Biochemical Pharmacology, Vol. 60, pp. 661–668, 2000.


ISSN 0006-2952/00/$–see front matter
PII S0006-2952(00)00357-9

# Site-Directed Mutagenesis Studies of Human $A_{2A}$ Adenosine Receptors

## INVOLVEMENT OF GLU[13] AND HIS[278] IN LIGAND BINDING AND SODIUM MODULATION

*Zhan-Guo Gao,* Qiaoling Jiang,† Kenneth A. Jacobson† and Adriaan P. IJzerman*‡*

*Division of Medicinal Chemistry, Leiden/Amsterdam Center for Drug Research, Leiden University, P.O. Box 9502, 2300 RA Leiden, The Netherlands; and †Molecular Recognition Section, Laboratory of Bioorganic Chemistry, NIDDK, NIH, Bethesda, MD 20982 U.S.A.

**ABSTRACT.** To provide insights into interactions between ligands and $A_{2A}$ adenosine receptors, site-directed mutagenesis was used to test the roles of a glutamic acid residue in the first transmembrane domain (Glu13) and a histidine residue in the seventh transmembrane domain (His278). The two residues, which have been suggested to be closely linked in molecular modeling studies, were mutated to glutamine (E13Q) and tyrosine (H278Y), respectively. Saturation experiments revealed that [$^3$H]ZM241385 (4-{2-[7-amino-2-(2-furyl)-1,2,4-triazolo[1,5-*a*][1,3,5]triazin-5-yl-amino]ethyl}phenol) bound wild-type and mutant receptors in membranes from COS-7 cells expressing human $A_{2A}$ adenosine receptors with high affinity and low non-specific binding. It was found from the competition experiments that the affinity of the $A_{2A}$ adenosine receptor agonists for the mutant receptors was 3- to 200-fold lower than for the wild-type receptor. Among antagonist competitors of binding at E13Q and H278Y mutant receptors, there was variation in the affinity depending on their different structures, although changes were relatively minor (<3-fold) except in the case of theophylline, whose affinity was decreased approximately 20 times on the H278Y mutant. The possible involvement of the two residues in sodium ion regulation was also tested. The agonist competition curves for [$^3$H]ZM241385 were shifted to the right in both wild-type and mutant receptors in the presence of 1 M sodium ions, but the extent of shift (2- to 27-fold) in wild-type receptor was generally larger than for the mutant receptors. Sodium ions also decreased [$^3$H]ZM241385 dissociation from both wild-type and mutant receptors, being more influential on the former than the latter. The results suggest that the two closely linked residues Glu13 and His278 in $A_{2A}$ adenosine receptor are most important for agonist recognition and are partly responsible for the allosteric regulation by sodium ions. BIOCHEM PHARMACOL **60**;5:661–668, 2000. © 2000 Elsevier Science Inc.

**KEY WORDS.** $A_{2A}$ adenosine receptor; mutation; glutamic acid; histidine; sodium ions

The $A_{2A}$ adenosine receptor is a member of the superfamily of G protein-coupled receptors that is involved in many important physiological processes [1–3]. Activation of $A_{2A}$ adenosine receptors results in vasodilation, and this effect has been examined as the end point for potential antihypertensive therapy using selective $A_{2A}$ agonists such as CGS21680§ [4]. In the brain, $A_{2A}$ adenosine receptors occur primarily in the striatum, where they are co-localized with $D_2$ dopamine receptors [5]. Adenosine acts in a manner opposite to that of dopamine and thus elicits locomotor depression [6]. Diseases in which the dopaminergic system is hyperactive such as schizophrenia [7] and Huntington's disease [6] may be improved by $A_{2A}$ adenosine receptor agonists. Parkinson's disease, in which the dopaminergic system is hyporesponsive, may be treated with $A_{2A}$ adenosine receptor antagonists [8, 9].

The pharmacological characterization of the receptor has been hampered because of the lack of a commercially available, selective high-affinity $A_{2A}$ adenosine receptor antagonist [10], particularly in a radiolabeled form. For instance, site-directed mutagenesis studies to probe the ligand binding site on the receptor have suffered from the limited usefulness of radiolabeled agonists such as [$^3$H]CGS21680 and antagonist [$^3$H]XAC, the latter having low affinity and high non-specific binding [11–15]. The antagonist radioligand [$^3$H]SCH58261 has recently been used to characterize the $A_{2A}$ adenosine receptor [16], but it has not been made available. Only very recently, the potent

‡ Corresponding author: Dr. Adriaan P. IJzerman, Division of Medicinal Chemistry, Leiden University, Leiden/Amsterdam Center for Drug Research, P.O. Box 9502, 2300 RA Leiden, The Netherlands. Tel. +31-71-527.46.51; FAX +31-71-527.45.65; E-mail: ijzerman@lacdr.leidenuniv.nl

§ *Abbreviations:* CGS15943, 5-amino-9-chloro-2-(2-furyl)-1,2,4-triazolo[1,5-*c*]quinazoline; CGS21680, 2-[4-[(2-carboxyethyl)phenyl]ethylamino]-5′-*N*-ethylcarboxamidoadenosine; CPA, $N^6$-cyclopentyladenosine; DPMA, $N^6$-[2-(3,5-dimethoxyphenyl)-2-(2-methylphenyl)ethyl]adenosine; NECA, 5′-*N*-ethylcarboxamidoadenosine; SCH58261, 5-amino-2-(furyl)-7(2-phenylethyl)pyrazolo[4,3-*e*]-1,2,4-triazolo[1,5-*c*]pyrimidine; XAC, 8-{4-[2-[2-amino ethyl(aminocarbonyl)]methoxy]-1,3-dipropyl-7-methylxanthine; and ZM241385, 4-{2-[7-amino-2-(2-furyl)-1,2,4-triazolo[1,5-*a*][1,3,5] triazin-5-yl-amino]ethyl}phenol.

