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# Hydrophilic Side Chains in the Third and Seventh Transmembrane Helical Domains of Human $A_{2A}$ Adenosine Receptors Are Required for Ligand Recognition

QIAOLING JIANG, A. MICHIEL VAN RHEE, JEONGHO KIM, SUSAN YEHLE, JÜRGEN WESS, and KENNETH A. JACOBSON

*Molecular Recognition Section (Q.J., A.M.v.R., J.K., S.Y., K.A.J.) and Drug Receptor Interaction Section (J.W.), Laboratory of Bioorganic Chemistry, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland 20892*

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## SUMMARY

Hydrophilic residues of the G protein-coupled human $A_{2A}$ adenosine receptor that are potentially involved in the binding of the ribose moiety of adenosine were targeted for mutagenesis. Residues in a $T_{88}QSS_{91}$ sequence in the third transmembrane helical domain (TM3) were individually replaced with alanine and other amino acids. Two additional serine residues in TM7 that were previously shown to be involved in ligand binding were mutated to other uncharged, hydrophilic amino acids. The binding affinity of agonists at T88 mutant receptors was greatly diminished, although the receptors were well expressed and bound antagonists similar to the wild-type receptor. Thus, mutations that are specific for diminishing the affinity of ribose-containing ligands (i.e., adenosine agonists) have been identified in both TM3 and TM7. The T88A and T88S mutant receptor fully stimulated adenylyl cyclase, with the dose-response curves to CGS 21680 highly shifted to the right. A Q89A mutant gained affinity for all agonist and antagonist ligands examined in binding and functional assays. Q89 likely plays an indirect role in ligand binding. S90A, S91A, and S277C mutant receptors displayed only moderate changes in ligand affinity. A S281N mutant gained affinity for all adenosine derivatives (agonists), but antagonist affinity was generally diminished, with the exception of a novel tetrahydrobenzothiophenone derivative.

Adenosine acts as a neuromodulator in the central and peripheral nervous systems and as a homeostatic regulator in a variety of other systems, including the cardiovascular, renal, and immune systems (1). Four pharmacologically distinct adenosine receptor subtypes, $A_1$, $A_{2A}$, $A_{2B}$, and $A_3$, have been cloned (2, 3). Activation of adenosine $A_{2A}$ receptors, in general, increases the energy supply in various organs. The regulation of blood pressure by centrally (4) and peripherally (5) mediated mechanisms involves $A_{2A}$ receptors. Activation of $A_{2A}$ receptors results in vasodilatation, and this effect has been examined as a potential antihypertensive therapy using selective $A_{2A}$ agonists such as CGS 21680 {2-[4-[(2-carboxyethyl)phenyl]ethyl-amino]-5′-*N*-ethylcarboxamidoadenosine} (6). $A_{2A}$ receptors, present in platelets, where they inhibit aggregation, and in the liver, have also been investigated for therapeutic applications. In the brain, $A_{2A}$ receptors occur primarily in the striatum, where they are colocalized with $D_2$ dopamine receptors (7). Adenosine acts in a manner opposite to dopamine and thus elicits locomotor depression (8). Diseases in which the dopaminergic system is hyperactive [e.g., schizophrenia (9) and Huntington's disease (8)] may be mitigated by $A_{2A}$ receptor activation. Parkinson's disease, in which the dopaminergic system is hyporesponsive, may be amenable to treatment with selective $A_{2A}$ receptor antagonists (10, 11).

We characterized $A_{2A}$ adenosine receptors through the design and use of novel ligand probes, including radioligands and affinity labels (1, 12), and biotinylated probes and fluorescent labels (13, 14). In addition, site-directed mutagenesis (15) and molecular modeling (16) were used for $A_{2A}$ receptor characterization. A rhodopsin-based model (15) of the human $A_{2A}$-receptor has been proposed. This model is highly consistent with mutagenesis results regarding orientation of individual amino acid residues within the central ligand-binding cavity, thus implicating residues in TM5, TM6, and TM7 of the $A_{2A}$ receptor in ligand recognition (15). In particular, two

**ABBREVIATIONS:** TM, (helical) transmembrane domain; ADAC, $N^6$-[4-[[[[4-[[[(2-aminoethyl)amino]carbonyl]methyl]anilino]carbonyl]methyl]-phenyl]adenosine; $BTH_4$, ethyl 3-benzylthio-4,5,6,7-tetrahydrobenzo[*c*]thiophen-4-one-1-carboxylate; CADO, 2-chloroadenosine; DPMA, $N^6$-[2-(3,5-dimethoxyphenyl)-2-(2-methylphenyl)ethyl]adenosine; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; GPCR, G protein-coupled receptor; HA, hemagglutinin; IB-MECA, $N^6$-(3-iodobenzyl)adenosine-5′-*N*-methyluronamide; NECA, 5′-*N*-ethylcarboxamidoadenosine; PCR, polymerase chain reaction; *R*-PIA, (*R*)-$N^6$-phenylisopropyladenosine; XAC, (8-[4-[[[[(2-aminoethyl)-amino]carbonyl]methyl]oxy]phenyl]-1,3-dipropylxanthine).

histidine residues (H250 and H278) and S277 are essential for ligand binding. The adenine moiety likely interacts with a pocket of aromatic amino acids in TM6, and the ribose moiety likely interacts with a hydrophilic region in TM7. Mutagenesis studies of $A_1$ adenosine receptors have identified some of the corresponding residues as necessary for ligand binding. The corresponding histidine residues (17), a hexapeptide region of TM5 (18), and I274 and S277 in TM7 of bovine $A_1$ receptors (19, 20) are involved in ligand binding. Thus, the position of the receptor-bound adenosine is likely to be similar in these subtypes.

In this study, we introduced the novel finding that residues of TM3 are essential for the binding of ligands to the human $A_{2A}$ receptor and further explore the role of the previously modified serine residues in TM7 (15). A long-range goal of this investigation is the design of more selective pharmacological agents based on structural differences in receptors.

## Experimental Procedures

**Materials.** Human $A_{2A}$ adenosine receptor cDNA ($pSVLA_{2A}$) was provided by Dr. Marlene A. Jacobson (Merck Research Labs, West Point, PA). *Taq* polymerase for the PCR was purchased from Perkin-Elmer Cetus (Norwalk, CT). All enzymes used in this study were obtained from New England Biolabs (Beverly, MA). The agonists CGS 21680, NECA, *R*-PIA, CADO, and DPMA and the antagonists XAC and CGS 15943 {9-chloro-2-(furyl)[1,2,4]triazolo[1,5-*c*]quinazolin-5-amine} were from RBI (Natick, MA). [$^3$H]CGS 21680 (41.2 Ci/mmol) and [$^3$H]XAC (118 Ci/mmol) were obtained from DuPont-New England Nuclear (Boston, MA), and [$^3$H]adenine (15 Ci/mmol) was purchased from American Research Chemicals Inc. (St. Louis, MO). IB-MECA was prepared as described previously (21). $BTH_4$ was obtained from Maybridge Chemicals (Trevillet, UK). Chemical structures of the agonist ligands used in this study may be found in Kim *et al.* (15). FBS and *o*-phenylenediamine dihydrochloride were purchased from Sigma Chemical Co. (St. Louis, MO). The Sequenase Kit, ATP, and cAMP were from United States Biochemical (Cleveland, OH). All oligonucleotides used were synthesized by Bioserve Biotechnologies (Laurel, MD). A monoclonal antibody (12CA5) against an HA epitope was purchased from Boehringer-Mannheim Biochemicals (Indianapolis, IN), and goat anti-mouse IgG ($\gamma$-chain specific) antibody conjugated with horseradish peroxidase was purchased from Sigma. DEAE-dextran was obtained from Pharmacia LKB (Piscataway, NJ). Rolipram was a gift from Schering AG (Berlin, Germany). SCH 58261 {5-amino-7(phenylethyl)-2-(2-furyl)-pyrazolo[4,3-*e*]1,2,4-triazolo[1,5-*c*]pyrimidine} was a gift from Dr. E. Ongini (Schering, Milan, Italy) and Prof. P. Baraldi (University of Ferrara, Italy). ZM 241385 {4-(2-[7-amino-2-(2-furyl)[1,2,4]triazolo[2,3$\alpha$][1,3,5]triazinyl-amino]ethyl)-phenol} was a gift from Dr. S. Poucher (Zeneca, Macclesfield, UK).

