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Ligand-Specific Binding and Activation of the Human Adenosine A_{2B} Receptor

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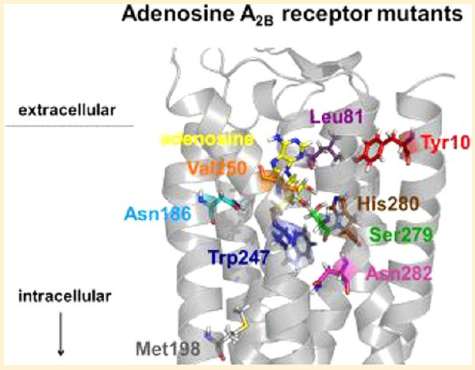
ABSTRACT: Adenosine A_{2B} receptors, which play a role in inflammation and cancer, are of considerable interest as novel drug targets. To gain deeper insights into ligand binding and receptor activation, we exchanged amino acids predicted to be close to the binding pocket. The alanine mutants were stably expressed in CHO cells and characterized by radioligand binding and cAMP assays using three structural classes of ligands: xanthine (antagonist), adenosine, and aminopyridine derivatives (agonists). Asn282$^{7.45}$ and His280$^{7.43}$ were found to stabilize the binding site by intramolecular hydrogen bond formation as in the related A_{2A} receptor subtype. Trp247$^{6.48}$, Val250$^{6.51}$, and particularly Ser279$^{7.42}$ were shown to be important for binding of nucleosidic agonists. Leu81$^{3.28}$, Asn186$^{5.42}$, and Val250$^{6.51}$ were discovered to be crucial for binding of the xanthine-derived antagonist PSB-603. Leu81$^{3.28}$, which is not conserved among adenosine receptor subtypes, may be important for the high selectivity of PSB-603. The N186$^{5.42}$A mutant resulted in an increased potency for agonists. The interactions of the non-nucleosidic agonist BAY60−6583 were different from those of the nucleosides: while BAY60−6583 appeared not to interact with Ser279$^{7.42}$, its interactions with Trp247$^{6.48}$ and Val250$^{6.51}$ were significantly weaker compared to those of NECA. Moreover, our results discount the hypothesis of Trp247$^{6.48}$ serving as a "toogle switch" because BAY60−6583 was able to activate the corresponding mutant. This study reveals distinct interactions of structurally diverse ligands with the human A_{2B} receptor and differences between closely related receptor subtypes (A_{2B} and A_{2A}). It will contribute to the understanding of G protein-coupled receptor function and advance A_{2B} receptor ligand design.



The endogenous purine nucleoside adenosine is a constituent of oligo- and polymeric nucleotides (RNAs) as well as small nucleotides such as ATP, cAMP, and NAD$^+$, which play a crucial role in a broad spectrum of physiological processes.[1] Adenosine itself is an important signaling molecule; it is able to activate four G protein-coupled adenosine receptor subtypes, namely, adenosine A_1, A_{2A}, A_{2B}, and A_3 receptors.[2,3] After heterologous expression of all human receptor subtypes, adenosine [**1** (Figure 1)] was shown to activate each subtype at submicromolar concentrations (0.3−0.7 μM), with the exception of adenosine A_{2B} receptors, which showed a micromolar EC_{50} value (24 μM).[4]

The human A_{2B} receptor's cDNA was first identified and cloned in 1992.[5] This receptor is coupled to G_s and G_q proteins, with an apparent preference for G_s coupling in many cells.[6,7] It was found to be ubiquitously expressed in the body, e.g., in cells of the lung, brain,[8] and cardiovascular system,[9] in cells of the immune system,[10] and in cells of the gastrointestinal and urogenital tract.[11] Because of the relatively low potency of adenosine at this receptor, it might not be fully activated by concentrations of adenosine in the normal physiological range, but rather by elevated adenosine concentrations, for example, as a result of ischemic or hypoxic conditions. In addition to an increased concentration of adenosine, hypoxia also leads to an enhanced expression of A_{2B} receptors, because of a hypoxia-responsive region in the promotor of the receptor gene.[12]

Although the receptor's physiological role is far from being completely understood, it has been implicated to play a role in various processes like modulation of arterial blood pressure and heart rate,[13] regulation of ciliary beat frequencies in airway epithelia[14] and ependymal cells,[15] colonic motility,[16] penile erection[17] and male fertility,[18] glucose homeostasis,[19] and sweet taste perception,[20] inflammatory response,[21,22] and inflammation-related or thermal pain.[23,24] The A_{2B} receptor is of considerable interest as a very promising new pharmacotherapeutical target.

There is much evidence for the A_{2B} receptor's involvement in the pathogenesis of asthma and chronic obstructive pulmonary disease (COPD).[25,26] Because of the A_{2B} receptor's ability to promote pulmonary inflammation, antagonists may be useful in the treatment of asthma and COPD.[27] Accordingly, in 2007 the first selective A_{2B} receptor antagonist, 3-ethyl-1-propyl-8-[1-[3-

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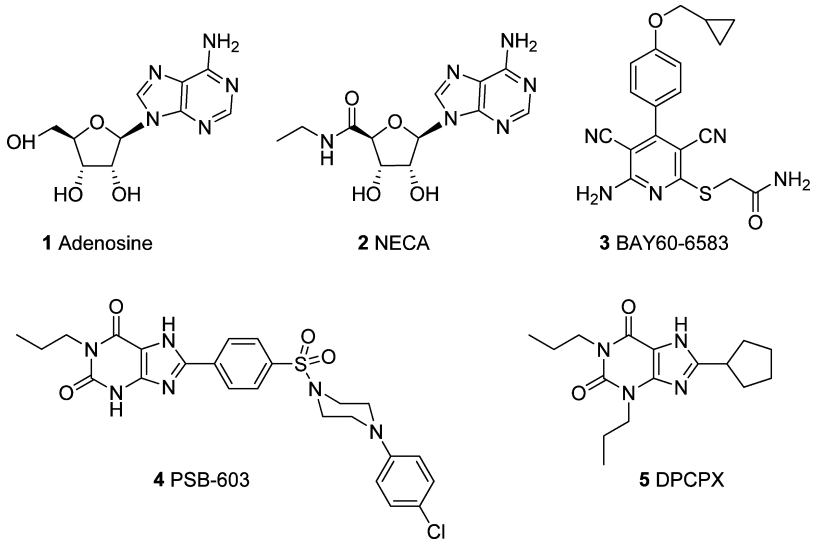

Figure 1. Structures of investigated agonists (**1**–**3**) and antagonists (**4** and **5**) used to characterize A_{2B} receptor mutants.

(trifluoromethyl)benzyl]-1*H*-pyrazol-4-yl]-1*H*-purine-2,6-(3*H*,8*H*)-dione (GS 6201, previously designated CVT-6883), was evaluated in a single ascending-dose phase I clinical study as a drug candidate in the treatment of chronic inflammatory airway diseases.[28]

Furthermore, inhibition of A_{2B} receptor activation might be a promising approach in the treatment of cancer. A_{2B} receptors have been shown to be overexpressed in several tumor cells, and because of the chronically hypoxic conditions in solid tumors, infiltrating immune cells also show increased levels of A_{2B} receptor expression.[26] A_{2B} receptor antagonists were shown not only to slow tumor growth[29] but also to reduce the level of tumor cell chemotaxis in vitro and the number of spontaneous lung metastases of breast tumors in vivo in a mouse model.[30] The A_{2B} receptor is also an important player in neovascularization, because it enhances the release of vascular endothelial growth factor (VEGF), resulting in an increased intratumoral vascular density.[31] A_{2B} receptor-mediated promotion of tumor growth might be dependent on the fact that A_{2B} receptor activation facilitates tumoral immunoevasion by impairing T cell proliferation and inducing differentiation of regulatory T cells.[32,33] Inhibition of A_{2B} receptor activation could furthermore prove to be useful in the treatment of other pathological conditions like pain,[23,24] priapism,[34] and type 2 diabetes.[35]

