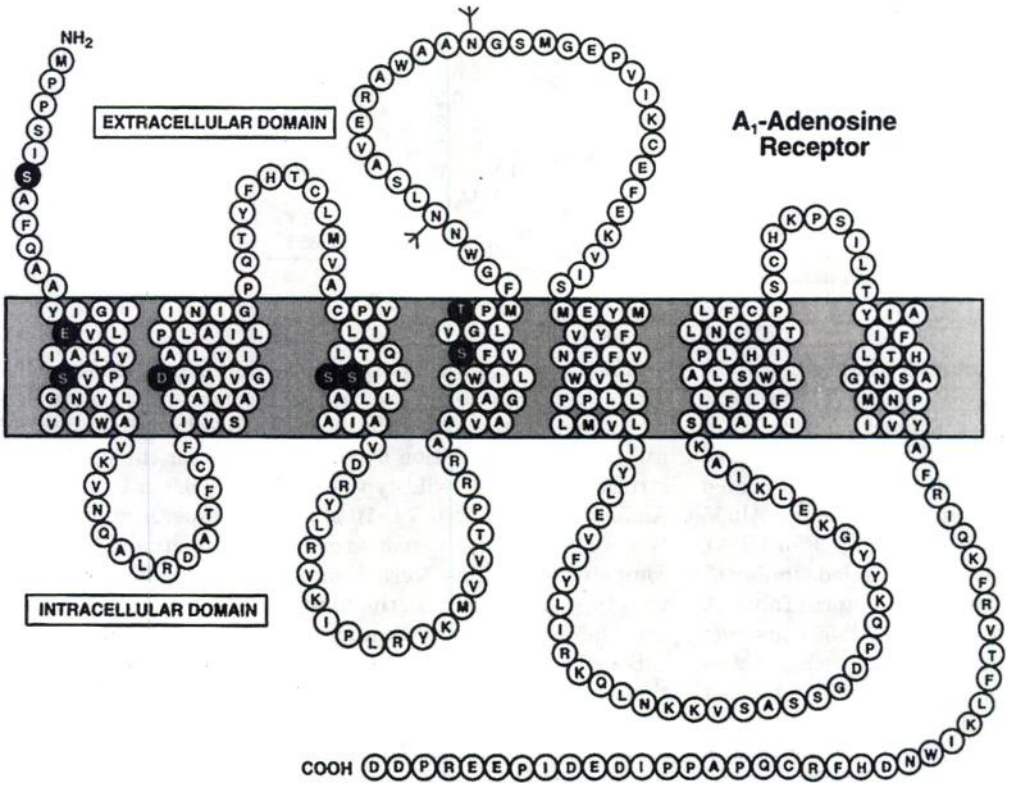

Fig. 1. Schematic representation of the human A_1 AR. *Black circles*, sites that were mutated.