**Plasmid construction and site-directed mutagenesis.** The coding region of $pSVLA_{2A}$ was subcloned into the pcD cDNA expression vector (22), yielding $pcDA_{2A}$. All mutations were introduced into $pcDA_{2A}$ by using standard PCR mutagenesis techniques (23). The accuracy of all PCR-derived sequences was confirmed by dideoxy sequencing of the mutant plasmids (24).

**Epitope tagging.** A 9-amino acid sequence derived from the influenza virus HA protein (TAC CCC TAC GAC GTC CCC GAC TAC GCC; peptide sequence: YPYDVPDYA) was inserted after the second methionine residue at the extracellular amino terminus of the $A_{2A}$ adenosine receptor gene (15). Oligonucleotides containing the HA-tag sequence were designed and used to generate PCR fragments, which were then used to replace the homologous wild-type $pcDA_{2A}$ sequences.

**Transient expression of mutant receptors in COS-7 cells.** COS-7 cells ($2 \times 10^6$) were seeded into 100-mm culture dishes containing 10 ml of Dulbecco's modified Eagle's medium supplemented with 10% FBS. Cells were transfected with plasmid DNA (4 $\mu$g of DNA/dish) according to the DEAE-dextran method (25) ~24 hr later and grown for an additional 72 hr at 37°.

**Membrane preparation and radioligand binding assay.** Cells were scraped into ice-cold lysis buffer (4 ml of 50 mM Tris, pH 6.8, at room temperature, containing 10 mM $MgCl_2$). Harvested cells were homogenized using a Polytron homogenizer (Brinkmann, Westbury, NY) and then spun at 27,000 × *g* for 15 min. Cell membranes (pellet) were resuspended in the same buffer.

For saturation and competition binding experiments, each tube contained 100 $\mu$l of membrane suspension (containing 2 units/ml adenosine deaminase (Boehringer-Mannheim Biochemicals), 50 $\mu$l of radioligand, and either 50 $\mu$l of buffer/competitor (50 mM Tris, pH 6.8, 10 mM $MgCl_2$) or 50 $\mu$l of 80 $\mu$M CADO in buffer (to determine nonspecific binding). The mixtures were incubated at 25° for 120 min, filtered, and washed three times with ~5 ml of ice-cold buffer/wash using a Brandel cell harvester (Gaithersburg, MD). Pharmacological parameters were analyzed using the KaleidaGraph program (version 3.01; Abelbeck/Synergy Software, Reading, PA). Statistical analysis was performed using the alternate *t* test (InStat version 2.04; GraphPad, San Diego, CA).

**cAMP determination.** cAMP levels were determined by measurement of the conversion of [$^3$H]ATP to [$^3$H]cAMP. One day after transfection, cells were transferred from 100-mm dishes into six-well dishes (~$3 \times 10^5$ cells/well) and incubated with culture media containing 2 $\mu$Ci/ml [$^3$H]adenine. After 24 hr, the cultures were washed and incubated with 1 ml/well Hanks' balanced salt solution containing 0.1 mM rolipram for 15 min at 37°. The cells were incubated with different concentrations of the agonist CGS 21680 (in culture media) for 30 min at 37°. The reaction was terminated by aspiration of the media and the addition of 1 ml of ice-cold 5% trichloroacetic acid containing 1 mM ATP and 1 mM cAMP. After a 30-min incubation at 4°, cell lysates were eluted through sequential chromatography on Dowex and alumina columns (26). cAMP formation is expressed as percentage of maximal stimulation of conversion of [$^3$H]ATP to [$^3$H]cAMP (27). At agonist concentrations of >100 $\mu$M, a stimulation was observed in nontransfected COS-7 cells (15), and these values were subtracted from values obtained in the transfected mutant receptor cells.

**ELISA.** For indirect cellular ELISA measurements, cells were transferred to 96-well dishes (4–5 × $10^4$ cells/well) 1 day after transfection. At ~48 hr after splitting, cells were fixed in 4% formaldehyde in phosphate-buffered saline for 30 min at room temperature. After washing with phosphate-buffered saline three times and blocking with Dulbecco's modified Eagle's medium (containing 10% FBS), cells were incubated with HA-specific monoclonal antibody (12CA5; 20 $\mu$g/ml) for 3 hr at 37°. Plates were washed and incubated with a 1:2000 dilution of a peroxidase-conjugated goat anti-mouse IgG antibody (Sigma) for 1 hr at 37°. Hydrogen peroxide and *o*-phenylenediamine (each 2.5 mM in 0.1 M phosphate/citrate buffer, pH 5.0) served as substrate and chromogen, respectively. The enzymatic reaction was stopped after 30 min at room temperature with 1 M $H_2SO_4$ solution containing 0.05 M $Na_2SO_3$, and the color development was measured bichromatically in the BioKinetics reader (EL 312; Bio Tek Instruments, Winooski, VT) at 490 nm and at 630 nm (base-line).

## Results

Sequence alignments for selected transmembrane regions of adenosine receptors and other GPCRs are shown in Fig. 1. The residues of the human $A_{2A}$ receptor selected as targets for site-directed mutagenesis are shown in bold; they include hydrophilic residues potentially involved in the binding of the ribose moiety, which was suggested in our previous studies (15, 16) to occur at TM7 (from mutagenesis results) and at TM3 (predicted by molecular modeling). Mutated residues

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TM3
                          8899
                          8901
hA2a     78  LFIACFVLVLTQSSIFSLLAIAIDR  102
rA2a     75  LFFACFVLVLTQSSIFSLLAIAIDR   99
hA2b     79  LFLACFVLVLTQSSIFSLLAVAVDR  103
hA1      81  LMVACPVLILTQSSILALLAIAVDR  105
hA3      84  LFMTCLLLIFTHASIMSLLAIAVDR  108
rA3      86  LFMSCVLLVFTHASIMSLLAIAVDR  110
m3      141  DLWLSLDYVASNASVMNLLVISFDR  165
β2      107  EFWTSIDVLCVTASIETLCVIAVDR  131
NK1     106  KFHNFFPIAAVFASIYSMTAVAFDR  130

TM7
                            2   2
                            7   8
                            7   1
hA2a    266  PLWLMYLAIVLSHTNSVVNPFIYAYRIREFR  296
rA2a    261  PPWLMYLTIILSHSNSVVNPFIYAYRIREFR  291
cA2a    266  PLWLMYLTIVLSHTNSVVNPFIYAYRIREFR  296
gpA2a   263  PPWLMSMTIILSHGNSVVNPLIYAYRIREFR  293
hA2b    268  PKWAMNMAILLSHANSVVNPIVYAYRNRDFR  298
hA1     266  PSILTYIAIFLTHGNSAMNPIVYAFRIQKFR  296
hA3     260  PQLVLYMGILLSHANSMMNPIVYAYKIKKFK  290
rA3     262  PEIAMCLGILLSHANSMMNPIVYACKNKKVQ  292
m3      230  PKTYWNLGYWLCYINSTVNPVCYALCNKTFR  256
β2      304  PKEVYILLNWLGYVNSAFNPLIYCRSPDFRI  334
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**Fig. 1.** Location of mutations carried out in this study, illustrated through an alignment of the TM3 and TM7 of selected receptor subtypes. *Bold*, residues mutated in $A_{2A}$ receptors (present study) and in a chimeric bovine $A_1$/rat $A_3$ construct (18). Accession numbers are hA2a (human) P29274, rA2a (rat) P30543, cA2a (dog) P11617, gpA2a (guinea pig) U04201, hA2b (human) P29275, hA1 (human) P30542, hA3 (human) P33765, rA3 (rat) P28647, m3 (rat) P08483, β2 (hamster) P04274, and NK1 (human) P25103. In TM3, the canine and guinea pig $A_{2A}$ sequences are identical to the rat $A_{2A}$ sequence.

include S91 and S281, which are highly conserved among GPCRs. Another mutated residue, T88, is conserved among all known adenosine receptor sequences. Other mutated residues include those that are conserved between $A_1$ and $A_2$ receptors (Q89, S90) or between $A_2$ and $A_3$ receptors (S277). Each of these amino acid residues was individually replaced with alanine and/or other amino acids (see below). In addition, each mutant contained an epitope-tag sequence included at the amino terminus for immunological detection (see below). The pharmacological properties were compared with those of the wild-type receptor that was similarly modified.