On the other hand, agonists of the A_{2B} receptor might be therapeutically beneficial for various indications. A_{2B} receptor activation mediates cardioprotective processes, resulting in a reduction in infarction size[36] and dampening of post-ischemic injuries of heart tissue.[37] Additionally, an A_{2B} receptor selective agonist could be shown to reduce the extent of atherosclerosis in mice.[38] Recently, A_{2B} receptor agonists have also been shown to protect against endotoxin-induced acute lung injury[39] and reduce mortality after excessive sepsis-induced inflammation in mice.[40]

However, the A_{2B} receptor still remains the most enigmatic adenosine receptor subtype, because only very few agonists have been described. Most of them, e.g., 5′-*N*-ethylcarboxamidoadenosine [NECA, **2** (Figure 1)], are adenosine analogues, nonselective, and even less potent at A_{2B} receptors than at the other adenosine receptor subtypes. 1-Deoxy-1-[6-[*N*′-(furan-2-carbonyl)hydrazino]-9*H*-purin-9-yl]-*N*-ethyl-β-D-ribofuranuronamide was the first described relatively potent adenosine analogue, which may have some A_{2B} selectivity.[41] A highly selective A_{2B} agonist is the non-nucleosidic aminopyridine BAY60−6583 [**3** (Figure 1)].[42] On the other hand, many selective antagonists have been described recently, most of them being xanthine derivatives with large 8-substituents.[2,43] One of the most potent and selective A_{2B} receptor antagonists known to date is the xanthine derivative PSB-603 [**4** (Figure 1)],[44] which has been obtained in radiolabeled form thus providing a useful pharmacological tool.[45−47]

Molecular models of receptor proteins, especially combined with mutagenesis data, can provide valuable information about the receptor's putative binding pocket(s).[48−51] Although there is no crystal structure available for the human A_{2B} receptor, several crystal structures of its closest relative, the human A_{2A} receptor, cocrystallized with non-xanthine or xanthine antagonists,[52−55] or with adenosine derivatives (agonists), including UK432097,[56] NECA (**2**), and adenosine (**1**), have been published.[57]

These structures are suitable as templates for the further refinement of A_{2B} receptor models. However, despite the similarity between the A_{2A} and A_{2B} receptors, there are still many differences between the two receptor subtypes, e.g., the longer extracellular loop 2 of the A_{2B} receptor or the fact that the A_{2A} receptor possesses four disulfide bonds in the extracellularly oriented part of the protein whereas the A_{2B} receptor has been found to have only one disulfide bond.[45]

Gaining deeper insight into the structure of the human A_{2B} receptor's binding pocket and identification of chemical structures mediating the interaction between the receptor and ligand can help in the design of ligands with improved pharmacological properties. It is of particular interest to identify ligand−receptor interactions that are unique for the adenosine A_{2B} receptor, as well as those that are conserved in all adenosine receptor subtypes to allow the design of selective ligands. On the other hand, identifying conserved amino acid residues involved in ligand binding can help in the understanding of general properties of ligand binding and receptor activation of the whole class of adenosine receptors and even of GPCRs in general. The goal of this study was to identify amino acids involved in the interaction with the receptor and the binding of structurally diverse agonists and antagonists and in receptor activation. On the basis of our A_{2B} receptor model,[58] eight amino acids predicted to be in the proximity of the docked ligands were chosen to be exchanged for alanine. To investigate their role in

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ligand binding and receptor activation, radioligand binding assays using the antagonist radioligand [^{3}H]PSB-603 and cAMP accumulation assays were performed. The obtained information will help to improve our understanding of interactions of the A_{2B} receptor with ligands and will be especially valuable for drug design.

EXPERIMENTAL PROCEDURES

Site-Directed Mutagenesis. The coding sequence for the human adenosine A_{2B} receptor was cloned into the plasmid vector pUC19. Point mutations leading to the desired amino acid exchanges were introduced through site-directed mutagenesis using whole plasmid recombination polymerase chain reaction (PCR).[59] Complementary oligonucleotide primers containing the corresponding mutations were designed. Therein, each mismatching base is flanked by 12−19 nucleotides at the 3′- and 5′-ends of the primer. The PCR mixture contained 20 ng of template DNA, 15 pmol of both sense and antisense primer, 10 mM dNTPs, 1× Thermopol reaction buffer, and 1 unit of $Vent_R$ polymerase (New England Biolabs). PCR was performed as follows: 4 min at 94 °C and 20 cycles consisting of 1 min at 94 °C, 1 min at 66 °C, and 10 min at 72 °C followed by a final elongation step of 10 min at 72 °C. The yielded PCR product was digested with the restriction enzyme DpnI (New England Biolabs) for 90 min to degrade any parental template DNA.[60] After transformation of the PCR product into competent *Escherichia coli* Top10 cells, plasmids from single individual clones were isolated and sequenced (GATC Biotech). Mutated receptor DNA was subsequently subcloned into the retroviral plasmid vector pLXSN, which contained a hemagglutinin (HA) tag, resulting in an N-terminally tagged receptor after translation. Transformation, isolation, and sequencing of the newly constructed plasmids were performed.

Cell Culture. GP+envAM12 packaging cells were cultured at 37 °C and 5% CO_2 in HXM medium containing Dulbecco's modified Eagle medium [DMEM (Invitrogen)], 10% fetal calf serum (FCS), 100 units/mL penicillin G, 100 μg/mL streptomycin, 1% ultraglutamine, 200 μg/mL hygromycin B, 15 μg/mL hypoxanthine, 250 μg/mL xanthine, and 25 μg/mL mycophenolic acid. Chinese hamster ovary (CHO) cells were maintained in DMEM-F12 medium (Invitrogen) with 10% FCS, 100 units/mL penicillin G, 100 μg/mL streptomycin, and 1% ultraglutamine under the same conditions. After the stable transfection of CHO cells, this medium was supplemented with 200 μg/mL geneticin (G418).

Retroviral Transfection of Chinese Hamster Ovary Cells. On the day before transfection, 1.5×10^6 GP+envAM12 packaging cells[61] were plated on 25 cm^2 cell culture flasks and cultured in 5 mL of DMEM supplemented with 10% FCS, 100 units/mL penicillin G, 100 μg/mL streptomycin, and 1% ultraglutamine. The medium was replaced with 5 mL of DMEM containing 10% FCS and 1% ultraglutamine 3 h before transfection. The packaging cells were then transfected with 10 μg of DNA using Lipofectamine 2000 (Invitrogen). To pseudotype the subsequently generated viruses and thus increase their infection efficiency, we cotransfected the cells with vesicular stomatitis virus G protein (VSV-G) DNA[62] in a ratio of 1:1.7 (VSV-G DNA:receptor DNA). After 15 h, the medium was exchanged for 3 mL of the previously used medium with 5 mM sodium butyrate added, and the cells were incubated for 48 h at 32 °C and 5% CO_2. The supernatant medium of the packaging cells containing the retroviral vector was filtered to remove any remaining cells. Polybrene (6 μL of a 4 mg/mL solution) was added to 3 mL of supernatant, and the mixture was transferred into a flask of ∼50% confluent CHO cells. The medium was discarded after incubation for 2.5 h and replaced with CHO culture medium. The selection for successfully infected cells (which had acquired geneticin resistance) was started after 48 h by the addition of 800 μg/mL G418 to the medium. After 1 week, the G418 concentration was reduced to 200 μg/mL.