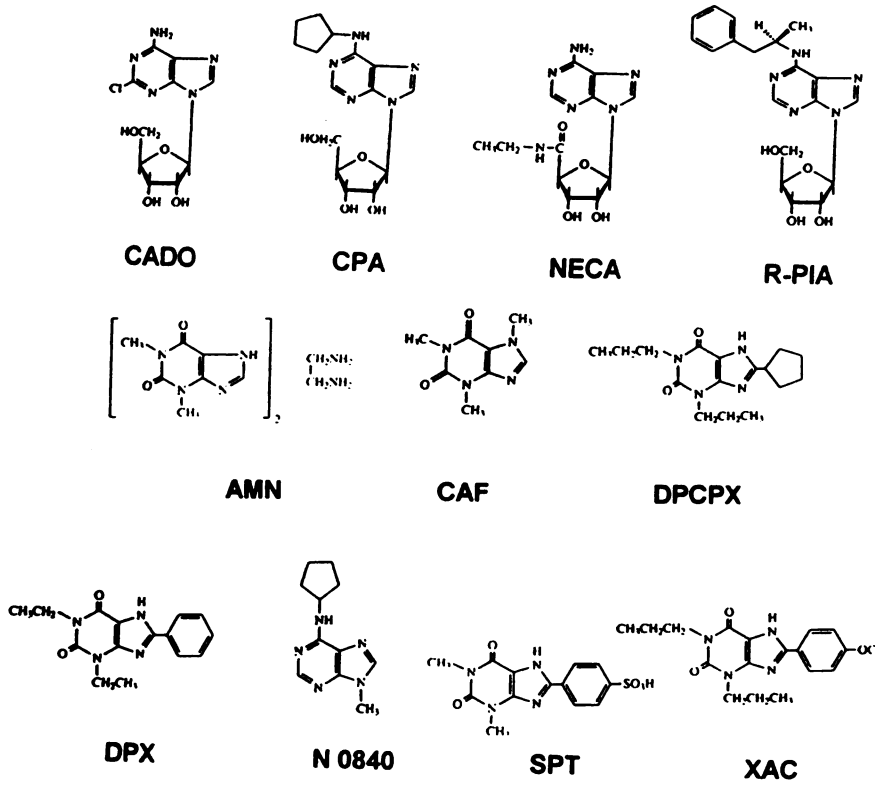

Fig. 2. Schematic representation of the ligands used in competition studies. *CADO*, 2-chloroadenosine; *AMN*, aminophylline; *CAF*, caffeine; *XAC*, xanthine amine congener.

TABLE 1

Binding affinities for [^{3}H]CCPA and [^{3}H]DPCPX in site-directed mutagenesis studies

All values are mean of three to six separate studies per construct.

Receptor construct	[^{3}H]CCPA			[^{3}H]DPCPX		
	K_d	B_{max}	Change (from wild-type A_1 AR)	K_d	B_{max}	Change (from wild-type A_1 AR)
	nM	*fmol/mg*	*–fold*	*nM*	*fmol/mg*	*–fold*
Wild-type A_1 AR	0.6 ± 0.15	550 ± 62		0.7 ± 0.2	565 ± 75	
Glu16→Ala16	5.3 ± 2.2	92 ± 13	8.8	1.3 ± 0.2	156 ± 112	1.3
Glu16→Gln16	9.3 ± 5*	63 ± 100	15.5	1.7 ± 0.2	252 ± 97	2.4
Ser50→Ala50	1.1 ± 0.2	112 ± 14	1.8	0.5 ± 0.3	90 ± 28	0.7
Asp55→Ala55	0.2 ± 0.1[a]	330 ± 67	0.3	0.5 ± 0.2	252 ± 87	0.7
Ser93→Ala93	1.0 ± 0.2	65 ± 13	1.6	0.4 ± 0.2	87 ± 21	0.6
Ser94→Ala94	N.D.			N.D.		
Ser135→Ala135	0.6	303	1.0	0.72	757	1.0
Thr141→Ala141	0.4	611	0.6	0.74	651	1.0

[a] $p < 0.05$ by ANOVA with Bonferroni post-test comparison versus wild type A_1 AR.
N.D., not determined.

the affinity for [^{3}H]DPCPX was similar to that of the wild-type A_1 AR (Table 1). Agonist competition studies similarly showed higher affinity for the Ala55 construct than the wild-type receptor (Table 2). Examination of competition curves for the Ala55 construct versus the wild-type A_1 AR revealed that the slopes of the curves were more acute for the Ala55 construct than for the wild-type A_1 AR (see Fig. 3 for CPA). Antagonist competition studies, however, revealed similar K_I values between the mutant and wild-type receptors (Table 2).

Next, we assessed whether changes in the Ala55 construct functionally affected the regulation of adenylyl cyclase activity. Stable CHO cell lines expressing the Ala55 construct were generated, and the cell line with the highest level of cell surface receptor expression (line I7; 325 ± 36 fmol/mg of protein at 1 nM [^{3}H]DPCPX) was tested in side-by-side experiments with the H6 (13) cell line that expressed the wild-type A_1 AR. Similar to the results of ligand-binding studies, we found that the IC_{50} for inhibiting forskolin-stimulated (10 nM) cAMP accumulation by CPA was less for the Ala55 construct than for the wild-type A_1 AR (IC_{50} 6.9 ± 0.2 E-11 for Ala55 versus 9.9 ± 0.3 E-10 M for wild-type; n = 3; Fig. 3). When DPCPX dose-response curves for functional and drug competition studies were compared for Ala55 versus the wild-type A_1 AR (Fig. 3), the curves were shifted to the left for the Ala55 construct by comparable orders of magnitude.