**Ligand binding properties of mutant human $A_{2A}$ adenosine receptors.** Radioligand saturation studies and competition binding experiments using a fixed concentration of either the agonist [$^3$H]CGS 21680 at a concentration of 15 nM (6) or the antagonist [$^3$H]XAC at a concentration of 2 nM (28) were carried out on the wild-type and mutant receptors. The agonists selected for competition (Table 1) included adenosine derivatives modified at the 2 position (CADO), the $N^6$ position (DPMA, *R*-PIA, and ADAC), the 5′ position (NECA), the 5′ and 2 positions (CGS 21680), and the 5′ and $N^6$ positions (IB-MECA). A diverse set of adenosine antagonists (Fig. 2), including XAC, a potent and nonselective nonxanthine (CGS 15943), several recently reported (29, 30) potent and highly selective $A_{2A}$ antagonists (SCH 58261 and ZM 241385), and a novel structure ($BTH_4$) that does not contain any nitrogen atoms (31), were studied in competition for [$^3$H]CGS 21680 binding.

Among TM3 mutations, amino acid substitution of T88 distinguished between agonist and antagonist ligands (Table 1). The specific binding of [$^3$H]CGS 21680 was greatly diminished (i.e., <2% of the specific binding of 15 nM [$^3$H]CGS 21680 observed with the wild-type receptor) in the T88A, T88S, and T88R mutant receptors, whereas binding of [$^3$H]XAC was similar to that of the wild-type receptor. The $K_d$ determined for [$^3$H]XAC at the wild-type receptor (8.83 ± 1.46 nM) was not significantly different from the $K_d$ at the T88 mutant receptors (4.2–10.8 nM). The receptor densities, $B_{max}$, in membranes of COS-7 cells transfected with the T88 mutant receptors (6–7 pmol/mg protein) were approximately half those of the COS-7 cells expressing wild-type receptors (14.9 ± 1.2 pmol/mg of protein). $K_i$ values for competition of binding of [$^3$H]XAC by agonists in T88A and T88R mutant receptors indicated a 60–830-fold lower affinity than in wild-type receptors. Even the T88S mutant receptor, which differs only in the absence of a methyl group, bound agonists with 6.6-fold (for CADO) to 41-fold (for NECA) lower affinity. Affinities of the antagonists XAC and ZM 241385 were un-

TABLE 1

**Binding characteristics of wild-type and mutant human $A_{2A}$-adenosine receptors using the antagonist radioligand [$^3$H]XAC**

Data are presented as mean ± standard deviation of two or three independent experiments, each performed in duplicate. Each sample contained 7–11 μg of membrane protein/tube. Agonist and antagonist binding affinities [$K_i$ values, structures in Fig. 2 and Jacobson *et al.*, (35)] were determined in [$^3$H]XAC (1.0 nM) competition binding studies using membrane homogenates prepared from transiently transfected COS-7 cells, as described in Experimental Procedures. $K_i$ values were calculated from $IC_{50}$ values by using the KaleidaGraph program. All constructs contain an HA-tag sequence at the amino terminus (15).

| | | Construct | | | |
|---|---|---|---|---|---|
| | Compound | Wild-type | T88A | T88S | T88R |
| $B_{max}$ (pmol/mg) | [$^3$H]XAC | 14.9 ± 1.2 | 6.54 ± 1.61[b] | 6.26 ± 0.70[b] | 5.54 ± 0.47[b] |
| $K_d$ (nM) | [$^3$H]XAC | 8.83 ± 1.46 | 10.8 ± 1.8[d] | 5.76 ± 1.82[d] | 4.21 ± 1.17[c] |
| $K_i$ (nM) | Agonists | | | | |
| | CADO | 118 ± 22 | 16,400 ± 3,200[c] | 776 ± 313[c] | 9,140 ± 2,590[c] |
| | DPMA | 65.5 ± 0.3 | 5,710 ± 2,520[c] | 655 ± 134[c] | 4,090 ± 760[c] |
| | NECA | 19.1 ± 1.7 | 1,590 ± 280[c] | 778 ± 224[c] | 2,660 ± 1,390[c] |
| | CGS 21680 | 26.7 ± 6.5 | 22,100 ± 2,900[b] | 422 ± 180[c] | 4,620 ± 620[b] |
| | Antagonists | | | | |
| | CGS 15943 | 1.20 ± 0.30 | 7.98 ± 1.71[c] | 6.28 ± 1.68[c] | 5.00 ± 0.52[b] |
| | ZM 241385 | 0.758 ± 0.135 | 3.47 ± 2.41[d] | 1.16 ± 0.20[d] | 2.00 ± 0.71[d] |

[a] $p$ <0.001.
[b] $p$ <0.01.
[c] $p$ <0.05.
[d] Not significant.