Cell Surface ELISA. CHO cells overexpressing the adenosine A_{2B} receptor or its mutants were transferred into 24-well plates (Sarstedt) at a density of 200000 cells/well 24 h prior to the ELISA. As a negative control, cells transfected with an empty pLXSN vector were used. Except for the incubation with the antibody, the cells were cooled on ice while being incubated with 500 μL of precooled solutions per well. After incubation with phosphate-buffered saline (PBS) for 10 min, nonspecific protein binding sites were blocked with 1% BSA in PBS for 5 min. Then, cells were incubated with 300 μL of a 1:1000 dilution of monoclonal anti-HA antibody (clone HA-7, Sigma) in DMEM, 1% BSA, 10 mM HEPES, and 1 mM $CaCl_2$ for 1 h at room temperature. After being washed three times with PBS, cells were fixed with 4% paraformaldehyde for 5 min and then washed again with PBS. Following a 10 min incubation period with 1% BSA in PBS, 300 μL of a 1:5000 dilution of a horseradish peroxidase-coupled goat anti-mouse antibody (Sigma) were added. After incubation for 1 h at room temperature, cells were washed four times with PBS and subsequently incubated with 300 μL of 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS, Calbiochem) reagent for 50 min at room temperature. For each well, 170 μL of the supernatant was transferred to a 96-well plate (Greiner). The solution's absorbance at 405 nm was measured using a FLUOstar plate reader (BMG Laboratory Technologies). One to three independent experiments were performed each in triplicates.

Determination of Intracellular cAMP Accumulation. Stably transfected cells expressing the wild-type (wt) receptor or its mutants were transferred into 24-well plates at a density of 200000 cells/well. After 24 h, the medium was removed and the cells were washed with 500 μL of 37 °C warm Hank's Balanced Salt Solution {HBSS [20 mM HEPES, 13 mM NaCl, 5.5 mM glucose, 5.4 mM KCl, 4.2 mM $NaHCO_3$, 1.25 mM $CaCl_2$, 1 mM $MgCl_2$, 0.8 mM $MgSO_4$, 0.44 mM KH_2PO_4, and 0.34 mM Na_2HPO_4 (pH adjusted to 7.3)]} with 1 unit/mL adenosine deaminase (ADA, Sigma). The cells were then incubated in 300 μL of HBSS with ADA at 37 °C and 5% CO_2 for 2 h. Then, 100 μL of the phosphodiesterase inhibitor Ro20−1724 (Hoffmann La Roche; final concentration of 40 μM) was added to each well, and the cells were incubated for 15 min at 37 °C and 5% CO_2. Then 100 μL of various dilutions of agonist 5′-*N*-ethylcarboxamidoadenosine (NECA, Sigma), BAY60−6583 (Bayer Schering Pharma), or adenosine (Sigma) in HBSS containing 5% DMSO was added in triplicate. Assays for determining the potency of adenosine were performed in the absence of ADA. After incubation for 15 min at 37 °C and 5% CO_2, the supernatant was removed and 500 μL of 90 °C hot lysis buffer consisting of 4 mM EDTA and 0.01% Triton X-100 (pH adjusted to 7.3) was added. After incubation for 1 h with gentle shaking on ice, cAMP amounts of the lysates were determined by competitive radioligand binding experiments. Competition experiments were performed in a final volume of 120 μL containing 50 μL of cell lysates, 30 μL of a [^{3}H]cAMP solution in lysis buffer (final concentration of 3 nM), and 40 μL of cAMP binding protein[63] diluted in the same buffer (50 μg of protein/vial). For determining cAMP concentrations, 50 μL of various

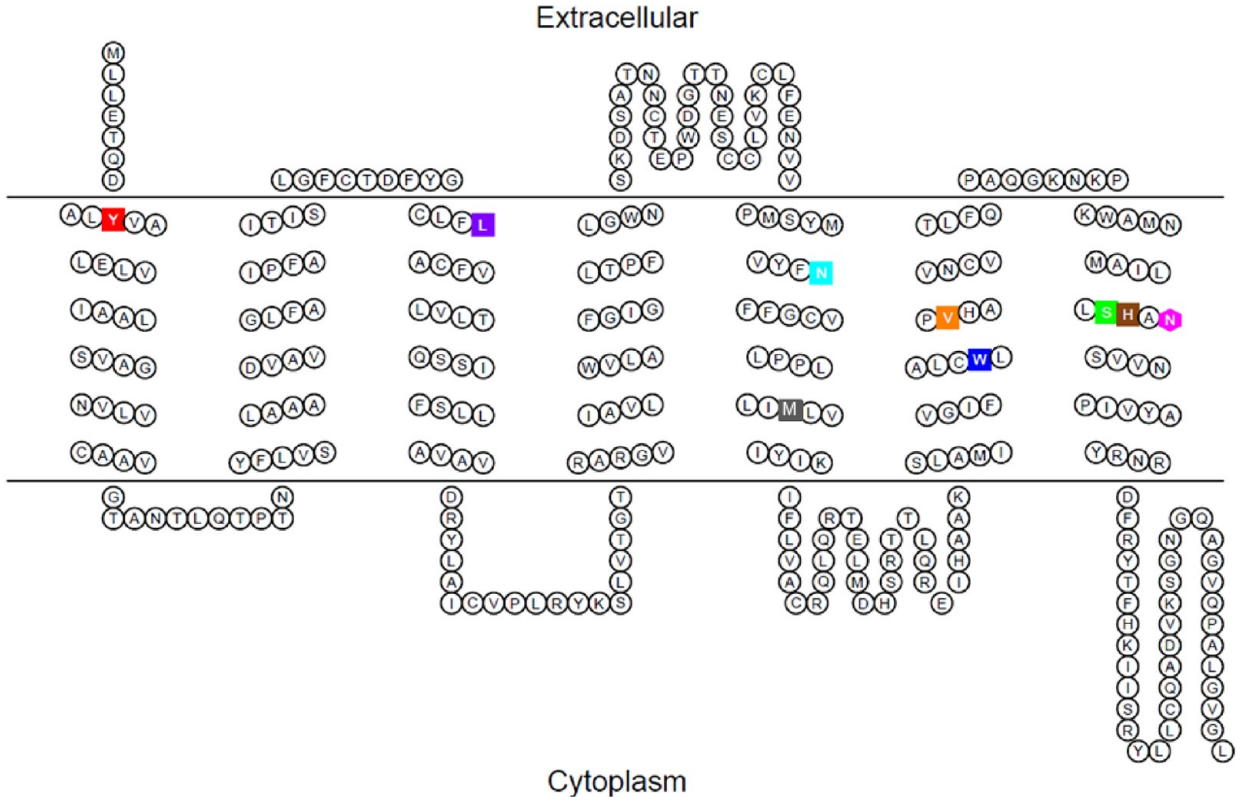

Figure 2. Snakelike plot of the human adenosine A_{2B} receptor. Exchanged amino acids are highlighted as follows: Tyr10$^{1.35}$ in red, Leu81$^{3.28}$ in purple, Asn186$^{5.42}$ in cyan, Met198$^{5.54}$ in gray, Val250$^{6.51}$ in orange, Trp247$^{6.48}$ in blue, Ser279$^{7.42}$ in green, His280$^{7.43}$ in brown, and Asn282$^{7.45}$ in magenta. Amino acids shown as squares were exchanged for alanine, while Asn282$^{7.45}$ (shown as a hexagon) was exchanged for aspartate.

cAMP dilutions were added instead of cell lysates to obtain a standard curve. Total binding was determined in the absence of cAMP, and nonspecific binding of the filter was determined without addition of binding protein. The mixture was incubated for 60 min on ice and filtered through GF/B glass fiber filters using a cell harvester (Brandel). The filters were washed three times with 2−3 mL of ice-cold 50 mM Tris-HCl buffer (pH 7.4) and subsequently transferred into scintillation vials. The liquid scintillation counting of the filters started after incubation for 9 h in 2.5 mL of scintillation cocktail (Lumac). Three separate experiments were performed. The amount of cAMP was determined by comparison to a standard curve generated for each experiment.