Received 28 October 1999; accepted 22 February 2000.

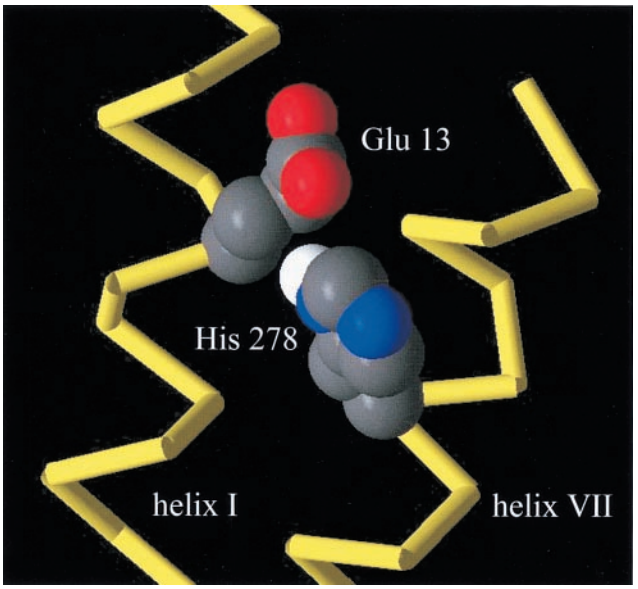


**FIG. 1. Molecular model for the human $A_{2A}$ adenosine receptor, showing helices I and VII with Glu13 and His278, respectively (see Materials and Methods). Atom colours: *gray*: carbon; *white*: hydrogen; *red*: oxygen; *blue*: nitrogen.**

and selective radiolabeled antagonist [$^3$H]ZM241385 has been generally introduced. Hence, we decided to use this label to further investigate the ligand binding site on the $A_{2A}$ adenosine receptor.

In a previous modeling study [17], it had been suggested that Glu13 in the first transmembrane domain and His278 in the seventh transmembrane domain of the $A_{2A}$ adenosine receptor are involved in agonist binding. In another study on human $A_{2A}$ adenosine receptor [13], it was proposed that there may be interactions between Glu13, His278, and the ribose moiety of agonist molecules in the activation of $A_{2A}$ adenosine receptors. A proton transfer over the ribose group could be a communication mechanism between Glu13 and His278. Recently, a molecular model for the $\alpha$-carbon positions in the seven transmembrane helices in the rhodopsin family of G protein-coupled receptors has been presented [18]. Overlaying the amino acid sequence of the human $A_{2A}$ adenosine receptor with this three-dimensional architecture suggests that Glu13 and His278 in the $A_{2A}$ adenosine receptor may even be closely linked (Fig. 1).

Chemical modification studies of $A_{2A}$ adenosine receptors indicated that histidine residues appear to be involved in ligand binding [19]. In bovine $A_1$ adenosine receptors, it has been demonstrated that His278 in the seventh transmembrane domain is important for both agonist and antagonist binding [20]. Site-directed mutagenesis studies of $A_{2A}$ adenosine receptors also suggest that His278 is important for ligand binding [11].

In this study, we characterized the binding of agonists and antagonists of different structures (Fig. 2) to wild-type and two mutant receptors, E13Q and H278Y, by using [$^3$H]ZM241385. Since Glu13 may be involved in the regulation of $A_{2A}$ adenosine receptors by sodium ions [13], the possible involvement of the two residues in the regulation of $A_{2A}$ adenosine receptors by sodium ions was also investigated.

## MATERIALS AND METHODS

### *Materials*

Human $A_{2A}$ adenosine receptor cDNA (expression vector pSVL-$A_{2A}$) was kindly provided by Dr. Marlene Jacobson (Merck Research Labs). Taq polymerase for the polymerase chain reaction (PCR) was purchased from Perkin Elmer. All enzymes used in this study were obtained from New England Biolabs. Oligonucleotides used were synthesized by Bioserve Biotechnologies. [$^3$H]ZM241385 (17 Ci/mmol) was from Tocris Cookson Ltd. GTP was purchased from Aldrich and BSA from Sigma. Adenosine deaminase was obtained from Boehringer Mannheim. CGS15943 and CGS21680 were gifts from Ciba-Geigy. CPA, NECA, DPMA, theophylline, and XAC were obtained from RBI. SCH58261 was a gift from Schering-Plough. All other compounds were obtained from standard commercial sources and were of analytical grade.

### *Plasmid Construction, Site-Directed Mutagenesis, and Transient Expression of Wild-Type and Mutant Receptors in COS-7 Cells*

This protocol has been described previously [11, 13].

### *Membrane Preparation*

Cells were scraped into ice-cold lysis Tris–HCl buffer (pH 7.4 at 20°, 50 mM). Harvested cells were homogenized using a Polytron homogenizer and then spun at 27,000 *g* for 15 min. The pellet (plasma membranes) was resuspended in the same buffer, incubated with 2 IU/mL of adenosine deaminase at 37° for 30 min. The membrane preparation was kept at −80° until use.

### *Radioligand Binding Assay*

For saturation experiments, membranes (6–12 μg of protein) were incubated with increasing concentrations (0.25–8.0 nM) of [$^3$H]ZM241385 in duplicate in a final volume of 0.4 mL of Tris–HCl buffer (50 mM, pH 7.4 at 20°), at 25° for 120 min. Non-specific binding was defined as that retained on the filter and membranes in the presence of 1 μM CGS15943. Where appropriate, NaCl (1 M) or GTP (1 mM) was added to analyze their effects on the binding process. Binding reactions were terminated by filtration through Whatman GF/B glass fiber filters under reduced pressure using an MT-24 cell harvester (Brandell). Filters were washed three times with ice-cold buffer and placed in scintillation vials with 5 mL scintillation fluid, and bound radioactivity was determined by using a liquid scintillation counter.