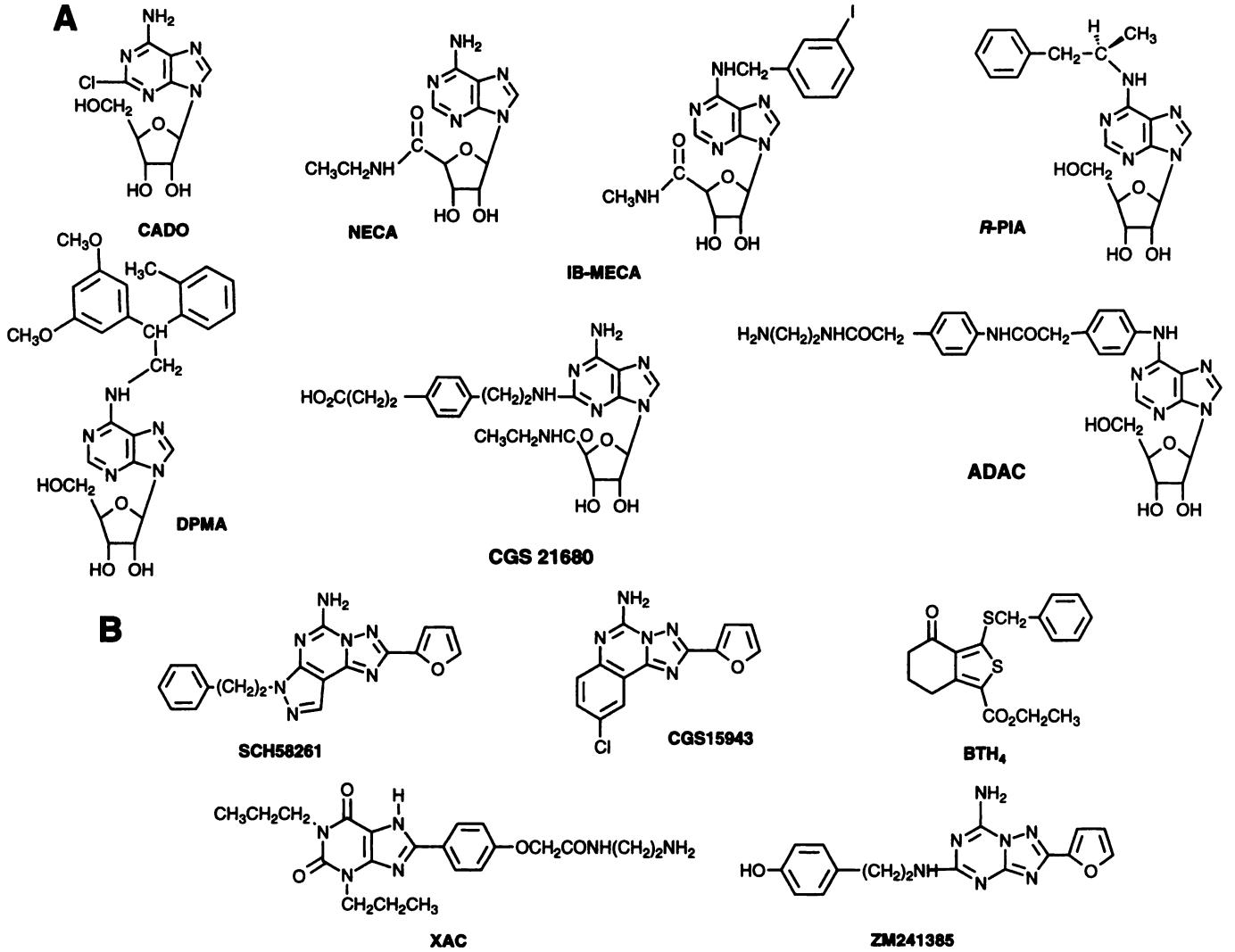


**Fig. 2.** Structures of agonist (A) and antagonist (B) ligands used in this study.

changed in the T88S mutant receptor, whereas CGS 15943 was 5.2-fold weaker than in wild-type receptors. In a comparison of T88S and T88A mutants, the largest contrast in ligand affinity occurred for the agonist CGS 21680. The removal of the serine hydroxyl group of the T88S mutant, already impaired in its ability to bind agonists, diminished by 52-fold the affinity of this adenosine derivative. Thus, the requirements for agonist binding at position 88 are highly specific, whereas the binding of antagonists was less dramatically affected by mutation at this site.

The T88 mutant receptors were found to be properly expressed on the cell surface using an ELISA (15). To estimate approximate levels of receptor protein present in the plasma membrane, a standard curve was constructed from different batches of transfected COS-7 cells expressing different levels of HA-tagged $A_{2A}$ wild-type receptors (see Experimental Procedures and Ref. 15). This ELISA procedure does not interfere with the intactness of the plasma membrane barrier; thus, the assay is specific for receptor molecules having the proper orientation (extracellular amino terminus). Expression levels for the various mutants (HA-tagged wild-type receptor = 100%) determined according to this method were as follows (six experiments): T88A, 77.3 ± 12.5%; T88S, 89.8 ± 43.0%; and T88R, 134 ± 38.3%. The combination of ELISA and radioligand binding results indicates that the T88 residue is important, either directly or indirectly, for the high affinity binding of agonist ligands.

In contrast, replacement of other amino acids, located carboxyl-terminally to T88 in TM3, did not have as detrimental an effect on binding of the agonist radioligand [$^3$H]CGS 21680 (Table 2). The S90A mutant receptor displayed a slight trend toward higher affinity of both agonists and antagonists relative to the wild-type receptor ($K_d$ of [$^3$H]CGS 21680 was 2-fold lower). In competition binding studies at the S91A mutant receptor, only slight changes were noted in $K_i$ values relative to the wild-type receptor. CADO, CGS 21680, and ADAC ($N^6$-substituted) were approximately half as potent in displacing radioligand binding as the wild-type receptor.

The mutation of Q89 gave an unanticipated enhancement of affinity for both agonist and antagonist ligands (Tables 2 and 3). From saturation binding, it was determined that the Q89A mutant receptor had a 4.6-fold greater affinity for [$^3$H]CGS 21680 (6.70 ± 0.86 nM) than the wild-type receptor (31.0 ± 1.0 nM). The affinities of the competing ligands at the Q89A mutant receptor (Table 2) were dramatically higher than in the wild-type receptor. IB-MECA had the greatest

TABLE 2

**Binding characteristics of wild type and mutant human $A_{2A}$-adenosine receptors using the agonist radioligand [$^3$H]CGS 21680**

Data are presented as mean ± standard deviation of two or three independent experiments, each performed in duplicate. Each sample contained 7–11 μg of membrane protein/tube. Agonist and antagonist binding affinities [$K_i$ values, structures in Fig. 2 and Jacobson *et al.* (35)] were determined in [$^3$H]CGS 21680 (15 nM) competition binding studies using membrane homogenates prepared from transiently transfected COS-7 cells, as described in Experimental Procedures. $K_i$ values were calculated from $IC_{50}$ values by using the KaleidaGraph program. All constructs contain an HA-tag sequence at the amino terminus (15).

| | | Construct | | | | | |
|---|---|---|---|---|---|---|---|
| | Compound | Wild-type | Q89A | S90A | S91A | S277C | S281N |
| $B_{max}$ (pmol/mg) | [$^3$H]CGS 21680 | 11.2 ± 0.3 | 13.2 ± 1.5[d] | 12.1 ± 1.2[d] | 14.3 ± 2.1[d] | 1.20 ± 0.01[a] | 6.26 ± 0.86[c] |
| $K_d$ (nM) | [$^3$H]CGS 21680 | 31.0 ± 1.0 | 6.70 ± 0.86[a] | 16.1 ± 3.5[c] | 59.7 ± 7.7[c] | 37.2 ± 1.2[b] | 12.3 ± 1.8[a] |
| $K_i$ (nM) | Agonists | | | | | | |
| | CADO | 100 ± 14 | 19.1 ± 6.2[b] | 19.4 ± 4.0[c] | 224 ± 7[b] | 384 ± 22[a] | 7.43 ± 1.80[b] |
| | DPMA | 77.3 ± 4.8 | 16.6 ± 3.0[a] | 11.1 ± 1.7[b] | 88.4 ± 28.6[d] | 355 ± 113[d] | 5.92 ± 2.00[b] |
| | NECA | 27.6 ± 0.9 | 1.90 ± 0.22[a] | 5.90 ± 0.40[a] | 35.5 ± 1.6[b] | 129 ± 12[b] | 4.50 ± 0.50[a] |
| | *R*-PIA | 299 ± 83 | 28.6 ± 10.0[c] | 76.1 ± 20.0[c] | 314 ± 13[d] | 1590 ± 570[d] | 17.6 ± 5.4[c] |
| | IB-MECA | 435 ± 100 | 3.21 ± 0.07[c] | 78.5 ± 13.6[c] | 614 ± 212[d] | 1755 ± 400[c] | 53.6 ± 15.1[c] |
| | ADAC | 1330 ± 50 | 41.2 ± 2.6[b] | 675 ± 83[c] | 2530 ± 560[d] | 3580 ± 168[b] | 156 ± 88[a] |
| | Antagonists | | | | | | |
| | SCH 58261 | 1.85 ± 0.15 | 0.246 ± 0.084[a] | 1.50 ± 0.50[d] | 2.30 ± 1.12[d] | 4.70 ± 0.50[c] | 7.85 ± 0.55[b] |
| | CGS 15943 | 1.95 ± 0.45 | 0.455 ± 0.165[b] | 0.925 ± 0.475[d] | 1.95 ± 0.56[d] | 1.53 ± 1.1[d] | 5.10 ± 0.90[c] |
| | XAC | 8.71 ± 1.60 | 2.01 ± 0.72[c] | 16.6 ± 1.6[b] | 18.1 ± 2.3[c] | 10.3 ± 4.6[d] | 82.3 ± 2.1[a] |
| | $BTH_4$ | 859 ± 14 | 133 ± 15[a] | 508 ± 101[c] | 1880 ± 30[a] | 279 ± 17[a] | 245 ± 23[a] |
| | $BTH_4$ | 859 ± 14 | 133 ± 15[a] | 508 ± 101[c] | 1880 ± 30[a] | 279 ± 17[a] | 245 ± 23[a] |
| | ZM 241385 | 0.525 ± 0.035 | 0.197 ± 0.041[b] | 0.360 ± 0.080[d] | 1.09 ± 0.11[c] | 1.35 ± 0.35[d] | 1.93 ± 0.23[b] |

[a] $p < 0.001$.
[b] $p < 0.01$.
[c] $p < 0.05$.
[d] Not significant.