Because studies of other G_i-linked GPCRs have revealed that the negatively charged aspartate residue in TM2 conserved among many receptors plays a role in allosteric modulation by sodium (2, 21, 28), we assessed whether Asp55 modulates allosteric effects of sodium. In parallel, we also

TABLE 2

K_I values from competition of [^{3}H]DPCPX binding

Values are mean of three or more separate studies per drug in which samples were tested in quadruplicate in each study in side-by-side studies with the wild-type human A_1 AR.

Drug	Ala16	Gln16	Ala55	Wild-type A_1 AR
		K_I M		
Agonist				
NECA	4.2 ± 0.4 E-5[a]	1.5 ± 0.6 E-4[a]	7.0 ± 3.6 E-9[a]	5.0 ± 3.3 E-6
2-Chloroadenosine	1.3 ± 2.0 E-4[a]	5.6 ± 1.4 E-5[a]	1.5 ± 1.5 E-7[a]	8.4 ± 2.1 E-6
(*R*)-PIA	1.3 ± 0.3 E-6	4.1 ± 0.4 E-6[a]	1.2 ± 0.1 E-9[a]	3.7 ± 0.8 E-7
CPA	5.3 ± 2.1 E-6	7.3 ± 3.3 E-5[a]	9.6 ± 4.8 E-10[a]	1.3 ± 1.2 E-7
Antagonist				
Caffeine	5.2 ± 4.2 E-4		6.3 ± 3.2 E-4	7.5 ± 4.4 E-4
N 0840	3.8 ± 0.8 E-5[a]	1.4 ± 0.8 E-5[a]	4.1 ± 1.0 E-6	1.3 ± 1.2 E-6
Aminophylline	8.3 ± 1.2 E-5		5.2 ± 0.6 E-5	7.7 ± 1.0 E-5
DPX			2.7 ± 0.8 E-7	1.8 ± 0.4 E-7
DPCPX	2.3 ± 1.1 E-9	3.3 ± 1.3 E-9	1.6 ± 0.3 E-9	2.2 ± 0.2 E-9

[a] $p < 0.05$ by ANOVA with Bonferroni post-test comparison versus wild-type A_1 AR.

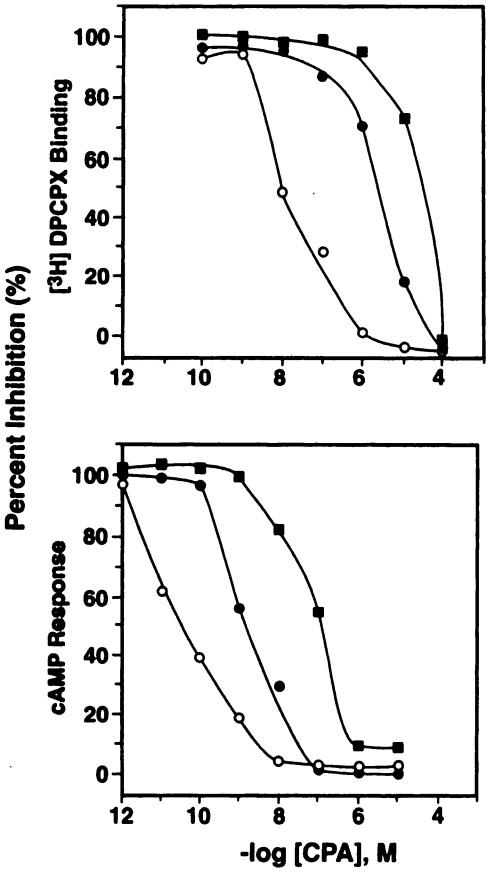

Fig. 3. Comparison of CPA competition studies and cAMP dose-response studies for the wild-type A_1 AR (●), and Gln16 (■), and Ala55 (○) constructs. *Top*, competition of [^{3}H]DPCPX [1 nM] binding by CPA. Binding is expressed as a percentage of total binding. *Bottom*, inhibition of forskolin-stimulated (10 nM) cAMP accumulation. cAMP levels are expressed as a percentage of maximal inhibition. Data are representative of three separate studies.

assessed whether the negatively charged Glu16 residue in TM1 modulated allosteric effects of sodium.