For competition experiments, membranes (6–12 μg of

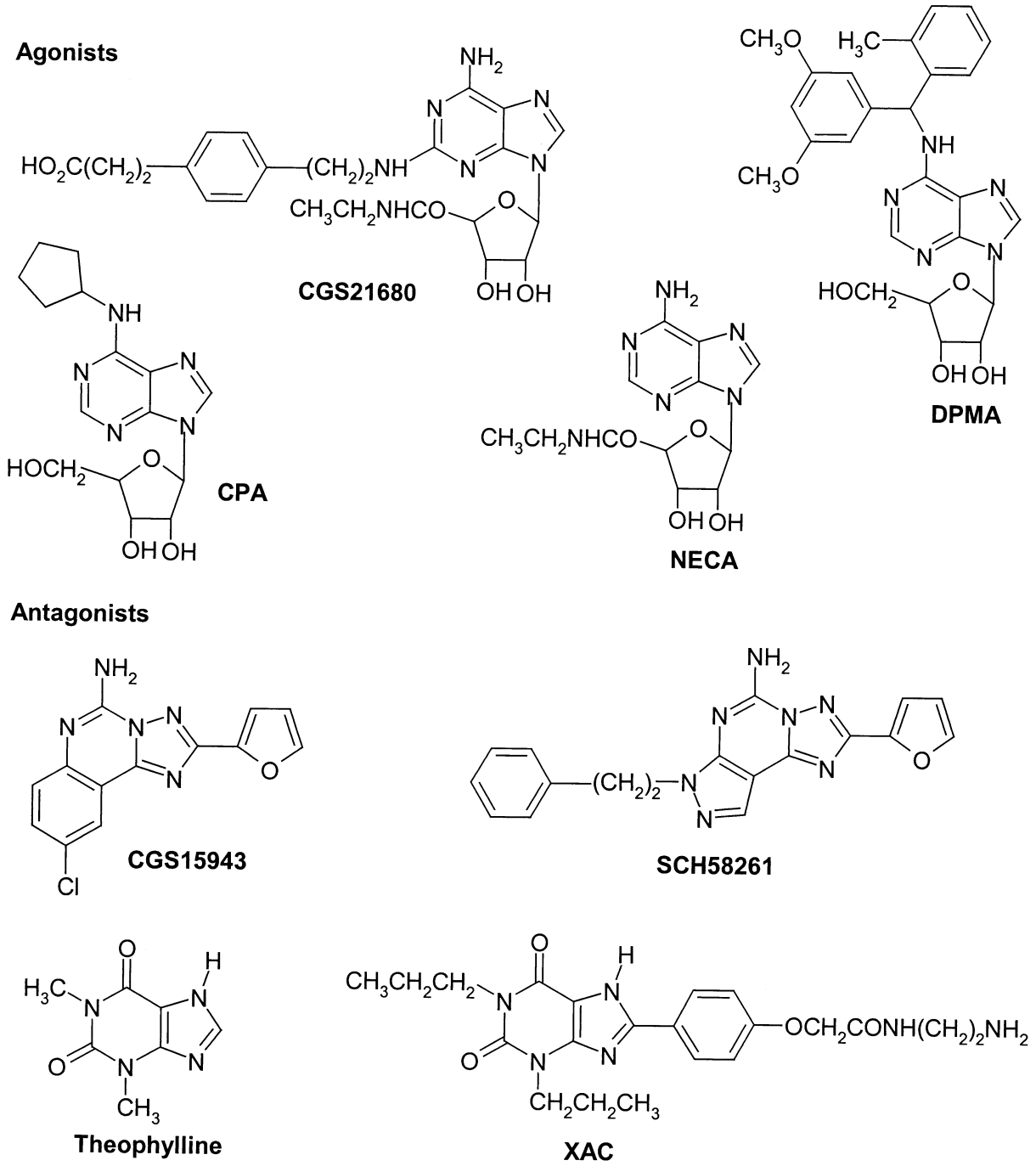


FIG. 2. Chemical structures of ligands tested in this study.

protein) were incubated with 2.0 nM [$^3$H]ZM241385 in duplicate, together with increasing concentrations of the competing compounds, in a final volume of 0.4 mL Tris–HCl buffer at 25° for 120 min.

In kinetic studies, a single concentration of [$^3$H]ZM241385 (2.0 nM) was used. The time–course of association of [$^3$H]ZM241385 was measured by adding the radioligand to the membrane fraction at 25° in a shaking water bath. At varying times, the reaction was stopped and the samples were filtered as described above. For dissociation kinetics, the membranes and radioligand were incubated with other assay constituents for 2 hr at 25°. The dissociation phase was initiated at 2 hr by the addition of 1 μM CGS15943, in the presence or absence of 1 M NaCl or 1 mM GTP. Duplicate samples were filtered at various times. Non-specific binding was determined in parallel samples with the addition of 1 μM CGS15943 before the initiation of dissociation.