TABLE 3

**Radioligand binding characteristics of Q89 mutant human $A_{2A}$-adenosine receptors using an agonist radioligand**

Data are presented as mean ± standard deviation of two or three independent experiments, each performed in duplicate. Each sample contained 7–11 μg of membrane protein/tube. Saturation of binding of [$^3$H]CGS 21680 using membrane homogenates prepared from transiently transfected COS-7 cells, as described in Experimental Procedures. All constructs contain the HA-epitope tag sequence at the amino terminus (15).

| Construct[a] | $K_d$ | $B_{max}$ |
|---|---|---|
| | *nM* | *pmol/mg* |
| Wild-type | 31.0 ± 1.0 | 11.2 ± 0.3 |
| Q89A | 6.70 ± 0.86[b] | 13.2 ± 1.5[e] |
| Q89N | 98.7 ± 23.6[d] | 8.71 ± 0.69[c] |
| Q89S | 93.3 ± 11.5[d] | 14.6 ± 3.4[e] |
| Q89L | 35.6 ± 0.7[c] | 8.68 ± 0.32[c] |
| Q89H | 46.8 ± 0.4[c] | 11.4 ± 0.2[e] |
| Q89R | 50.8 ± 10.8[e] | 4.68 ± 0.00[b] |

[a] Specific binding versus [$^3$H]XAC (5.5 nM) showed levels comparable to HA-tagged wild-type receptors for Q89A, Q89N, Q89S, Q89L, and Q89H mutant receptors. For the Q89R mutant, <8% of the specific binding found for wild-type receptors was observed for Q89R.
*** p <0.001; ** p <0.01; * p <0.05; n.s. not significant.
[b] $p < 0.001$.
[c] $p < 0.01$.
[d] $p < 0.05$.
[e] Not significant.

ratio of affinities (136-fold versus the wild-type receptor; Fig. 3A), whereas the potencies of ADAC (Fig. 3B) and NECA were enhanced 32- and 15-fold, respectively. Other $N^6$-modified analogues, such as *R*-PIA and DPMA, and the C2-modified analogue CADO were 5–12-fold more potent in binding at the Q89A mutant receptor. The antagonists generally displayed a 4–5-fold enhancement of affinity at the Q89A mutant receptor versus the wild-type receptor.

Other amino acids were substituted at position 89 to probe the contribution to ligand recognition of factors such as size, polarity, aromaticity, or charge (Table 3). Substitution of Q89 by residues, charged or uncharged, larger than alanine (Q89S, Q89N, Q89L, Q89H, and Q89R) did not preclude the high affinity binding of [$^3$H]CGS 21680. Histidine was chosen because it occurs at this position in $A_3$ receptors; however, no selective enhancement of the affinity of the $A_3$ receptor-selective agonist IB-MECA was observed. The mutant receptors Q89S and Q89N had a lower affinity (3-fold) for the radioligand than wild-type receptors. The mutant receptor Q89A was unique in having increased affinity for [$^3$H]CGS 21680. Thus, for binding of the agonist radioligand, there existed a great deal of tolerance for substitution at position 89.

Competition binding was studied for three ligands (NECA, IB-MECA, and XAC) at this set of mutant receptors at position 89 (Table 3). The affinities showed an approximate dependence on the size of the amino acid side chain. Solvent-accessible surface, a theoretical value reported for each amino acid (36) that was used as a relative steric indicator for ordering the amino acids (Fig. 4), fell within the range of 224 Å$^2$ for A to 355 Å$^2$ for R, and affinity varied over several orders of magnitude. For each competitor, the $K_i$ value tended to increase as a function of the size of residue 89. The effects on affinity relative to wild-type receptors ranged from a large enhancement (for the two agonists NECA and IB-MECA at alanine, serine, asparagine, and leucine mutants) to substantial reduction (for the antagonist XAC at H and R mutant receptors).

The TM7 mutant receptors S277C and S281N, both small polar substitutions capable of hydrogen bonding, were also constructed (Table 2). In a previous study (15), these serine residues were mutated to alanine and found to be required for ligand recognition (S277 for agonists and S281 for all ligands). Changes in the affinities of agonists at the S277C mutant receptor were relatively modest (i.e., ≤5-fold lower than at the wild-type receptor). Some antagonists (XAC and CGS 15943) were approximately equipotent at S277C mutant receptors and at wild-type receptors. The nonxanthine $A_{2A}$-receptor antagonists SCH 58261 and ZM 241385 were

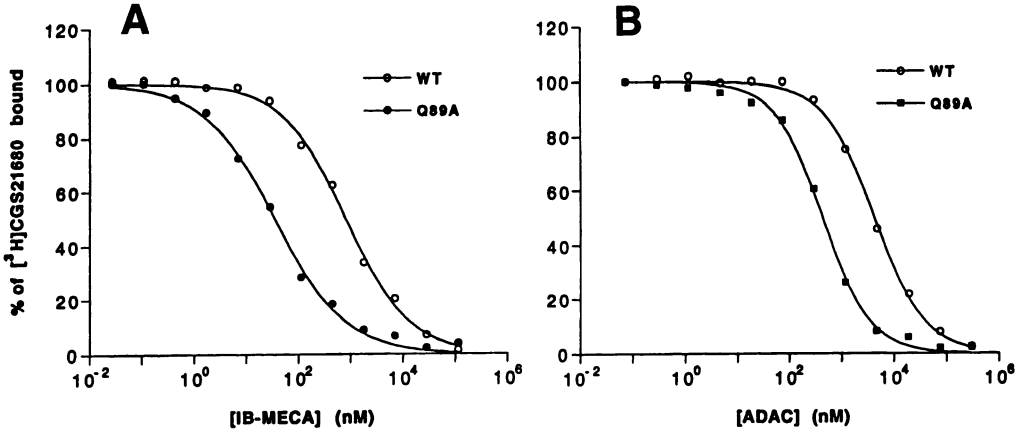


**Fig. 3.** Displacement of binding of the agonist radioligand [$^3$H]CGS 21680 from HA-tagged $A_{2A}$ wild-type (*WT*) and Q89A mutant receptors expressed in COS-7 cells. Competitors used were IB-MECA (A) and ADAC (B). Competition binding studies were carried out using membrane homogenates prepared from transfected COS-7 cells, as described in Experimental Procedures. Data are from a representative experiment performed in duplicate.

moderately diminished in affinity, whereas the novel tetrahydrobenzothiophenone derivative $BTH_4$ displayed 3-fold enhanced affinity at S277C mutant receptors. At S281N mutant receptors, the affinities of agonists, including those substituted at $N^6$, C2, or C5′ positions, were 6–17-fold higher than at wild-type receptors. The agonist order (decreasing) for enhancement of affinity produced by the S281N mutation was *R*-PIA (17.0-fold) > CADO (13.5-fold) = DPMA (13.1-fold) > ADAC (8.5-fold) = IB-MECA (8.1-fold) > NECA (6.1-fold) > CGS 21680 (2.5-fold). Antagonist affinity at the S281N mutant receptor was generally diminished (3–10-fold) compared with wild-type receptors, except for $BTH_4$ (3.5-fold enhancement).