Generation and Expression of the N282D Mutant. The N282D mutation occurred accidentally during subcloning of the A_{2B} receptor's coding DNA. The mutant was not tagged by an HA epitope; thus, receptor expression was not assessed by an ELISA. As a reference, the untagged receptor was used. The receptor mutant was stably expressed in Flp-In CHO cells (Invitrogen).

Membrane Preparation for Radioligand Binding Assays. The medium from transfected CHO cells plated on cell culture dishes was removed, and the dishes were immediately frozen at −80 °C overnight. After thawing, cells were scraped off and taken up into a buffer containing 5 mM Tris-HCl and 2 mM EDTA (pH 7.4). After homogenization of the cell suspension for 1 min with an Ultra-Turrax (setting 1, 11000 rpm) and subsequently for 1 min with a glass Teflon homogenizer, the homogenate was centrifuged for 10 min at 1000*g* and 4 °C. To sediment membrane fragments containing the receptor, the supernatant of the previous centrifugation was spun at 48000*g* and 4 °C for 1 h. Pellets were resuspended in 10 mL of 50 mM Tris-HCl buffer (pH 7.4), and centrifugation was repeated under the same conditions. The resuspended and homogenized pellet was aliquoted and stored at −80 °C until it was used. The protein concentration of the membrane preparation was determined using the method described by Lowry et al.[64]

Radioligand Binding Studies. Experiments were performed using the antagonist radioligand [^{3}H]PSB-603.[44] A final volume of 200 μL, in some cases 500 μL, contained 50 μL of test compound diluted in 10% DMSO/90% Tris-HCl buffer (50 mM, pH 7.4), 50 μL of radioligand diluted in Tris-HCl buffer (50 mM, pH 7.4, final radioligand concentration of 0.3 nM), and 100 μL of membrane suspension (50 μg of protein/vial), which was preincubated with ADA (2 units/mL) for 20 min. Total binding was determined in the absence of any test compound, and nonspecific binding was measured in the presence of 10 μM 8-cyclopentyl-1,3-dipropylxanthine (DPCPX, **5** Figure 1). After incubation for 75 min at room temperature, the mixture was filtered through GF/B glass fiber filters. Filters were washed with ice-cold buffer containing 50 mM Tris-HCl (pH 7.4) and 0.1% bovine serum albumin (BSA). Washing and counting of the filters were performed as described for the determination of the cAMP accumulation assays. Three separate experiments were performed each in duplicate. Data were analyzed using GraphPad Prism 5 (GraphPad Software, La Jolla, CA) with one-site and two-site competition; the one-site competion model of analysis for the figures (the two-site model led to ambiguous results). K_D values for [^{3}H]PSB-603 at the (mutant) receptors were calculated using homologous competition data.

Receptor Homology Model. For the generation of the homology models of the adenosine A_{2B} receptor, the X-ray

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structures 3EML, 3QAK, and 2YDV of the human adenosine A_{2A} receptor were used as templates.[52,56,57] In detail: for antagonist docking the inactive model, based on the A_{2A} X-ray structure 3EML, was used as previously published.[58] This model was used for the predictions for the mutagenesis studies. For agonist docking, two A_{2B} homology models were generated on the basis of the 2YDV and 3QAK A_{2A}AR X-ray structures using Modeler (Accelerys, San Diego, CA). The generated models were energy minimized by applying molecular dynamics simulations using GROMACS.[65] For generating ligand−receptor complexes, molecular docking was applied. Therfore, an automated docking procedure employing FlexX (BioSolveIT GmbH, St. Augustin, Germany) and the MD package Amber (University of California, San Francisco, CA) were used to create suitable starting structures for subsequent MD simulations of the complexes in an attempt to improve the binding mode and to predict the energetically most favorable binding mode for the respective ligand.

RESULTS

Mutagenesis Study. The goal of this mutagenesis study was to identify amino acids of transmembrane domains (TM) involved in ligand binding. On the basis of the predictions of a computer-generated homology model,[58] eight different amino acids were selected to be exchanged for alanine. Altered genetic sequences of the receptor encoding mutants Y10A (TM1), L81A (TM3), N186A (TM5), M198A (TM5), W247A (TM6), V250A (TM6), S279A (TM7), and H280A (TM7) were subcloned into retroviral expression vector pLXSN and expressed in CHO cells. In addition, the accidentally obtained receptor mutant N282D (TM7) was expressed. The replaced amino acid residues are highlighted in a snakelike plot of the A_{2B} receptor (Figure 2).

Homology Modeling and Molecular Docking. To analyze and explain the effects of the mutagenesis results, we performed molecular docking with the ligands used for the experiments. The antagonist PSB-603 was docked into the 3EML-based[52] A_{2B}AR model. Agonists NECA, adenosine, and BAY60−6583 were docked into two different A_{2B}AR homology models, based on NECA-bound A_{2A}AR X-ray structure 2YDV[57] and UK-432097-bound structure 3QAK.[56] Ligand−receptor interactions were compared (data not shown).

Determination of Expression Levels. Receptors were N-terminally tagged with an HA epitope. Whole cell ELISAs showed that the level of expression of most mutant receptors was 44−105% of the wild-type (wt) receptor's expression level (Table 1). Only expression level of the receptor mutant H280A was lower (31%), while expression level of the mutants W247A, V250A, and S279A was significantly higher (152−202%) compared to that of the wt receptor (Table 1). B_{max} values of all mutants, determined by homologous radioligand competition assays (where specific binding was sufficient to perform radioligand binding studies), ranged from 1000 to 1750 fmol/mg of protein [wt, 1240 fmol/mg of protein (data not shown)].

Table 1. Cell Surface Expression of Adenosine A_{2B} Receptor Mutants[a]

receptor	expression ± SEM (%)
Y10A	43.7 ± 2.1
L81A	105 ± 15
N186A	65.2 ± 5.6
M198A	45.0 ± 9.5
W247A	202 ± 18
V250A	152 ± 14
S279A	186 ± 7
H280A	31.1 ± 14.4

[a]Data are means ± SEM of independent experiments. The expression level of the wt receptor was defined as 100%.

Pharmacology of the Wild-Type Adenosine A_{2B} Receptor. cAMP accumulation assays were performed using two structurally different A_{2B} agonists, the adenosine derivative NECA and the non-nucleosidic agonist BAY60−6583 (for structures, see Figure 1). Agonist-induced cAMP accumulation was assessed using cells expressing the wt and generated mutant receptors (Figure 3 and Table 2). At the wt receptor, NECA showed an EC_{50} value (83.5 nM) similar to that of BAY60−6583 (80.2 nM). For comparison of the efficacies, forskolin (100 μM) was investigated in the same assay. The maximal amounts of cAMP that accumulated in each assay were comparable for all functional mutants, reaching a value slightly below that of forskolin for the agonist NECA. This indicates full activatability of the mutant receptors. BAY60−6583 appeared to be slightly less efficacious than NECA, but the difference was not statistically significant. For radioligand binding studies, the tritiated antagonist PSB-603 ([^{3}H]PSB-603, 0.3 nM)[44] was incubated with membrane preparations of CHO cells expressing the adenosine A_{2B} receptor (wt or mutants). The nucleosidic agonist NECA and the unlabeled xanthine-derived antagonist PSB-603 were examined in competition experiments (Figure 4). PSB-603 showed a K_i value of 3.59 nM (K_D = 4.40 nM) at the wt receptor (Table 3). For NECA, a K_i value of 5850 nM was determined in binding studies at the wt receptor.