### *Molecular Modeling*

The α-carbon template for human $A_{2A}$ adenosine receptor was retrieved from the G protein-coupled receptor database

**TABLE 1. $K_d$ values (nM) of [$^3$H]ZM241385 binding to wild-type and mutant human $A_{2A}$ adenosine receptors in the presence and absence of sodium ions and GTP**

| | WT | E13Q | H278Y |
|---|---|---|---|
| Control | 1.8 ± 0.20 | 3.0 ± 0.34 | 4.3 ± 0.49 |
| +NaCl | 0.95 ± 0.08* | 1.9 ± 0.37* | 2.7 ± 1.2* |
| +GTP | 1.8 ± 0.23 | 2.8 ± 0.24 | 3.7 ± 0.79 |

Membranes (6–12 μg of protein) were incubated with increasing concentrations (0.5–8.0 nM) of radioligand, in a final volume of 0.4 mL of Tris–HCl buffer, at 25° for 120 min. Non-specific binding was defined as that retained on the filter and membranes in the presence of 1 μM CGS15943. The data are expressed as means ± SE from three independent experiments performed in duplicate. Binding parameters were obtained from non-linear regression analysis of the saturation curve using a one-site binding isotherm.

*$P < 0.05$ vs control.

on the WWW (*http://www.gpcr.org/7tm/*). It was graphically displayed on a Silicon Graphics 4000XZ workstation using the molecular modeling software Biograf 3.2 (Molecular Simulations Inc.).

### *Data Analysis*

Binding parameters were estimated by GraphPad Prism software (GraphPad). $IC_{50}$ values obtained from competition curves were converted to $K_i$ values by using the Cheng–Prusoff equation [21]. Data were expressed as means ± SE for the number of experiments indicated.

## RESULTS

### *Saturation Binding of [$^3$H]ZM241385 to Human Wild-Type and Mutant $A_{2A}$ Adenosine Receptors and the Effects of Sodium Ions and GTP*

Saturation experiments were carried out with both wild-type and mutant receptors using [$^3$H]ZM241385 as a radioligand. The experiments revealed that [$^3$H]ZM241385 bound to a single class of wild-type receptors ($K_d$ = 1.8 ± 0.20 nM; $B_{max}$ = 7617 ± 858 fmol/mg protein) displaying low non-specific binding (<10%). The $K_d$ values for [$^3$H]ZM241385 binding at the E13Q and H278Y mutant receptors were 2–3 times higher than for the wild-type receptor. NaCl (1 M) produced a <2-fold decrease in $K_d$ values in both the wild-type and mutant receptors. By comparison, GTP did not influence the $K_d$ value. The binding parameters are summarized in Table 1.

### *Kinetics of [$^3$H]ZM241385 Binding to Wild-Type and Mutant $A_{2A}$ Adenosine Receptors*

Binding of [$^3$H]ZM241385 to the wild-type receptor was rapid, with half-maximal binding occurring at 8 min and reaching equilibrium within 30 min. The data were fit to a single exponential model with an association rate constant of 0.15 ± 0.08 $nM^{-1}$ $min^{-1}$. The time–course for association of [$^3$H]ZM241385 to E13Q and H278Y mutant receptors was even more rapid than that for the wild-type receptor. Sodium ions increased the association rates of [$^3$H]ZM241385 binding to wild-type receptor, but the association to E13Q and H278Y mutant receptors was not significantly influenced. The values of the association rates in the presence and absence of sodium ions are summarized in Table 2.

The dissociation rate for [$^3$H]ZM241385 binding was determined by addition of an excess amount of CGS15943 (1 μM) after equilibrium was reached. The reversal of binding was complete after 10 to 20 min. Calculation of the $K_d$ from the kinetic experiments ($k_{-1}/k_1$) (Table 2) gave an apparent $K_d$ value for [$^3$H]ZM241385 of 2.0 ± 0.7 nM, which is in good agreement with the equilibrium $K_d$ value (1.8 ± 0.20 nM). $Na^+$ decreased the [$^3$H]ZM241385 dissociation rate from both wild-type and mutant receptors, but to a different extent, being more influential on the wild-type than on the two mutant receptors. The rates of [$^3$H]ZM241385 dissociation from the wild-type and the E13Q and H278Y mutant receptors were decreased 2.6, 1.5, and 1.4 times, respectively. GTP did not affect the dissociation rate of [$^3$H]ZM241385 significantly (data not

**TABLE 2. [$^3$H]ZM241385 association and dissociation rates in the presence and absence of sodium ions**

| | $k_1$ ($nM^{-1}$ $min^{-1}$) | | | $k_{-1}$ ($min^{-1}$) | | |
|---|---|---|---|---|---|---|
| | WT | E13Q | H278Y | WT | E13Q | H278Y |
| Control | 0.15 ± 0.08 | 0.32 ± 0.07 | 0.25 ± 0.04 | 0.29 ± 0.03 | 0.60 ± 0.05 | 0.51 ± 0.09 |
| +NaCl | 0.096 ± 0.013* | 0.31 ± 0.04 | 0.27 ± 0.04 | 0.11 ± 0.02* | 0.40 ± 0.14* | 0.35 ± 0.06* |
| +GTP | ND | ND | ND | 0.27 ± 0.04 | 0.57 ± 0.09 | 0.52 ± 0.08 |

Time–course of [$^3$H]ZM241385 association binding to human wild-type and mutant $A_{2A}$ adenosine receptors in the absence and presence of 1 M NaCl was determined as described in Materials and Methods. After the pre-equilibration of [$^3$H]ZM241385 with membranes, the dissociation was started by addition of 1 μM CGS15943 mixed with 1 M NaCl or 1 mM GTP. The $k_1$ and $k_{-1}$ values are expressed as means ± SE from three independent experiments performed in duplicate.

*$P < 0.05$ vs control. ND, not determined.