**Functional assay.** To determine whether T88A mutant receptors that lacked high affinity radioligand binding were still functional at high agonist concentrations, their ability to mediate increases in intracellular cAMP levels in transfected COS-7 cells was studied. Rolipram was used as an inhibitor of phosphodiesterases. The T88A and T88S mutant receptors showed a dose-dependent stimulation of cAMP production after treatment with CGS 21680, with $EC_{50}$ values of 14.4 ± 0.423 and 0.323 ± 0.048 μM, respectively. Thus, functional potency of CGS 21680 was far less at the T88A and T88S mutant receptors than at wild-type receptors ($EC_{50}$ = 0.915 ± 0.213 nM) by factors of 15,700 and 353, respectively. A maximal response of these mutant receptors was reached at ~$10^{-4}$ M. The T88R mutant receptor however, showed minimal stimulation of adenylyl cyclase even at 1 mM CGS 21680 (Fig. 5).

The 2- and 5′-disubstituted adenosine agonist CGS 21680 acting at the Q89A mutant receptor elicited a dose-dependent stimulation of cAMP production (Fig. 6, *top*), with an $EC_{50}$ value of 0.223 ± 0.054 nM [i.e., 4-fold more potent ($p$ < 0.05) than at wild-type receptors ($EC_{50}$ = 0.915 ± 0.213 nM)]. A similar gain of potency was observed for the $N^6$-substituted compound DPMA ($EC_{50}$ = 10.8 ± 1.4 nM at the wild-type and 2.73 ± 0.59 nM at the Q89A mutant receptor; a 4-fold increase, $p$ < 0.05; Fig. 6, *bottom*). This effect was even more pronounced for the 5′-substituted compound NECA ($EC_{50}$ = 44.5 ± 6.2 nM at the wild-type and 2.91 ± 0.54 nM at the Q89A mutant receptor), whereas the Q89A mutation induced a 15.3-fold increase in potency ($p$ < 0.01, Fig. 6, *middle*). Thus, there was a parallel between the enhanced binding affinities of agonists at the Q89A mutant receptor (Table 2) and their functional potencies. No increase in basal adenylyl cyclase activity was observed for this mutant receptor.

## Discussion

**Ligand binding and stimulation of adenylyl cyclase in TM3 mutant human $A_{2A}$ adenosine receptors.** The current study demonstrates clearly that hydrophilic residues of TM3 of the human $A_{2A}$ receptor are involved in ligand binding. Several TM3 mutants prepared in this study are highly unnatural in their ligand binding properties, having either enhanced (Q89A) or greatly diminished (T88A and T88R) affinity for various ligands. Alanine scanning mutagenesis showed that T88 is essential for high affinity agonist binding, whereas only moderate changes in ligand binding affinity occur on replacement of S90 and S91. T88A and T88R mutations had similar detrimental effects on agonist binding, whereas high affinity antagonist binding was still observed in these mutant receptors. Affinity in the T88S receptor mutant was decreased as well but to a lesser extent. Thus, a section of TM3 seems to be involved in ligand (especially agonist) recognition. This is consistent with a molecular model based on a rhodopsin template (15), which predicted that T88 and Q89 are in proximity to the ribose moiety of adenosine.

At high agonist concentrations, the T88A and T88S mutant receptors were active functionally in the stimulation of adenylyl cyclase (Fig. 5), with the dose-response curves right-shifted by 15,700- and 350-fold, respectively ($p$ < 0.01). This change in potency reflects the relative agonist affinity shifts in the binding assays. The T88R mutant receptor seemed to be functionally impaired (Fig. 5), even at agonist concentrations that clearly displaced bound [$^3$H]XAC (Table 1).

For single amino acid replacements of Q89, the ligand affinity varied from dramatic increases (e.g., IB-MECA at Q89A) to decreases for the larger amino acid substitutions. The trend of inverse dependence of ligand affinity on steric bulk of the side chain at position 89 (Fig. 4) was shown for a number of structurally divergent amino acids substituted at this position through mutagenesis. Because electronic factors of the amino acids seemed to be not as important as steric factors and because three selected competing ligands were similarly affected, it is hypothesized that residue Q89 affects the size of the crevice that constitutes the binding site. Diminishment of the size of a sterically limiting side chain (i.e., in the Q89A mutant receptor) would increase the accessibility of the binding site, thus lowering the $K_i$ values. This mechanism either might involve the side chain acting as an energy barrier to a ligand occupying the binding site or may