Competition studies were therefore performed in 50 mM Tris·HCl buffer, pH 7.4, that contained 0, 140, or 400 mM NaCl. Studies of wild-type receptors revealed significantly lower affinities for CPA at 400 mM NaCl than at lower salt concentrations (0 mM NaCl, K_I = 4.1 ± 3.2 E-6; 140 mM NaCl, K_I = 4.6 ± 3.8 E-7; 400 mM NaCl, K_I = 2.2 ± 0.2 E-5; $p <$ 0.001, ANOVA) (Fig. 4). Studies of the Ala16 construct also revealed that an allosteric effect of sodium was present because the affinity for CPA was lowest at high salt concentrations (0 mM NaCl, K_I = 3.3 ± 1.9 E-6; 140 mM NaCl, K_I = 1.8 ± 1.6 E-5; 400 mM NaCl, K_I = 4.3 ± 1.3 E-5; $p < 0.05$, ANOVA). It is also interesting to note that the wild-type receptor showed highest affinity for CPA at 140 mM NaCl, whereas the Ala16 construct showed the highest affinity for CPA at 0 mM NaCl.

In contrast to the above receptors, studies of the Ala55 mutation failed to reveal an influence of sodium concentration on affinity for CPA. K_I values were similar from 0 to 400 mM NaCl (0 mM NaCl, K_I = 4.0 ± 1.4 E-8; 140 mM NaCl, K_I = 5.4 ± 2.3 E-8; 400 mM NaCl, K_I = 5.1 ± 1.7 E-8; $p > 0.05$, ANOVA) (Fig. 4), suggesting that Asp55 exerts an prominent role in allosteric regulation of binding by sodium.

We also tested for allosteric effects of sodium on binding of the antagonist DPCPX. However, in contrast to CPA studies, the K_I values for DPCPX were similar over the range of NaCl concentrations tested for the wild-type A_1 AR (0 mM NaCl, K_I = 2.3 ± 0.4 E-6; 140 mM NaCl, K_I = 2.8 ± 0.2 E-7; 400 mM NaCl, K_I = 2.2 ± 0.3 E-7; $p > 0.05$, ANOVA). An effect of sodium on DPCPX binding also was not observed for the Ala55 receptor (0 mM NaCl, K_I = 2.0 ± 0.4 E-7; 140 mM NaCl, K_I = 3.1 ± 0.2 E-7; 400 mM NaCl, K_I = 2.2 ± 0.3 E-7; $p >$ 0.05, ANOVA).

Although each of the above tested mutations yielded functional binding, conversion of Ser94 to Ala94 resulted in the loss of detectable binding by [^{3}H]CCPA or [^{3}H]DPCPX, raising the possibilities that receptor affinity is markedly reduced or ligand binding is lost by this change. Thus, to test for the possibility that the Ala94 construct encoded for receptors with very low affinity for adenosine agonists, several stable CHO cell lines (L2, L19, L25) were generated that expressed Ala94 mRNA by dot-blotting. However, although three separate cell lines were tested, an influence of CPA (10 μM) or NECA (10 μM) on basal or forskolin-stimulated (10 μM) cAMP levels could not be detected.

Because we did not detect functional binding sites, we next tested whether the Ala94 construct encoded for a receptor protein. Using immunocytochemistry, A_1 AR immunoreactivity was examined in the stable cells lines in parallel with cell lines expressing the wild-type A_1 AR (H6 cell line). Nontransfected CHO cell lines were also examined in parallel. We

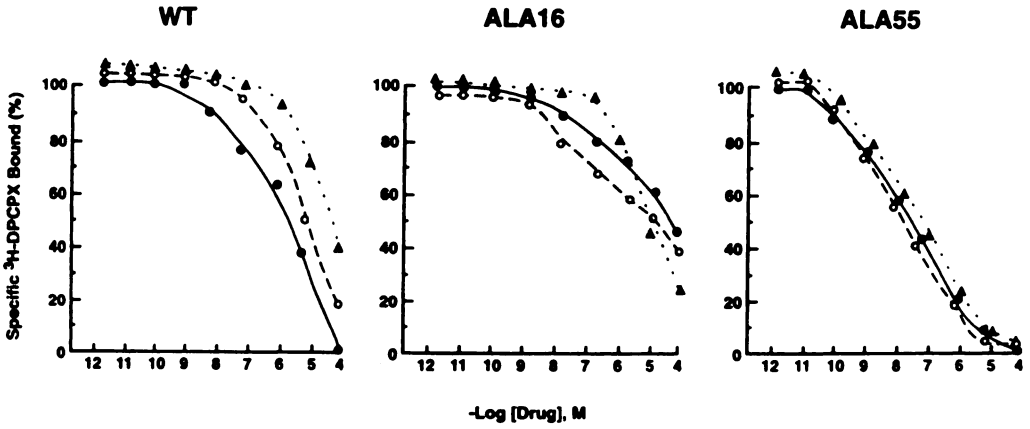

Fig. 4. Studies of allosteric affects of NaCl. Competition of [^{3}H]DPCPX [1 nM] binding by CPA to the wild-type (*WT*) A_1 AR, the Glu16-to-Ala16 A_1 AR construct, and the Asp55-to-Ala55 A_1 AR construct is shown in the presence of (○) 0, (●) 140, and (▲) 400 mM NaCl. Binding is expressed as a percentage of total binding. Data are representative of three separate studies.