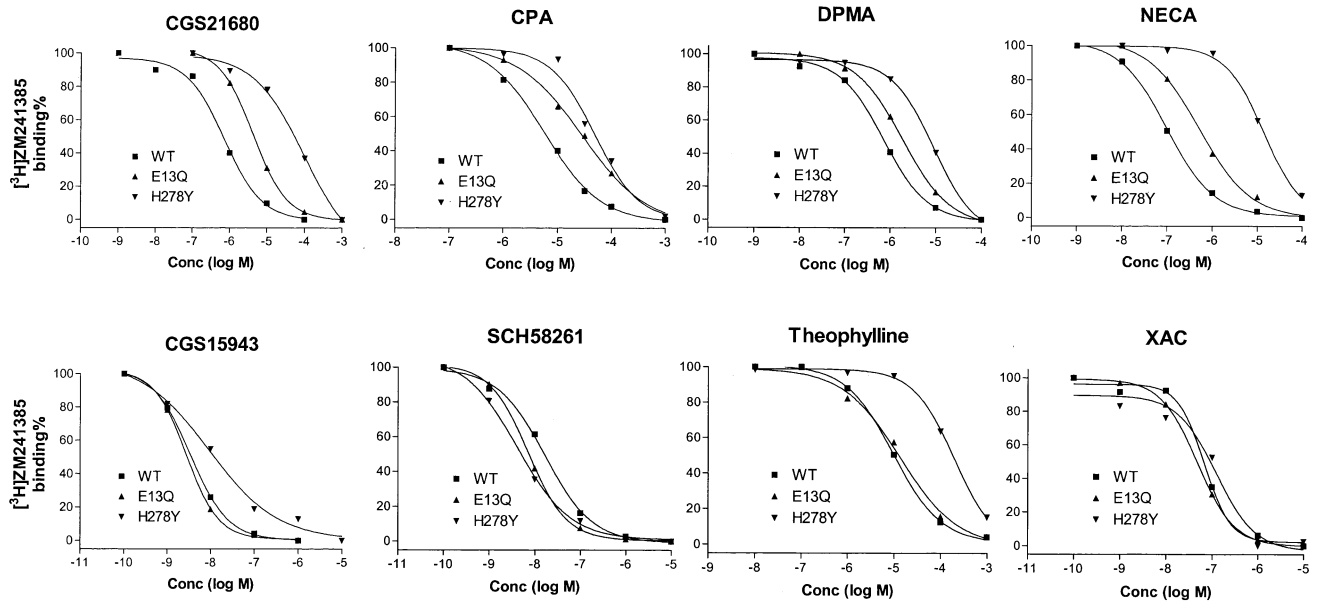


**FIG. 3. Displacement of the binding of antagonist radioligand [$^3$H]ZM241385 from human wild-type and E13Q and H278Y mutant $A_{2A}$ adenosine receptors expressed in COS-7 cells. Competitors include four agonists, CGS21680, CPA, DPMA, and NECA and four antagonists, CGS15943, SCH58261, theophylline, and XAC. Competition binding studies were carried out using membrane homogenates as described under Materials and Methods. Data are from one experiment representative of three independent experiments, each performed in duplicate.**

shown). The dissociation rate constants in the presence and absence of sodium ions or GTP are summarized in Table 2.

### *Competitive Binding of Various Agonists and Antagonists to Human Wild-Type and Mutant $A_{2A}$ Adenosine Receptors and the Effects of Sodium Ions and GTP*

The agonists selected for competition included adenosine derivatives modified at the $N^6$-position (DPMA, CPA), at the 5′-position (NECA), and at both the 5′- and 2-positions (CGS21680). The affinity of all the agonist competitors tested was 3 to 10 times lower for the E13Q mutant and 10–200 times lower for the H278Y mutant than for the wild-type receptor (Fig. 3). The $K_i$ values are summarized in Table 3.

Figure 3 also shows the competition of the diverse set of adenosine receptor antagonists for [$^3$H]ZM241385 binding to wild-type and mutant receptors. Among the antagonists were two xanthines, XAC and theophylline; a potent and non-selective non-xanthine, (CGS15943); and a potent and selective non-xanthine, SCH58261. The affinity of CGS15943 and XAC for both the wild-type and the E13Q mutant receptor was similar, with a modest decrease in affinity for the H278Y mutant receptor. Theophylline had similar affinity for the wild-type and the E13Q mutant receptor, but its affinity for the H278Y mutant was about 20 times lower than for the wild-type receptor. The affinity of SCH58261 for the mutant receptors was 2–3 times higher than for the wild-type receptor. The $K_i$ values are summarized in Table 3.

The shift of agonist competition curves for [$^3$H]ZM241385 by GTP (only for DPMA) was determined in both the wild-type and mutant receptors (Table 3). GTP shifted the DPMA competition curve for [$^3$H]ZM241385 1.5 to 2 times to the right in all membrane preparations tested. We next examined the influence of sodium ions on the affinity of CGS21680, DPMA, and NECA. The shifts induced by sodium ions were dependent on the different structural classes of the compounds and the receptors used in the experiments. Sodium ions caused a rightward shift in the CGS21680 competition curves of approximately 8, 2, and 2 times for the wild-type and the E13Q and H278Y mutant receptors, respectively. The DPMA competition curves were shifted to the right 10 and 8 times in the wild-type and the E13Q mutant receptor, respectively, whereas only a $<2$ times shift was observed in H278Y mutant receptor. NECA's affinity was most affected, up to over 25-fold. The apparent $K_i$ values of the compounds in the presence and absence of sodium ions and the ranges of shift are summarized in Table 3.