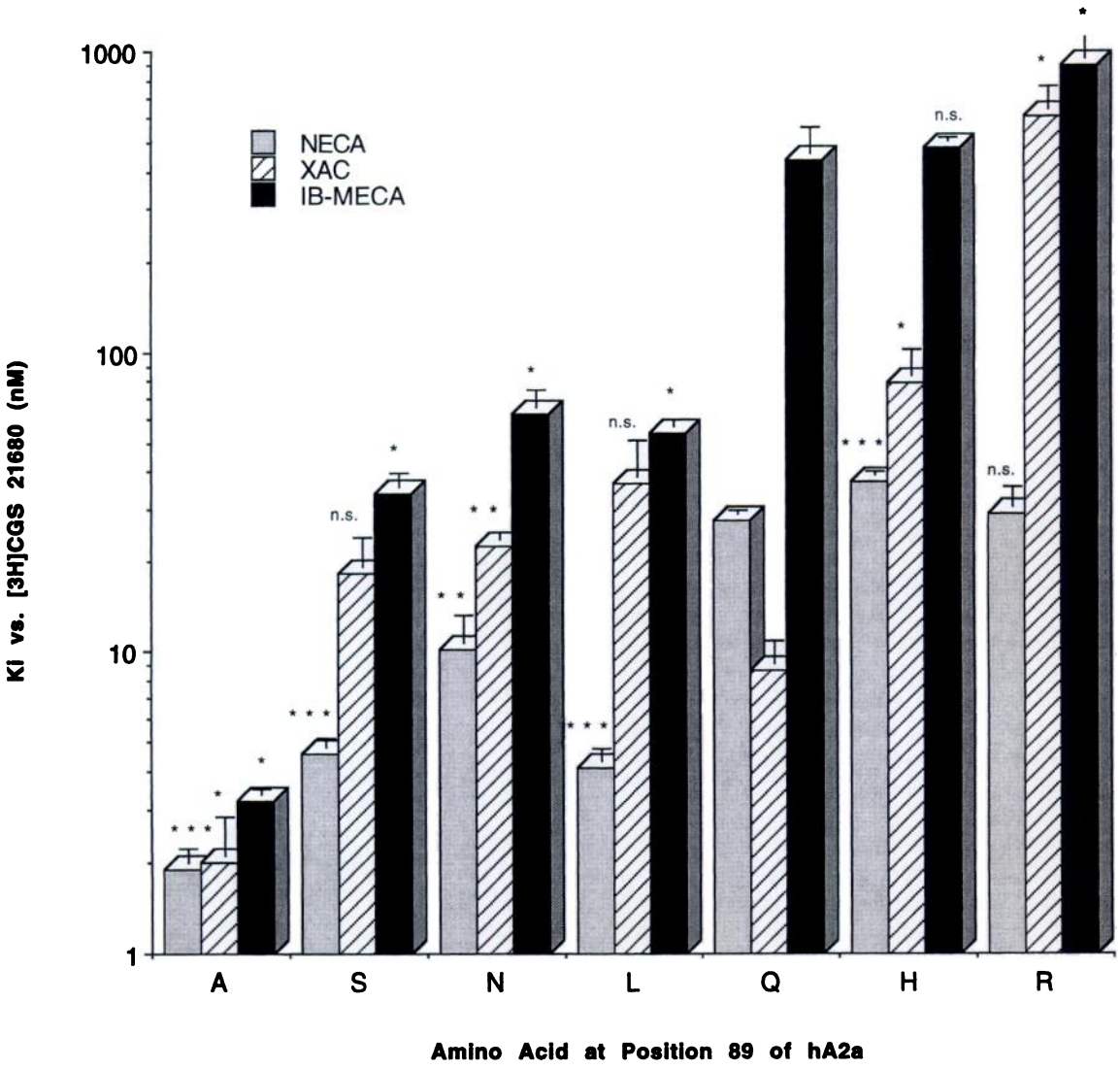


**Fig. 4.** Plot of affinity ($K_i$ in nM) in competition binding experiments of three adenosine receptor ligands at Q89 mutant receptors as a function of the amino acid residue. The amino acids were arranged in order of increasing solvent-accessible surface area, using theoretical values for each isolated amino acid as calculated by Hubbard *et al.* (36). $K_i$ values for the agonist (NECA and IB-MECA) and antagonist (XAC) ligands (structures in Fig. 2) were determined in [$^3$H]CGS 21680 (15 nM) competition binding studies using membrane homogenates prepared from transiently transfected COS-7 cells, as described in Experimental Procedures. $K_i$ values were calculated from $IC_{50}$ values by using the KaleidaGraph program. All constructs contain the HA epitope tag sequence at the amino terminus (15). ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$; *n.s.*, not significant.

be more indirect (e.g., by changing interhelical distances). Several other residues of the human $A_{2A}$ receptor (i.e., Y271) have been postulated to affect ligand binding through interhelical contacts (15), although alanine substitution decreased agonist affinity in that case.

The finding of enhanced affinity for all ligands, both agonists and antagonists, on single amino acid replacement, as in the Q89A mutant receptor, is a rather rare finding. An increase in agonist affinity, but not antagonist affinity, is often observed in constitutively active mutants (32, 33). The Q89A mutation, however, did not affect basal adenylyl cyclase levels. Even though affinity and potency of agonists were both increased in the Q89A mutant receptor versus the wild-type receptor, this mutant receptor was not constitutively active.

In the S90A mutant $A_{2A}$ receptor, both agonist and antagonist affinities were only slightly affected. Mutation of S91 to alanine had virtually no effect on ligand affinity.

**Structurally related differences in ligand binding in TM7 mutant human $A_{2A}$ adenosine receptors.** In our previous study (15), we reported that residue S277 is important for agonist recognition. We now present data regarding a new mutant, S277C, in which ligand binding affinities are only slightly affected (agonist affinity was unaffected for CGS 21680 and decreased by 3–5-fold for other agonists). This mutant to a certain extent rescues ligand binding compared with the earlier S277A mutant, in which agonist affinity decreased 43–1070-fold relative to the wild-type receptor.

This study has revealed differences in affinity shifts between agonists and antagonists [e.g., S281N (agonists become more potent and antagonists less potent) and mutations of T88 (agonists selectively become much less potent)]. Therefore, a partially different set of amino acid residues in the receptor is involved in agonist versus antagonist binding. The results of chemical modification and conformational analysis (34, 35) as well as mutagenesis studies of adenosine

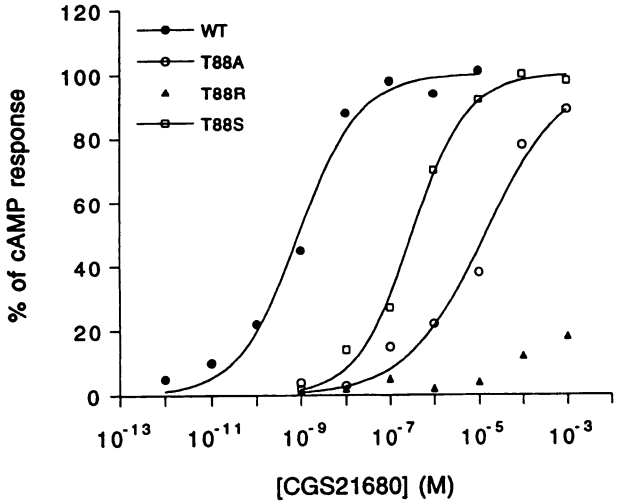


**Fig. 5.** Stimulation of adenylyl cyclase in COS-7 cells transiently expressing HA-tagged $A_{2A}$ wild-type (*WT*) or mutant $A_{2A}$-adenosine receptors in the presence of 2 units/ml adenosine deaminase and 0.1 mM rolipram. The following receptors were studied: wild-type, T88A, T88R, and T88S mutant receptors. Transfected COS-7 cells were incubated for 30 min at 37° (for details, see Experimental Procedures) with increasing concentrations of CGS 21680. Data are presented as percentage of maximal increase in cAMP above basal levels in the absence of CGS 21680 for a representative experiment. For each curve, the maximal stimulation represents a 4–5-fold stimulation over basal levels. At agonist concentrations of ≥100 μM, the stimulation observed in nontransfected COS-7 cells (15) was subtracted. $EC_{50}$ values (average of three independent experiments, each carried out in duplicate) were wild-type receptor, 0.915 ± 0.213 nM; T88A, 14.4 ± 0.423 μM; and T88S, 0.323 ± 0.048 μM.

receptors (15, 17) have suggested that the ribose moiety is coordinated to the histidine residue of TM7, common to all adenosine receptors. Furthermore, changes in affinity of the ribose-modified agonist NECA at $A_1$ receptors are associated with mutation of an adjacent threonine residue (19, 20). Mutations that are specific for diminishing the affinity of ribose-containing ligands (i.e., adenosine agonists) have been identified in both TM3 and TM7, consistent with the previously reported molecular models of human $A_{2A}$ receptors that predicted that the ribose moiety of adenosine may bridge these domains (15, 16).

It was proposed (15) that S281 probably is not directly involved in ligand binding, yet in the current study the S281N mutant receptor distinguishes between agonists and antagonists. All agonists examined displayed an increased affinity (2.5-fold for CGS 21680 to 17-fold for *R*-PIA) toward the S281N mutant receptor, whereas affinity for most antagonists was moderately decreased (3–9-fold). The $A_1$-selective antagonist XAC showed the largest decrease in affinity. The $A_2$-selective antagonists SCH 58261 and ZM 241385 exhibited an intermediate decrease, and the nonselective antagonist CGS 15943 had the smallest decrease. Interestingly, the novel tetrahydrobenzothiophenone derivative $BTH_4$ showed a moderate increase in affinity for the S281N mutant receptor. Unlike other antagonists, $BTH_4$ displayed enhanced affinity at both TM7 mutant receptors examined in this study (Table 2). Thus, the binding mode of this antagonist seems to be unique, as is its chemical structure. It is among the least purine-like of known adenosine antagonists, which are almost exclusively nitrogen-containing heterocycles.