Pharmacology of Mutants with Exchanged Amino Acid Residue in TM1 and TM3. Both NECA and BAY60−6583 exhibited a significant reduction in potency shown by an increase in their EC_{50} values with the Y10A mutant (top of TM1). With this mutant, the decrease in potency was more pronounced for BAY60−6583 (1160 nM, 15-fold) than for NECA (426 nM, 5-fold) (Figure 3 and Table 2). The K_i value of NECA, determined in binding studies, was also increased for the Y10A mutant (2-fold, from 5850 to 11500 nM), while the K_i of the antagonist PSB-603 was not significantly different compared to that at the wt receptor (K_D = 5.85 nM). At the L81A mutant (top of TM3), NECA was less potent (909 nM, 11-fold higher EC_{50} value compared to that of the wt receptor) than BAY60−6583 (EC_{50} of 398 nM, 5-fold higher than that of the wt). The increase of both agonists' EC_{50} values was significant. The specific binding of the radioligand (0.3 nM) at the L81A mutant was not sufficient to perform radioligand binding studies (data not shown).

Pharmacology of Mutants with an Exchanged Amino Acid Residue in TM5. N186A was the only mutant that exhibited a significantly increased potency for NECA (EC_{50} of 43.1 nM, 2-fold) and BAY60−6583 (5.33 nM, 15-fold) compared to that of the wt receptor (Table 2 and Figure 3). However, the N186A mutant lacked specific binding of the xanthine-derived radioligand PSB-603. The M198A mutant showed a slightly reduced potency for NECA (165 nM, 2-fold) but no difference in radioligand binding studies. The EC_{50} value for BAY60−6583 was unaltered, as was the affinity of PSB-603 [K_D = 3.33 nM (Table 3 and Figure 4)].

Pharmacology of Mutants with an Exchanged Amino Acid Residue in TM6. The mutants W247A and V250A exhibited significantly reduced potencies for both examined

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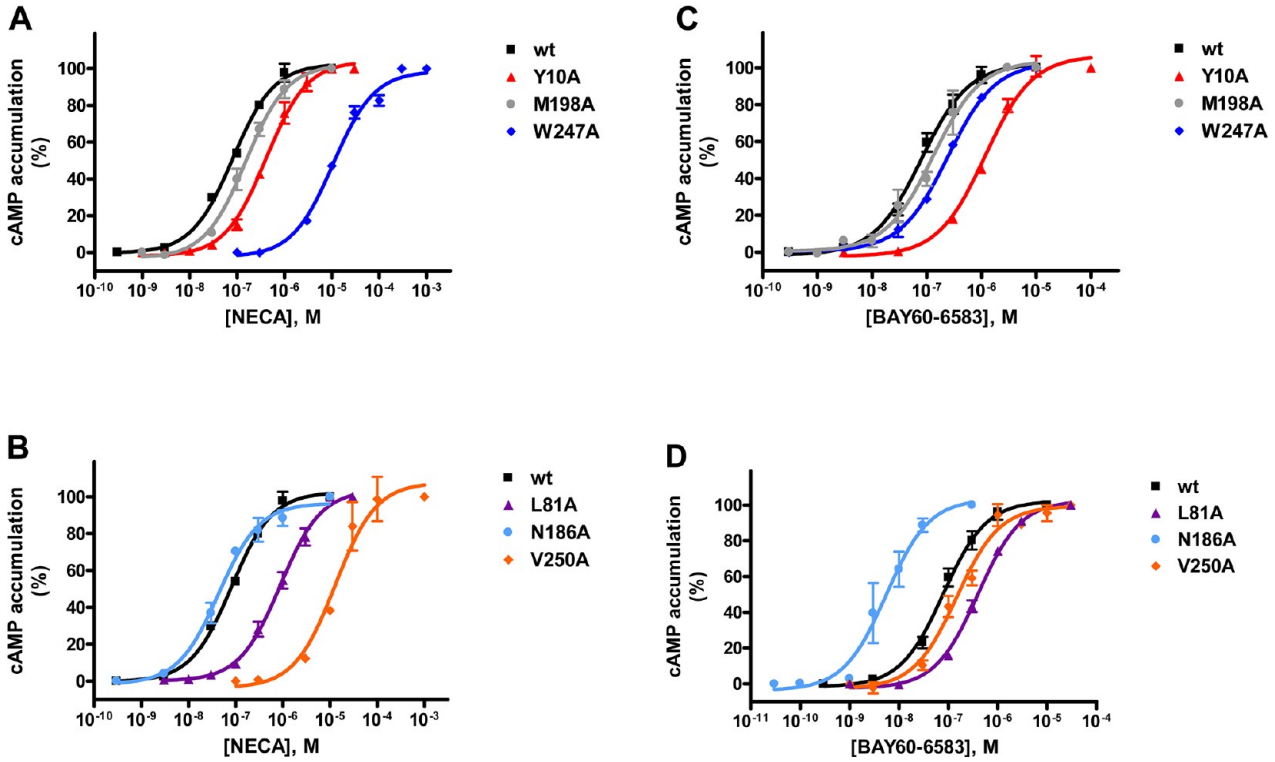

Figure 3. Agonist activation of human adenosine A_{2B} receptor mutants. Concentration−response curves show mean cAMP accumulation in retrovirally transfected CHO cells after receptor activation by NECA (A and B) and BAY60−6583 (C and D) (in % of maximally accumulated cAMP amounts by NECA and BAY, respectively, ±SEM). Corresponding EC_{50} values are listed in Table 2.

Table 2. Activity of Agonists at Mutants of the Human Adenosine A_{2B} Receptor Determined in cAMP Accumulation Assays[a]

	NECA			BAY60−6583		
receptor	EC_{50} ± SEM (nM)[b]	*x*-fold shift[c]	E_{max}[d] (%)	EC_{50} ± SEM (nM)[b]	*x*-fold shift[c]	E_{max}[d] (%)
wt	83.5 ± 5.8			80.2 ± 11.9		
Y10A	426 ± 55**	5	120	1160 ± 38***	15	70
L81A	909 ± 164**	11	100	398 ± 32***	5	100
N186A	43.1 ± 3.2**	0.5	90	5.33 ± 1.09**	0.07	50
M198A	165 ± 25*	2	90	128 ± 16[ns]	2	90
W247A	10400 ± 1100***	125	150	241 ± 7***	3	110
V250A	13900 ± 1650**	166	180	164 ± 2*	2	130

[a]Data are means ± SEM of three independent experiments. [b]Results of a two-tailed *t* test: [ns], not significantly different from that of the wild type; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. [c]The shift represents the EC_{50} (mutant):EC_{50} (wt) ratio. [d]Maximal amounts of cAMP accumulated, with that of the wild type set to 100%.

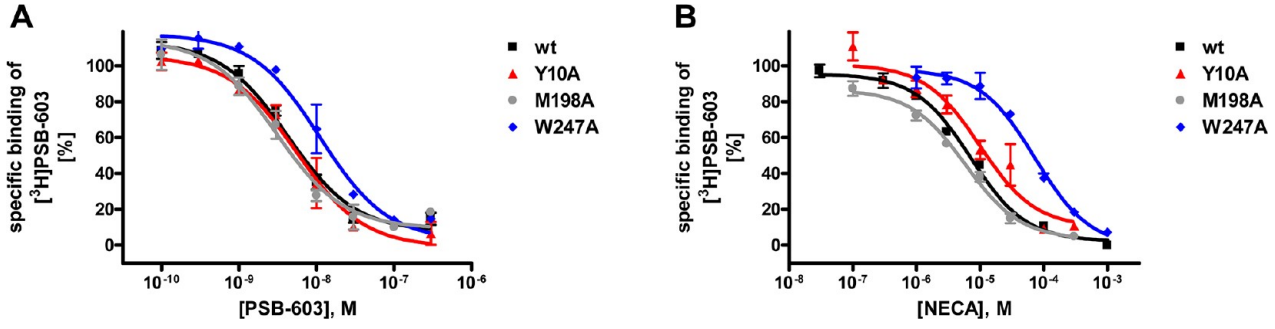

Figure 4. Homologous and heterologous competition at human adenosine A_{2B} receptor mutants. Competition of [^{3}H]PSB-603 vs antagonist PSB-603 (A) or agonist NECA (B). Mean values ± SEM were determined in radioligand binding studies using membrane preparations of retrovirally transfected CHO cells expressing adenosine A_{2B} receptor mutants. Corresponding K_i values are listed in Table 3.