## DISCUSSION

In the present study, the interactions between the human $A_{2A}$ adenosine receptor and its ligands were investigated by site-directed mutagenesis. In a previous molecular modeling study [17], it had been suggested that a glutamic acid in the first transmembrane domain (Glu13) and a histidine in the seventh transmembrane domain (His278) are somehow linked and that they are involved in agonist binding to the $A_{2A}$ adenosine receptor and its ligands. We followed up on this study by analyzing the binding behavior of the wild-type and the E13Q and H278Y receptors to agonists and

**TABLE 3. Apparent $K_i$ values of various ligands binding to wild-type and mutant human $A_{2A}$ adenosine receptors**

| | apparent $K_i$ (μM) | | |
|---|---|---|---|
| | **WT** | **E13Q** | **H278Y** |
| Agonists | | | |
| DPMA | 0.31 ± 0.04 | 0.94 ± 0.27 | 3.0 ± 1.3 |
| DPMA + NaCl | 3.2 ± 0.22 | 8.2 ± 4.1 | 5.1 ± 1.8 |
| DPMA + GTP | 0.54 ± 0.06 | 1.5 ± 0.81 | 6.0 ± 1.1 |
| CGS21680 | 0.28 ± 0.09 | 2.5 ± 0.51 | 19.6 ± 2.0 |
| CGS21680 + NaCl | 2.2 ± 0.48 | 5.3 ± 0.14 | 44.0 ± 21.7 |
| NECA | 0.06 ± 0.01 | 0.33 ± 0.06 | 13.8 ± 3.3 |
| NECA + NaCl | 1.6 ± 0.25 | 6.7 ± 1.2 | 340 ± 119 |
| CPA | 2.7 ± 0.30 | 21.9 ± 2.7 | 37.6 ± 6.5 |
| Antagonists | | | |
| CGS15943 | 0.002 ± 0.0002 | 0.002 ± 0.0001 | 0.006 ± 0.002 |
| SCH58261 | 0.008 ± 0.003 | 0.003 ± 0.001 | 0.003 ± 0.0003 |
| Theophylline | 4.7 ± 0.43 | 9.9 ± 3.7 | 98.5 ± 17.4 |
| XAC | 0.032 ± 0.006 | 0.031 ± 0.005 | 0.065 ± 0.016 |

Membranes (6–12 μg of protein) were incubated with 2.0 nM [$^3$H]ZM241385 in duplicate, together with increasing concentrations of the competing compounds, in a final volume of 0.4 mL Tris–HCl buffer (50 mM, pH 7.4 at 20°) at 25° for 120 min. Results were expressed as means ± SE from three independent experiments performed in duplicate.

antagonists under various conditions. A newly available tritiated antagonist, [$^3$H]ZM241385, was used for this purpose.

Significant differences in affinity shifts between agonists and antagonists were observed. All agonists tested had diminished affinity for the two mutant receptors, which suggests that both Glu13 and His278 are involved in agonist recognition of the $A_{2A}$ adenosine receptor, either directly or indirectly. It was found that the extent of affinity shifts was dependent on the structures of the compounds. The 5′-substituted agonist NECA showed the largest decrease in affinity for the H278Y mutant receptor, whereas the $N^6$-substituted adenosine derivative DPMA exhibited the smallest decrease. The adenosine derivative modified at both the 5′- and 2-positions (CGS21680) displayed an intermediate decrease.

Affinity changes were less pronounced for antagonist competitors. The affinities of the non-selective antagonists, CGS15943 and XAC, were similar for the wild-type and the E13Q mutant receptor, but their affinities for the H278Y mutant receptor were modestly decreased. Theophylline had similar affinities for the wild-type and the E13Q mutant receptor, but its affinity for the H278Y mutant receptor was decreased 20 times. Interestingly, the potent and selective antagonist SCH58261 showed a moderate increase in affinity for both the E13Q and H278Y mutant receptors. Thus, the binding mode of this antagonist seems to be unique.

In a previous site-directed mutagenesis study on $A_1$ adenosine receptors, it was demonstrated that agonist affinity fell 10- to 100-fold after the mutation of the corresponding glutamic acid residue (Glu16 in the human $A_1$ adenosine receptor). The affinity for $A_1$ adenosine receptor antagonists was not affected significantly, which is consistent with the present result [22]. In the $A_1$ adenosine receptor, the corresponding histidine (His278) appeared important for both agonist and antagonist binding [20], which is also in line with the result of the current study.

The addition of 1 mM GTP caused only a 2-fold shift of the DPMA competition curve for [$^3$H]ZM241385, due to a presumed transition from high- to low-affinity states of the receptor. The relatively small GTP effect was anticipated, as a similar small shift had been observed with antagonists [$^3$H]KF17837S and [$^3$H]SCH58261 on rat striatal membranes [16, 23]. A similar shift had also been observed in our previous experiments using [$^3$H]XAC as a radiolabeled antagonist [13]. The small range of the GTP shift suggests that human $A_{2A}$ adenosine receptors are poorly coupled to G proteins, as noted previously [24]. As the ranges of the GTP shift in wild-type and mutant receptors are similar, it is suggested that the mutations do not affect agonist binding by influencing G protein coupling.

It has been suggested that Glu13 may be involved in the modulation of agonist binding to human $A_{2A}$ adenosine receptors by sodium ions [13]. This was again demonstrated and further explored in the present study. As Glu13 and His278 are closely linked, we also assessed whether His278 in TM7 was influenced by sodium ions. It was demonstrated in the present study that the affinity of agonists for both wild-type and mutant receptors was decreased in the presence of 1 M NaCl. Since NaCl is more influential on wild-type than on mutant receptors, these two sites may influence sodium effects on binding of various agonists to different extents.