have previously shown that preimmune serum does not label the H6 cell line (17).

Results of immunocytochemistry studies revealed comparable levels of A_1 AR immunoreactivity for the H6, L2, L19, and L25 cell lines (Fig. 5). Indicating that there was cell surface A_1 AR expression, the outlines of cells were clearly apparent. In contrast, there was no labeling over nontransfected CHO cells or when the primary antisera was omitted from incubations with the H6, L2, L19, and L25 cell lines. The Ala94 construct thus seems to encode for a receptor with A_1 AR immunoreactivity that does not bind adenosine and xanthine analogs.

Although the above studies suggest that Ala94 encodes for a receptor protein, we considered that substitution of alanine for serine altered receptor conformation, leading to the loss of binding. Thus, to further examine the role of Ser94, we next tested whether substitution of serine by threonine altered ligand-binding properties. For these studies, COS cells, acutely transfected with either the wild-type A_1 AR or the Thr94 constructs were examined in side-by-side experiments. When different agonists were tested [NECA, (*R*)-PIA, CPA], we found similar affinities for the Thr94 construct and the wild-type receptor. In contrast, the affinity for ethyl and propyl N1- and N3-substituted antagonists was reduced 3–4 fold, whereas the affinity for caffeine and aminophylline was not altered (Table 3).

Discussion

In studies of chimeric A_1/A_{2a} ARs, TMs 1–4 of the human A_1 AR were shown to confer specificity for A_1-selective ligands (10).

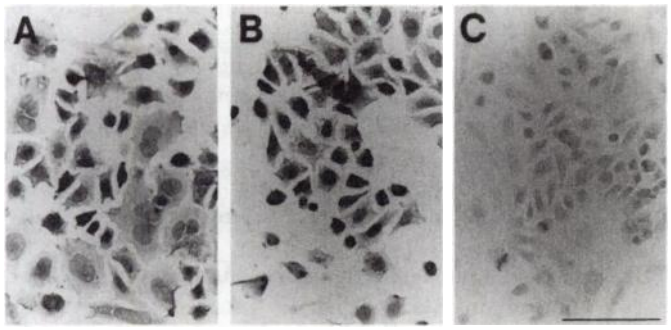

Fig. 5. Patterns of A_1 AR immunoreactivity over stably transfected CHO cell lines. A, Wild-type A_1 AR (line H6). B, Thr94 mutant (line L2). C, Nontransfected CHO cells. *Black staining*, specific labeling. *Scale bar*, 50 mm.

Because single-residue substitution studies have not previously involved amino acids in the first four TM domains of A_1 ARs, we sought to identify sites within TMs 1–4 that could potentially influence ligand/A_1 AR interactions. For other GPCRs that bind small molecules, charged amino acids and those with hydroxyl groups influence ligand binding (11, 12). We therefore tested the role of several such amino acids in the first four TM domains of the A_1 AR that were common to all adenosine or A_1 ARs. Of the eight residues tested, only modification of Glu16, Asp55, and Ser94 resulted in altered binding characteristics. In contrast, modification of Ser6, Ser23, Ser93, Ser135, and Thr141 did not alter ligand binding.

Studies of acidic amino acids Glu16 and Asp55 suggested a role for these sites in agonist binding. When these sites were mutated to alanine, broad changes were seen in affinities for agonists, whereas altered affinity for antagonists was hardly observed. Mutagenesis studies of other receptors have shown that sites that influence agonist but not antagonist binding generally play a role in maintaining the conformation of the agonist-bound state of the receptor (2, 29). Thus, our findings suggest that Glu16 and Asp55 mutations may influence ligand/receptor conformation.