Although there are several models for mapping the xan-

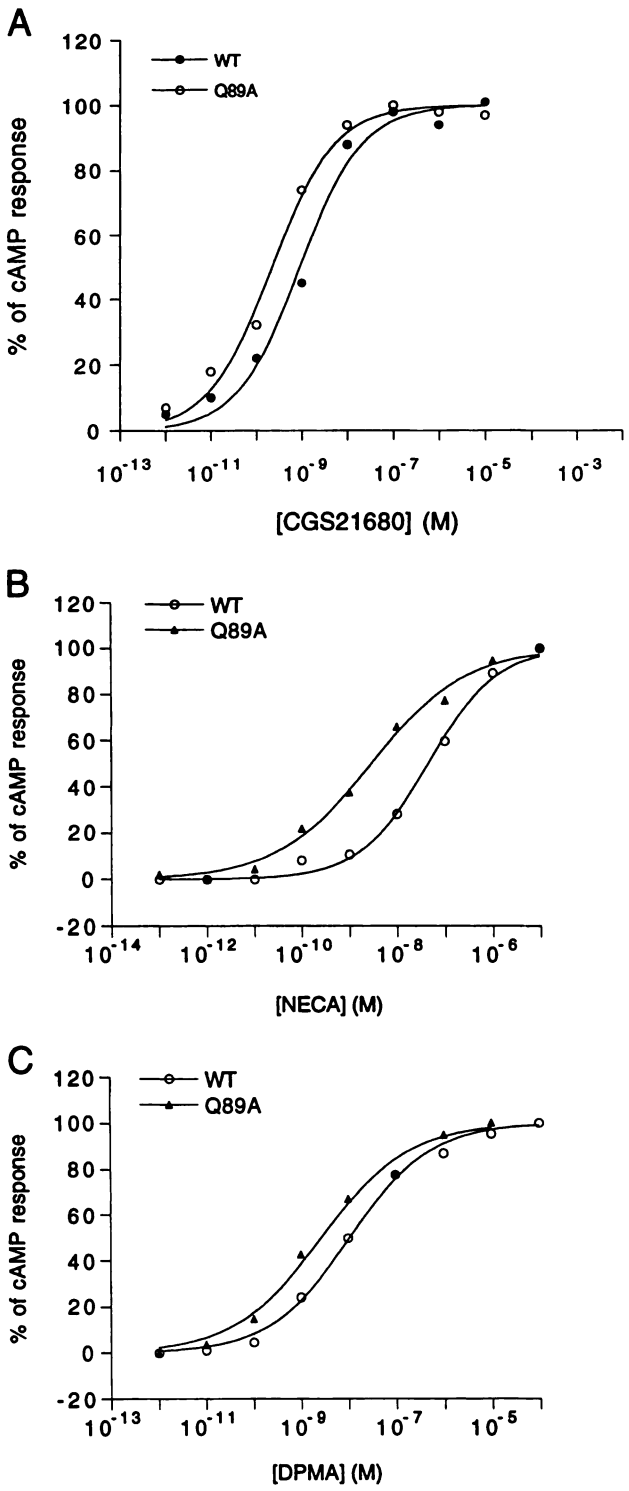


**Fig. 6.** Stimulation of adenylyl cyclase in COS-7 cells transiently expressing HA-tagged $A_{2A}$ wild-type (*WT*) or mutant $A_{2A}$-adenosine receptors in the presence of 2 units/ml adenosine deaminase and 0.1 mM rolipram. The following receptors were studied: wild-type and Q89A mutant receptors with CGS 21680 (*top*), NECA (*middle*), or DPMA (*bottom*). Transfected COS-7 cells were incubated for 30 min at 37° (for details, see Experimental Procedures) with increasing concentrations of agonist. Data are presented as percentage of maximal increase in cAMP above basal levels in the absence of agonist for a representative experiment. For each curve, the maximal stimulation represents a 4–5-fold stimulation over basal levels. $EC_{50}$ values were calculated averaged over three independent experiments, each carried out in duplicate.

thine binding site onto the partially overlapping adenosine binding site (34, 37), no such exercises have been performed for the nonxanthine antagonists SCH 58261, ZM 241385, and CGS 15943. The current data suggest that the mode of nonxanthine antagonist binding to the receptor may differ significantly from the modes proposed for xanthine-based antagonists.

**Structural homology of human $A_{2A}$ adenosine receptors to other GPCRs.** In the biogenic amine GPCRs, there is an essential, conserved aspartate residue in TM3 that coordinates the positively charged secondary nitrogen group of the endogenous ligand. This has been demonstrated for adrenoceptors (38, 39) and receptors for dopamine (40, 41), histamine (42), serotonin (43, 44), and acetylcholine (45, 46). Adenosine, being uncharged at physiological pH, would not benefit from a strong electrostatic interaction with the receptor at this position. In all adenosine receptors, the residue homologous to the above-mentioned aspartate corresponds to a valine (V84 in the human $A_{2A}$ adenosine receptor). The current study has identified important residues for ligand recognition at positions in the human $A_{2A}$ adenosine receptor deeper in the membrane than V84.

The essential T88 of $A_{2A}$ receptors would be expected to be facing approximately the same direction as that of the essential aspartate residue involved in recognition of the secondary nitrogen of biogenic amines at their receptors, being four residues (i.e., one helical turn) closer to the cytoplasmic side. Residue C118 of the human $D_2$ receptor, equivalent to T88, reacts with a thiol reagent, indicating that this residue is solvent exposed and thus faces the central binding cavity (41).

The S505R mutant of the human thyrotropin receptor (position equivalent to T88) was constitutively active (47), whereas none of the T88 mutants in the current study were constitutively active. Residue T88 of the $A_{2A}$ receptor aligns with V116 of the NK1 receptor (Fig. 1). Mutation of this residue to leucine reversed the selectivity of antagonists (48). There is no direct evidence that Q89 is pointing directly into the binding cavity. By analogy with rhodopsin, residue E122, the equivalent to Q89, is facing the binding cavity. This residue was found to coordinate and neutralize the Schiff base of retinal (formed with K296), based on a blue shift in the absorption spectrum of the E122Q mutant (49). Residue A120 of the human $D_2$ receptor (41), the position equivalent to S90, was found not to be exposed to the ligand binding cavity because the A120C mutant receptor did not react with thiol reagents.

Almost one full helical turn down from T88 is S91. This residue is conserved as a serine in 98 of 156 GPCR sequences probed (e.g., m3 muscarinic receptor, hamster $\beta_2$-adrenoceptor: Fig. 1) and therefore is not likely to confer ligand binding specificity. Mutations at this site in the hamster $\beta_2$-adrenoceptor led to decreased receptor expression and improper post-transcriptional processing (S120A; Ref. 50), although the S91A mutant $A_{2A}$ adenosine receptor had a $B_{max}$ value for [$^3$H]CGS 21680 binding similar to wild-type receptors. This site was shown to be facing the binding cavity in the human $D_2$ receptor (S121C; Ref. 41).

Also in TM7, homology to amino acids known to be involved in ligand binding was found. In the rat m1 muscarinic receptor, the mutant receptor C407S, corresponding to residue S277 of the $A_{2A}$ receptor, had decreased agonist affinity (51). Residue S281 corresponds to the essential S319 in the hamster $\beta_2$-adrenergic receptor (50). In the human luteinizing hormone receptor, the naturally occurring mutation at the same site, S616Y, results in hypogonadism and decreased agonist affinity (52).

**Conclusions.** Results of the current study strongly imply that residues of TM3 are involved in ligand recognition and extend the findings of Kim *et al.* (15) concerning TM7. Thus, it seems that hydrophilic residues in both TM3 and TM7 are important for recognition of the ribose moiety. The binding affinity and functional activity of agonists at T88 mutant receptors were greatly diminished. A Q89A mutant gained affinity for all agonist and antagonist ligands examined. Q89 likely plays an indirect role in ligand binding. Furthermore, the fact that all of the residues examined in this study are conserved among most adenosine receptors (species and subtypes) suggests that the proposed mode of binding of agonists is common or very similar in these receptors. Divergent effects of mutagenesis within TM3 and TM7 on the binding of adenosine derivatives, xanthines, and nonxanthine antagonists suggest nonidentical mechanisms of binding.

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**Send reprint requests to:** Dr. K. A. Jacobson, Chief, Molecular Recognition Section, Building 8A, Room B1A-17, NIDDK, NIH, Bethesda, MD 20892. E-mail: kajacobs@helix.nih.gov