agonists, NECA and BAY60−6583, in cAMP assays. While the difference was moderate for BAY60−6583 (W247A, EC_{50} of 241 nM, 3-fold; V250A, EC_{50} of 164 nM, 2-fold), it was dramatic for NECA with EC_{50} values increasing to the micromolar range

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Table 3. Affinity of Antagonist PSB-603 and Agonist NECA at the Adenosine A_{2B} Receptor Mutants Determined in Radioligand Binding Studies versus [^{3}H]PSB-603 (0.3 nM)[a]

	PSB-603		NECA	
receptor	$K_i \pm$ SEM (nM)[b]	*x*-fold shift[c]	$K_i \pm$ SEM (nM)[b]	*x*-fold shift[c]
wt	3.59 ± 0.59		5850 ± 888	
Y10A	5.73 ± 1.45[ns]	2	11500 ± 1500*	2
L81A	nd[d]		nd[d]	
N186A	nd[d]		nd[d]	
M198A	2.89 ± 0.23[ns]	0.8	5530 ± 710[ns]	1
W247A	12.8 ± 1.9**	4	78500 ± 2100***	13
V250A	nd[d]		nd[d]	

[a]Data are means ± SEM of three independent experiments. [b]Results of a two-tailed *t* test: [ns], not significantly different from that of the wild type; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. [c]The shift represents the K_i (mutant):K_i (wt) ratio. [d]Could not be determined.

(W247A, EC_{50} of 10400 nM, 125-fold difference with respect to that of the wt; V250A, EC_{50} of 13900 nM, 166-fold difference) (Table 2 and Figure 3A,C). For the W247A mutant, this reduction in affinity for NECA could also be shown in radioligand binding assays (K_i of 78500 nM, 13-fold) (Table 3 and Figure 4B). The K_i value of PSB-603 at the W247A mutant was only moderately increased (12.8 nM, 4-fold, K_D of 13.3 nM) (Table 2 and Figure 3). However, at the V250A mutant, specific binding of the radioligand was not sufficient to perform radioligand binding assays.

Pharmacology of Mutants with an Exchanged Amino Acid Residue in TM7. Results for mutant S279A are listed in Table 4. Mutant S279A showed a drastically reduced potency for NECA (EC_{50} > 100000 nM, >1000-fold). The additionally tested endogenous agonist adenosine exhibited a complete loss of activity at the S279A mutant (Table 4). In contrast, BAY60−6583 showed a 7-fold increase in potency (EC_{50} = 12.1 nM) compared to that at the wt receptor (see Figure 5A). With the S279A mutant, it was not possible to generate a competition curve for NECA in radioligand binding studies: at 100 μM NECA, the inhibition of radioligand binding amounted to less than 10% (Table 4). The affinity of adenosine could not be determined in radioligand binding assays because adenosine deaminase has to be present to remove endogenous adenosine. BAY60−6583 binding was unaltered at the S279A mutant, and the displacement curve of BAY60−6583 best fit a one-site model of analysis. The K_i value of antagonist PSB-603 was only 2-fold higher with the S279A mutant than with the wt receptor [K_D = 4.39 nM (Figure 5B)]. At the H280A mutant,The Ki value of PSB-603 with the N282D mutant was not even at the highest possible agonist concentration (for NECA, 1000 μM), no cAMP production was observed. In addition, specific binding of the antagonist radioligand was abolished.

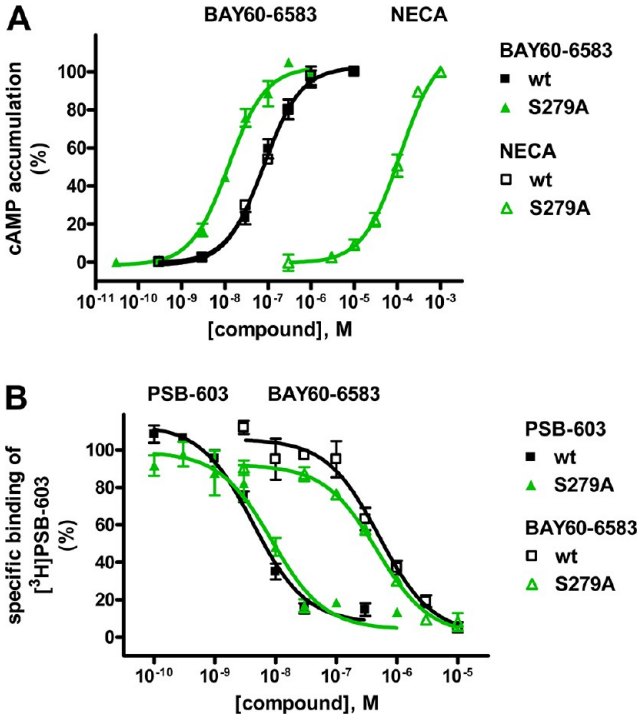

Figure 5. Pharmacological characterization of the human adenosine A_{2B} receptor mutant S279A. (A) Agonist-induced activation of human adenosine A_{2B} receptor mutant S279A. Dose−response curves show mean cAMP accumulation in retrovirally transfected CHO cells after receptor activation by BAY60−6583 and NECA (in % of maximally accumulated cAMP amounts, ±SEM). (B) Competition of [^{3}H]PSB-603 vs PSB-603, BAY60−6583, and NECA with the receptor mutant. Mean values ± SEM were determined in radioligand binding studies using membrane preparations of retrovirally transfected CHO cells overexpressing the receptor mutant. Corresponding EC_{50} and K_i values are listed in Table 4.

The N282D mutant (Table 5) showed a dramatic decrease in potency (EC_{50} of 15500 nM, 580-fold) and affinity (K_i of >100000, 16-fold) for NECA (Figure 6A and Table 5). The same trend was observed with BAY60−6583, but it was far less pronounced (26-fold higher EC_{50} value compared to that of wt).

Table 4. Potency of Agonists and Affinity of Ligands at the Adenosine Receptor Mutant S279A Determined in cAMP Accumulation Assays and Radioligand Binding Studies versus [^{3}H]PSB-603 (0.3 nM)[a]

		EC_{50} or $K_i \pm$ SEM (nM)[b]		
	compound	wild type	S279A	*x*-fold shift[d]
cAMP assays	adenosine	806 ± 36[c]	>5000000[c,e],***	>6200
	NECA	83.5 ± 5.8	>100000**	>1000
	BAY60−6583	80.2 ± 11.9	12.1 ± 1.2**	0.2
binding studies	PSB-603	3.59 ± 0.59	6.35 ± 0.28*	2
	NECA	5850 ± 888	(8.5 ± 1.5%)[f]	
	BAY60−6583	548 ± 35	436 ± 27[c,ns]	0.8

[a]Data are means ± SEM of three independent experiments. [b]Results of a two-tailed *t* test: [ns], not significantly different from that of the wild type; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. [c]Means ± SEM of four independent experiments; assays were performed in the absence of ADA. [d]The shift represents the EC_{50} (mutant):EC_{50} (wt) or K_i (mutant):K_i (wt) ratio. [e]Results of extrapolated curves (ffor extrapolation the accumulated cAMP amount produced by receptor activation with 1000 μM NECA was used). [f]Percent of inhibition at 100 μM NECA.