Ligand binding to many members of the G protein-coupled receptors is regulated allosterically by monovalent cations. Prominent among this group are receptors linked to inhibition of adenylyl cyclase such as $A_1$ adenosine [25], $\alpha_2$-adrenergic [26, 27], and $D_2$ dopamine receptors [28, 29] as well as certain types of muscarinic receptors [30]. Monovalent cation modulation of binding has also been demonstrated for β-adrenergic receptors [31], which are

linked to stimulation of adenylyl cyclase as are the $A_{2A}$ adenosine receptors [32]. An aspartate residue in the second transmembrane domain conserved in almost all G protein-coupled receptors appears to be involved, as originally demonstrated for the $\alpha_2$-adrenergic receptor [26]. This residue has also been identified by site-directed mutagenesis of the human $A_1$ adenosine receptor [22]. Mutation of Asp55 to Ala eliminated allosteric regulation of ligand binding by sodium ions. Thus, Asp55 in the $A_1$ adenosine receptor appears to play a critical role in sodium ion modulation. It was demonstrated in the present study that mutation of Glu13 to glutamine and His278 to tyrosine influenced the allosteric regulation by sodium ions to a limited extent only. This suggests that Glu13 and His278 in the $A_{2A}$ adenosine receptor are probably not as important in sodium ion regulation as the aspartate in helix II, which is also present in the $A_{2A}$ adenosine receptor. Sodium ions increased the antagonist radioligand [$^3$H]yohimbine dissociation from $\alpha_2$-adrenergic receptors [26]. However, the dissociation of the antagonist [$^3$H]ZM241385 from $A_{2A}$ adenosine receptors was significantly decreased in the presence of sodium ions, indicating the mode of regulation of $A_{2A}$ adenosine receptors by sodium ions to be different from that of the $\alpha_2$-adrenergic receptor.

As demonstrated in the present study, the affinity of all agonists for the mutant receptors decreased. The affinity of the antagonists for the mutant receptors was more variable with both decreases and increases, which may be consistent with the fact that all agonists are chemically closely related to adenosine, whereas the antagonists show greater structural diversity [32]. As there is variation in the affinity of the antagonists for the mutant receptors, there may be more than just one antagonist binding site on the $A_{2A}$ adenosine receptor. An alternative explanation would be that the two amino acids, through direct interaction, are stabilizing the receptor conformation that binds agonists with high affinity.

It is interesting that among agonists NECA is most affected by mutations and NaCl. This might be due to the 5′-*N*-ethylcarboxamide substitution on its ribose moiety. It has been demonstrated on the $A_1$ adenosine receptor that a serine residue adjacent to His278 (Ser277) also has greatest impact on NECA's affinity [33].

In summary, the two closely linked residues, Glu13 and His278, are important for agonist recognition on the human $A_{2A}$ adenosine receptor. The allosteric modulation of $A_{2A}$ adenosine receptors by sodium ions is influenced by these two residues, but to a rather limited extent.

*We thank Miriam de Groote, Jacobien K. von Frijtag Drabbe Künzel, and David S. Johnson for technical assistance.*

## References

1. Jacobson M, Molecular biology of adenosine receptors. In: *Adenosine and Nucleotides: From Molecular Biology to Integrative Physiology* (Eds. Belardinelli L and Pelleg A), pp. 5–14. Kluver, Norwell, MA, 1995.
2. Barraco RA, Martens KA, Parizon M and Normile HJ, Role of adenosine $A_{2A}$ receptors in the nucleus accumbens. *Prog Neuropsychopharmacol Biol Psychiatry* **18:** 545–553, 1994.
3. Olsson RA and Pearson JD, Cardiovascular purinoceptors. *Physiol Rev* **70:** 761–845, 1990.
4. Jarvis MF, Schulz R, Hutchison AJ, Do UH, Sills MA and Williams M, [$^3$H]CGS21680, a selective $A_2$ adenosine receptor agonist directly labels $A_2$ receptors in rat brain. *J Pharmacol Exp Ther* **251:** 888–893, 1989.
5. Ferre S, O'Connor WT, Snaprud P, Ungerstedt U and Fuxe K, Antagonistic interaction between adenosine $A_{2A}$ receptors and dopamine $D_2$ receptors in the ventral striopallidal system. Implications for the treatment of schizophrenia. *Neuroscience* **63:** 765–773, 1994.
6. Nikodijevic O, Jacobson KA and Daly JW, Acute treatment of mice with high-doses of caffeine: An animal model for choreiform movement. *Drug Dev Res* **30:** 121–128, 1993.
7. Martin GE, Rossi DJ and Jarvis MF, Adenosine agonists reduce conditioned avoidance responding in the rat. *Pharmacol Biochem Behav* **45:** 951–958, 1993.
8. Schiffman SN and Vanderhaegen JJ, Adenosine $A_2$ receptor regulation of striatal gene expression. In: *Adenosine and Nucleotides: From Molecular Biology to Integrative Physiology* (Eds. Belardinelli L and Pelleg A), pp. 71–76. Kluver, Norwell, MA, 1995.
9. Kanda T, Shiozaki S, Shimada J, Suzuki F and Nakamura J, KF17837: A novel selective adenosine $A_{2A}$ receptor antagonist with anticataleptic activity. *Eur J Pharmacol* **256:** 263–268, 1994.
10. Fredholm BB, Abbracchio MP, Burnstock G, Daly JW, Harden TK, Jacobson KA, Leff P and Williams M, Nomenclature and classification of purinoceptors. *Pharmacol Rev* **46:** 143–146, 1994.
11. Kim J, Wess J, van Rhee AM, Schoneberg T and Jacobson KA, Site-directed mutagenesis identifies residues involved in ligand recognition in the human $A_{2A}$ adenosine receptor. *J Biol Chem* **270:** 13987–13997, 1995.
12. Kim J, Jiang Q, Glashofer M, Yehle S, Wess J and Jacobson KA, Glutamate residues in the second extracellular loop of the human $A_{2A}$ adenosine receptor are required for ligand recognition. *Mol Pharmacol* **49:** 683–691, 1996.
13. IJzerman AP, Von Frijtag Drabbe Kunzel JK, Kim J, Jiang Q and Jacobson KA, Site-directed mutagenesis of the human adenosine $A_{2A}$ receptor. Critical involvement of Glu13 in agonist recognition. *Eur J Pharmacol* **310:** 269–272, 1996.
14. Jiang Q, van Rhee AM, Kim J, Yehle S, Wess J and Jacobson KA, Hydrophilic side chains in the third and seventh transmembrane helical domains of human $A_{2A}$ adenosine receptors are required for ligand recognition. *Mol Pharmacol* **50:** 512–521, 1996.
15. Jiang Q, Lee BX, Glashofer M, van Rhee AM and Jacobson KA, Mutagenesis reveals structure–activity parallels between human $A_{2A}$ adenosine receptors and biogenic amine G protein-coupled receptors. *J Med Chem* **40:** 2588–2595, 1997.
16. Dionisotti S, Ongini E, Zocchi C, Kull B, Arslan G and Fredholm BB, Characterization of human $A_{2A}$ adenosine receptors with the antagonist radioligand [$^3$H]-SCH58261. *Br J Pharmacol* **121:** 353–360, 1997.
17. IJzerman AP, van der Wenden EM, van Galen PJ and Jacobson KA, Molecular modeling of adenosine receptors. The ligand binding site on the rat adenosine $A_{2A}$ receptor. *Eur J Pharmacol* **268:** 95–104, 1994.
18. Baldwin JM, Schertler GF and Unger VM, An alpha-carbon template for the transmembrane helices in the rhodopsin family of G-protein-coupled receptors. *J Mol Biol* **272:** 144–164, 1997.