When Glu16 was changed to Ala16 or Gln16, we found that the affinity for agonists was reduced >10-fold. Of the antag-

TABLE 3

K_i values from competition of [^{3}H]DPCPX binding of the Thr94 construct versus wild-type A_1 AR

Values are mean of three or more separate studies per drug in which samples were tested in quadruplicate in each study. [^{3}H]DPCPX K_d = 2.5 ± 0.2 E-10 M, B_{max} = 657 ± 57 fmol/mg of protein for wild-type; 5.5 ± 1.2 E-10 M, B_{max} = 322 ± 43 fmol/mg of protein for Thr94 in three separate side-by-side studies.

Drug	K_i		Change from wild-type
	Thr94	Wild-type A_1 AR	
	M		*-fold*
Agonist			
NECA	1.0 ± 0.2 E-6	5.0 ± 2.3 E-7	0.5
(*R*)-PIA	1.2 ± 0.2 E-6	1.2 ± 0.2 E-6	1.0
CPA	1.0 ± 0.3 E-8	6.0 ± 2.0 E-7	0.6
Antagonist			
Caffeine	5.9 ± 1.5 E-5	5.4 ± 1.2 E-5	1.1
Aminophylline	3.7 ± 1.0 E-5	6.2 ± 0.8 E-5	0.5
DPX	4.2 ± 0.4 E-7	1.3 ± 0.2 E-7	3.2*
DPCPX	3.7 ± 0.4 E-7	1.0 ± 0.1 E-7	3.7*
SPT	7.2 ± 2.0 E-8	2.3 ± 0.8 E-8	3.1
Xanthine amine congener	4.9 ± 0.4 E-9	1.1 ± 0.2 E-9	4.4*

[a] $p < 0.05$ by paired *t* test, Thr94 versus wild-type A_1 AR.

onists tested, affinity was reduced only for N 0840, which consists of a modified adenine core rather than the modified xanthine core present in the other antagonists tested (Fig. 2). Thus, it is possible that the Glu16 position influences interactions with the adenine group.

Because the CHO cells transfected with the Gln16 construct functionally regulated adenylate cyclase, it is unlikely that the observed reductions in agonist affinity were mediated by impaired receptor/G protein coupling. Furthermore, receptor/G protein coupling is poor in acutely transfected COS cells, and changes in ligand-binding properties seen with COS cells generally reflect changes in receptor affinity (29). Thus, mutation of Glu16 seems to induce true decreases in receptor affinity. Furthermore, the rightward shifts in the CPA dose-response curves for competition and functional studies were similar.

It has also been suggested that Glu16 plays a role in receptor activation (26, 27). Analogous to opsin/rhodopsin interactions (30), it has been theorized that AR activation involves proton transfer from the ligand to the glutamate in TM1 (26, 27). However, the observation that the Gln16 construct functionally regulates adenylate cyclase challenges this contention.

In addition to generating an A_1 AR with lower affinity for agonists, we developed an A_1 AR with higher affinity for agonists than the wild-type A_1 AR. Mutations of aspartic acid residues in other receptors have been shown to result in decreases or increases in receptor affinity (20, 21, 29, 31, 32). Similarly, we found that the affinity for agonists increased with conversion of Asp55 to Ala55 by both ligand binding and studies of cAMP accumulation.

We also found that the Asp55 site was responsible for allosteric regulation of ligand binding by sodium. At high-salt concentrations, agonist affinity for the wild-type A_1 AR fell by 1 order of magnitude. This degree of change is consistent with that observed for several GPCRs (20, 21). However, when Asp55 was converted to Ala55, receptor affinity did not change over sodium concentrations of 0–400 mM. Interestingly, although Glu16 also is an acidic amino acid, allosteric regulation of A_1 AR binding by sodium continued to be present when this site was changed to Ala16. There were, however, some differences in the rank order of receptor affinity for CPA between the Ala16 construct ($0 < 140 < 400$ mM NaCl) compared with the wild-type A_1AR ($140 < 0 < 400$ mM NaCl). Thus, it is possible that this site may influence sodium effects on binding to a limited extent.

Of all of the constructs tested, only mutation of Ser94 resulted in the loss of detectable ligand binding. Using stably transfected cells expressing the Ala94 construct, we failed to detect regulation of cAMP levels even at high agonist concentrations. We were, however, able to detect robust A_1 AR immunoreactivity in these cells. Thus, mutation of Ser94 to Ala94 results in the inability of both agonists and antagonists to bind to A_1 AR.