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Table 5. Potency of Agonists and Affinity of Ligands at the Adenosine Receptor Mutant N282D Determined in cAMP Accumulation Assays and Radioligand Binding Studies versus [^{3}H]PSB-603 (0.3 nM)[a]

		EC_{50} or K_i ± SEM (nM)[b]		
	compound	wild type[d]	N282D[d]	*x*-fold shift[e]
cAMP assays	NECA	26.9 ± 4.5	15500 ± 500***	576
	BAY60−6583	37.5 ± 12.3[c]	908 ± 57***	26
binding studies	PSB-603	2.45 ± 0.79	1.38 ± 0.18[ns]	0.6
	NECA	6320 ± 1010	>100000***	>16
	BAY60−6583	727 ± 135	1800 ± 260*	3

[a]Data are means ± SEM of three independent experiments. [b]Results of a two-tailed *t* test: [ns], not significantly different from that of the wild type; **p < 0.01; ***p < 0.001. [c]Means ± SEM of four independent experiments. [d]Receptors are not HA-tagged; coding DNA was transfected with a different method (see Experimental Procedures). [e]The shift represents the EC_{50} (mutant):EC_{50} (wt) or K_i (mutant):K_i (wt) ratio.

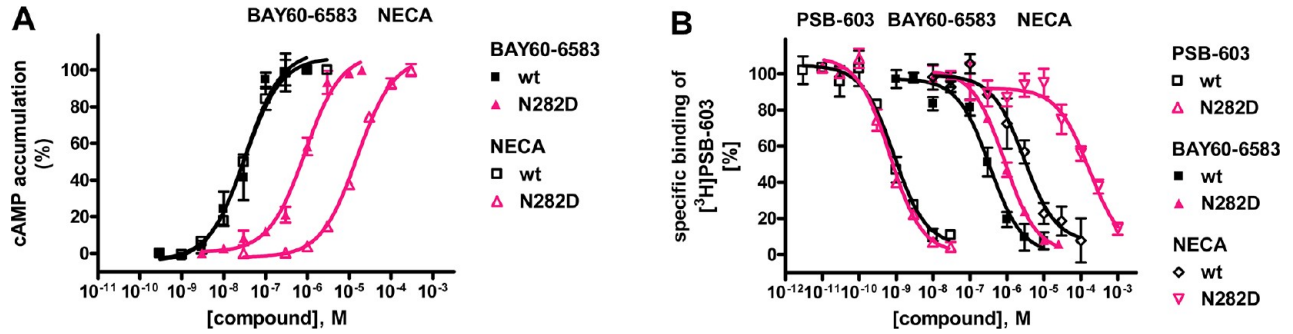

Figure 6. Pharmacological characterization of the human adenosine A_{2B} receptor mutant N282D. (A) Agonist-induced activation of human adenosine A_{2B} receptor mutant N282D. Dose−response curves show mean cAMP accumulation in retrovirally transfected CHO cells after receptor activation by BAY60−6583 and NECA (in % of maximally accumulated cAMP amounts, ±SEM). (B) Competition of [^{3}H]PSB-603 vs PSB-603, BAY60−6583, and NECA with the receptor mutant. Mean values ± SEM were determined in radioligand binding studies using membrane preparations of retrovirally transfected CHO cells overexpressing the receptor mutant. Corresponding EC_{50} and K_i values are listed in Table 5.

The K_i value of PSB-603 at the N828D mutant was not significantly different compared to that at the wt receptor (Figure 6B). It should be noted that this accidentally created mutant and the associated wt receptor were expressed under conditions somewhat different than those used for the other presented mutants (see Experimental Procedures).

DISCUSSION

The A_{2B} receptor represents an important new drug target.[66,67] The goal of this study was to identify amino acid residues involved in ligand binding and receptor activation focusing on the most prominent chemical classes of adenosine A_{2B} receptor agonists, namely, adenosine derivatives and non-nucleosidic aminopyridines, and xanthine-derived antagonists. Amino acids were chosen to be exchanged on the basis of the predictions of a computer-generated homology model.[58] Corresponding codons of the coding DNA sequence for the human A_{2B} receptor were altered by site-directed mutagenesis to encode alanine, the simplest chiral amino acid. The HA-tagged wt and mutant A_{2B} receptors were subsequently expressed in CHO cells. Non-transfected CHO cells showed no NECA-induced cAMP production and no specific binding of [^{3}H]PSB-603, but both could be observed for cells expressing the wt receptor with or without the HA tag, as expected. We previously showed that the HA tag does not influence the pharmacological properties of the human A_{2B} receptor.[45]

Expression levels of the receptors were quantified by whole cell ELISAs. cAMP accumulation assays with CHO cells expressing the receptors and radioligand binding studies with membrane preparations of these cells were performed. The comparison of obtained EC_{50} and K_i values at the wt receptor with those of the mutant receptors allowed the evaluation of each exchanged amino acid's involvement in ligand binding and receptor activation. It should be noted that the xanthine-derived antagonist [^{3}H]PSB-603 was employed as a radioligand, labeling a conformational state of the receptor, for which agonists show a relatively low affinity.

Role of Mutated Amino Acids Localized in TM1 and TM3. The Y10A mutant exhibited a rightward shift of its concentration−response curves for NECA (5-fold) and BAY60−6583 (15-fold). This might partly be due to a decreased level of cell surface expression of this receptor mutant. However, in binding studies, both agonists also showed a moderate decrease in affinity at the Y10A receptor mutant, while the binding of PSB-603 to the mutant receptor was unchanged. In accordance with the experimental data, the receptor model does not predict any direct interaction between the assayed ligands and the receptor. However, the side chains of His280$^{7.43}$ (TM7) and Glu14 (TM1) are predicted to form a salt bridge, thus constraining the receptor in the active state. Tyr10$^{1.35}$, which is located in the vicinity of the potential salt bridge, is involved in a hydrogen bonding interaction with Glu14$^{1.39}$, thereby stabilizing its interaction with His280$^{7.43}$. Therefore, Tyr10$^{1.35}$ is involved in maintaining the high-affinity agonist binding pocket showing a moderate contribution but is not involved in antagonist binding.

Leu81$^{3.28}$ was found to be essential in binding the antagonist radioligand [^{3}H]PSB-603, and specific high-affinity binding of the radioligand (0.3 nM) at the corresponding alanine mutant was abolished. According to our receptor model, Leu81$^{3.28}$ hydrophobically interacts with the phenyl ring attached to the xanthine core of PSB-603. A loss of this interaction results in a severe reduction in the affinity of the receptor. Possibly, Leu81$^{3.28}$

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is one of the amino acids responsible for A_{2B} receptor selectivity as shown for PSB-603,[44] because this amino acid is not conserved in the other adenosine receptors. Because of the radioligand's loss of affinity, the role of Leu81$^{3.28}$ for agonist binding could only be assessed in functional studies comparing the EC_{50} values of the agonists at the wt versus the mutant receptor. This is also true for the other amino acids whose exchange led to a loss of radioligand binding, namely, Asn186$^{5.42}$, Val250$^{6.51}$, and His280$^{7.43}$. The L81A mutant showed, apart from the obvious affinity loss of the tested antagonist, also significant rightward shifts in the concentration−response curves for NECA and BAY60−6583 by factors of 11 and 5, respectively. For both agonists, an interaction with Leu81$^{3.28}$ had been predicted with our original model,[58] which was based on the inactive conformation of the A_{2A} receptor (3EML[52]); while the 2′-hydroxyl group of NECA is connected to the backbone carbonyl residue of Leu81$^{3.28}$ via a hydrogen bond, the cyclopropyl residue of BAY60−6583 hydrophobically interacts with the alkyl residue of Leu81$^{3.28}$. However, according to the A_{2B} model based on the active A_{2A} structure 3QAK,[56] an indirect effect of Leu81$^{3.28}$ is more likely. This is in good agreement with the finding from the most recent A_{2A}AR X-ray structure, 4EIY,[55] which shows several structured water molecules involved in stabilizing the antagonist-bound inactive conformation. In the extracellular water cluster, which is located in the orthosteric binding pocket, one water molecule is stabilizing the non-proline kink in TM3 found in the inactive A_{2A}AR structures by forming H-bonds with Ile80$^{3.28}$, which is in the same position as Leu81$^{3.28}$ in the A_{2B}AR and Val84$^{3.32}$ that corresponds to Val85$^{3.32}$ in the A_{2B}AR.[55] When the leucine residue is lost, the water molecule that might be in the same position in the A_{2B}AR as it is in the A_{2A}AR, the water-stabilizing effect is also lost and antagonists cannot bind with high affinity. Weakening this intramolecular interaction might lead to a conformational change in the receptor, a rearrangement of the water molecules in this extracellular cluster, and therefore the disruption of the ligand binding pocket. This might especially affect larger ligands like PSB-603 extending into extracellularly facing parts of the receptor. For smaller ligands, like the agonist NECA, one would not expect a decrease in potency in the observed range when Leu81$^{3.28}$ is exchanged for alanine. However, the disruption of the intramolecular bond, in which Leu81$^{3.28}$ is involved, might not directly hamper the binding of NECA but rather prevent the conformational changes required in the activation process, which involves an upward movement of TM3 toward the extracellular space, which also straightens the kink. This is also well in agreement with the ligand-induced rearrangement of the water cluster involved in the activation process.[55] To conclude, Leu81$^{3.28}$ not only plays an important role in antagonist binding but also contributes indirectly to the potency of agonists, with greater importance for the nucleosidic agonist NECA.