19. Jacobson KA, Stiles GL and Ji XD, Chemical modification and irreversible inhibition of striatal $A_{2A}$ adenosine receptors. *Mol Pharmacol* **42:** 123–133, 1992.
20. Olah ME, Ren H, Ostrowski J, Jacobson KA and Stiles GL, Cloning, expression, and characterization of unique bovine $A_1$ adenosine receptor. Studies on the ligand binding site by site-directed mutagenesis. *J Biol Chem* **267:** 10764–10770, 1992.
21. Cheng Y and Prusoff WH, Relationship between the inhibition constant ($K_i$) and the concentration of inhibitor which causes 50 per cent inhibition ($I_{50}$) of an enzymatic reaction. *Biochem Pharmacol* **22:** 3099–3108, 1973.
22. Barbhaiya H, McClain R, IJzerman AP and Rivkees SA, Site-directed mutagenesis of human $A_1$ adenosine receptor: Influences of acidic and hydroxy residues in the first four transmembrane domains on ligand binding. *Mol Pharmacol* **50:** 1635–1642, 1996.
23. Nonaka H, Mori A, Ichimura M, Shindou T, Yanagawa K, Shimada J and Kase H, Binding of [$^3$H]KF17837S, a selective adenosine $A_2$ receptor antagonist, to rat brain membranes. *Mol Pharmacol* **46:** 817–822, 1994.
24. Luthin DR, Olsson RA, Thompson RD, Sawmiller DR and Linden J, Characterization of two affinity states of adenosine $A_{2A}$ receptors with a new radioligand, 2-[2-(amino-3-[$^{125}$I]iodophenyl)ethylamino]adenosine. *Mol Pharmacol* **47:** 307–313, 1995.
25. Linden J, Patel A, Earl CQ, Craig RH and Daluge SM, $^{125}$I-labeled 8-phenylxanthine derivatives: Antagonist radioligands for adenosine $A_1$ receptors. *J Med Chem* **31:** 745–751, 1988.
26. Horstman DA, Brandon S, Wilson AL, Guyer CA, Cragoe EJ Jr and Limbird LE, An aspartate conserved among G-protein receptors confers allosteric regulation of $\alpha_2$-adrenergic receptors by sodium. *J Biol Chem* **265:** 21590–21595, 1990.
27. Jagadeesh G, Cragoe EJ Jr and Deth RC, Modulation of bovine aortic $\alpha_2$ receptors by $Na^+$, 5′-guanylylimidodiphosphate, amiloride and ethylisopropylamiloride: Evidence for receptor G-protein precoupling. *J Pharmacol Exp Ther* **252:** 1184–1196, 1990.
28. Neve KA, Regulation of dopamine $D_2$ receptors by sodium and pH. *Mol Pharmacol* **39:** 570–578, 1991.
29. Hoare SR and Strange PG, Regulation of $D_2$ dopamine receptors by amiloride and amiloride analogs. *Mol Pharmacol* **50:** 1295–1308, 1996.
30. Hou X, Wehrle J, Menge W, Ciccarelli E, Wess J, Mutschler E, Lambrecht G, Timmerman H and Waelbroeck M, Influence of monovalent cations on the binding of a charged and an uncharged (′carbo′-)muscarinic antagonist to muscarinic receptors. *Br J Pharmacol* **117:** 955–961, 1996.
31. Ganz MB, Pachter JA and Barber DL, Multiple receptors coupled to adenylate cyclase regulate $Na^+$-$H^+$ exchange independent of cAMP. *J Biol Chem* **265:** 8989–8992, 1990.
32. Jacobson KA, van Galen PJ and Williams M, Adenosine receptors: Pharmacology, structure–activity relationships, and therapeutic potential. *J Med Chem* **35:** 407–422, 1992.
33. Tucker AL, Robeva AS, Taylor HE, Holeton D, Bockner M, Lynch KR and Linden J, $A_1$ adenosine receptors. Two amino acids are responsible for species differences in ligand recognition. *J Biol Chem* **269:** 27900–27906, 1994.