Because the Ala94 construct failed to yield functional binding sites, we also tested whether conversion of Ser94 to Thr94 altered ligand binding. Similar strategies have been successfully applied to study the role of Thr277 in TM7 of the human A_1 AR (3, 6). With this substitution, we found that conversion of Ser94 to Thr94 resulted in impaired affinity for several antagonists.

When PST, xanthine amine congener, DPX, and DPCPX were tested, each had a 3–4-fold lower affinity for the Thr94 construct than for the Ser94 construct. Although modest, the changes in affinity for the Thr94 versus the wild-type A_1 AR receptor were comparable to results observed in other serine/threonine substitution studies (9). Because these compounds have considerably different substitutions at the C8 position, it is unlikely that Ser94 interact with the C8 region. Each of these compounds, however, contains an ethyl or propyl group at the N1 and N3 positions. Because Thr94 did not have reduced affinity for caffeine and aminophylline that are not similarly modified at the N1 or N3 positions, we postulated that steric hindrance introduced by the conversion of Ser94 to Thr94 modified interactions with the N1 or N3 groups.

Because the N1 and N3 positions of the antagonists tested do not have a free hydrogen, it is unlikely that the serine position will directly interact with these sites by hydrogen bonding.[1] Rather, it is more likely that Ser94 will interact with the oxy group at the C2 and C6 positions. Ser94 may also interact with the N7 or N9 position. However, because C8 is close to the N7 and N9 sites, it is more likely that Ser94 interactions will be with the C2 or C6 site.

Currently, it is believed that the N6 positions of agonists and the C8 positions of antagonists have a similar orientation in the receptor-binding site (33, 34). Analogous to antagonist studies, we did not observe reduction in agonist affinity among several different N6-substituted compounds [CPA, (*R*)-PIA, (*S*)-PIA]. Thus, it is also unlikely that Ser94 will interact with the N6 adenine position. Rather, it is more likely that Ser94 interacts with an adenine ring site.

The adenine ring contains nitrogen at the 1, 3, 7, and 9 positions (33). Replacement of the N1 or N3 nitrogens reduces but does not eliminate the ability of adenosine to bind to A_1 ARs (33, 35). Selective removal of the N7 or N9 atoms, on the other hand, results in a ligand that does not bind to A_1 or A_{2a} receptors (33, 35). Because conversion of Ser94 to Ala94 also results in a complete loss of detectable agonist binding, we postulate that Ser94 may interact with the N7 or N9. Because of the lack of commercially available compounds modified at these positions, it was not possible for us to further pharmacologically define which of these agonist sites interacts with Ser94. Thus, it is not surprising that changes in affinity for agonists tested were not observed.

There is considerable precedent for TM serine residues in binding small molecule ligands. In adrenergic receptors, important serine residues are contained within SSXZS motifs present in TM5 (36–38). The later two serines influence ligand binding, whereas the first serine is not important (37). Examination of the primary structure of all cloned A_{2a}, A_{2b}, and A_3 ARs reveals a similar SSXZS motif in TM3, whereas A_1 ARs contain a S93S94XYA97 motif in TM3 (GenBank). Analogous to that observed for adrenergic receptors, our studies thus suggest that the first serine (Ser93) is not essential for ligand binding. The second serine (Ser94), however, is essential for both agonist and antagonist interactions.

In contrast to other small molecules (e.g., catecholamines and serotonin), which typically contain only a few reactive sites (11, 12), adenosine and xanthine analogs contain several sites that can potentially interact with receptors. Agonists have five ring nitrogens, one ring oxygen, and three hydroxyl residues. Antagonists have four ring nitrogens and two oxy groups. Thus, we anticipate that AR interactions will be quite complex. Currently, models of adenosine/AR interactions are based largely on ligand interactions with sites in

TMs 6 and 7 (26, 39, 40). It has also been suggested that Ser94 is in the ligand binding site (26). Our observations now directly demonstrate that the first three TM domains of the A_1 AR receptor are important for ligand/receptor interactions. Glu16 in TM1 and Asp55 in TM2 influence agonist/A_1 AR interactions, and we identify Ser94 in TM3 as a site that is important for interaction with both agonists and antagonists. With the continued identification of specific residues that are important for ligand/receptor interactions, we anticipate that it will be possible to develop new models of A_1 ARs binding adenosine and its analogs in the future.

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