Role of Mutated Amino Acids Localized in TM5. Asn186$^{5.42}$ appears to be crucial for high-affinity binding of xanthine antagonist PSB-603 because of the fact that specific binding of the radioligand was abolished at the N186A mutant. In cAMP studies, the N186A mutant showed a 2-fold increase in the potency for NECA, and a 14-fold increase for BAY60−6583. The efficacy was comparable for NECA [90% (Table 2)], while the efficacy for BAY60−6583 was surprisingly reduced to 50% (Table 2) compared to that of the wt receptor, meaning that BAY60−6583 behaved as a partial agonist at this receptor mutant. Thus, Asn186$^{5.42}$ appears to interfere with the binding of both agonists, although to a different degree. Beukers et al. had previously been searching for amino acid residues responsible for the A_{2B} receptor being a so-called "low-affinity" adenosine receptor compared to the closely related A_{2A} receptor at which adenosine exhibits a much higher potency.[68] Asn186$^{5.42}$ did not fit their criteria of such an amino acid that was not to be conserved in the A_{2A} receptor. However, the conformations of both receptors might be different because of nonconserved amino acids in positions distant from the binding pocket itself, causing the conserved amino acids to occupy different positions in both receptor molecules. Thus, Asn186$^{5.42}$ could still be one of the amino acids responsible for the relatively low potency of adenosine and other adenosine derivatives at the A_{2B} receptor compared to the A_{2A} receptor. To the best of our knowledge, an alanine mutant of the A_{2A} receptor's Asn181$^{5.42}$ has not been published. According to the hypothesis concerning the non-conserved function of Asn186$^{5.42}$, one would expect that an N181A mutant of the A_{2A} receptor would not exhibit any increase in potency for agonists or have major effects on antagonist binding. In fact, Kim et al. characterized an N181S mutant of the A_{2A} receptor and could not find an affinity increase for NECA at this mutant receptor.[69] Furthermore, the antagonist affinity of the N181S mutant was only slightly reduced (2-fold).[69] This latter result is in agreement with the current results for the N186A A_{2B} receptor mutant, if one assumes Asn5.42 to have the same function in both receptors and that the less pronounced effect at the N181S mutant was due to the fact that serine is still able to maintain the receptor's integrity by forming a hydrogen bond, which would be lost in the alanine mutant. According to the receptor model, a direct interaction of Asn186$^{5.42}$ with PSB-603, NECA, and BAY60−6583 is unlikely. However, Asn186$^{5.42}$ is predicted to act as a hydrogen bond donor by interacting with the backbone carbonyl moiety of Tyr181$^{5.37}$. According to the recently published high-resolution structure of the A_{2A}AR receptor, 4EIY,[55] Asn5.42 is involved in the coordination of a small cluster of three water molecules underneath the bound antagonist ZM241385 together with His6.52 and Gln3.37. Those interactions seem to be important for ligand selectivity and are possibly involved in receptor activation.[55] This is in good agreement with our hypothesis that the disruption of this intramolecular bond may have effects on the structure of the ligand binding pocket, leading to conformational changes inhibiting antagonist binding and facilitating agonist binding. To clarify whether Asn186$^{5.42}$ of the A_{2B} receptor is responsible for low nucleoside agonist affinity, radioligand binding studies using an agonist radioligand would be highly desirable. However, an agonist radioligand for binding studies at the A_{2B} receptor is currently not available.[67] Furthermore, the N181A mutant of the A_{2A} receptor would be of interest in clarifying the role(s) of the amino acid in both receptor subtypes.

In the study presented here, the M198A mutant of the A_{2B} receptor was found to be quite similar to the wt receptor with regard to its pharmacological properties, with a slight but, in the case of NECA, significant effect on agonist potency (2-fold decrease), which might be due to the mutant receptor's decreased level of cell surface expression (Table 1). Xanthine-derived PSB-603 showed no significant difference in affinity at the M198A mutant versus the wt receptor. Thus, Met198$^{5.54}$ does not appear to be involved in the binding of xanthine-derived antagonists. The concentration needed to half-maximally activate the M198A mutant with NECA was moderately higher, while the affinity of NECA for its low-affinity binding site determined in binding studies versus [^{3}H]PSB-603 remained unchanged compared to that for the wt receptor. These results are consistent

with the model, which does not predict any interaction with the investigated agonists and antagonist PSB-603. Furthermore, the homologous amino acid Met193$^{5.54}$ of the human adenosine A_{2A} receptor was postulated to play a role in receptor homodimerization.[70] Assuming a similar function of Met198$^{5.54}$ in the A_{2B} receptor as the corresponding Met193$^{5.54}$ in the A_{2A} receptor, one might argue that our finding of slightly decreased agonist potencies might be due to a loss of cooperative effects via the disruption of receptor oligomerization leading to a weakened ability of the agonist to activate the receptor mutant. However, another explanation of the observed moderate effect might be that the slightly reduced potency of NECA at the mutant is due to its reduced cell surface expression level. This in turn might be explained by the disruption of receptor oligomerization: as described for other GPCRs, cell surface trafficking of the receptor protein might be dependent on receptor homodimerization.[71,72] Because the role of homodimerization for the A_{2B} receptor is not well-studied, further experiments are required to explore this aspect in more detail. To conclude, Met198$^{5.54}$ appears not to play any major role in agonist or antagonist binding.

Role of Mutated Amino Acids Localized in TM6. Trp247$^{6.48}$ is highly conserved in most class A GPCRs and has been suggested to be a "toggle switch", performing a rotamer transition during activation. However this toggle switch model has been challenged by data obtained from crystal structures, e.g., of the human adenosine A_{2A} receptor.[56] For the W247A mutant of the A_{2B} receptor, the potency of NECA was dramatically reduced by a factor of 125, but it was only 2-fold reduced for BAY60−65830, as determined in functional studies. Both agonists showed increased efficacies [NECA, 150%; BAY60−6583, 110% (Table 2)], which might be due to the higher expression level of this mutant. While Trp247$^{6.48}$ appeared not to be essential for the potency of BAY60−6583, the amino acid's importance for NECA binding was further supported by a highly significant, 13-fold shift in its K_i value as determined in radioligand binding assays. Trp247$^{6.48}$ was predicted to interact with BAY60−6583 by an aromatic stacking interaction with its aminopyridine ring and its phenyl moiety, while it was proposed to interact with NECA by hydrophobic interactions with the ribose moiety. Surprisingly, BAY60−6583 showed a much lower potency reduction at the W247A mutant than NECA. The same was true for PSB-603, which was predicted to hydrophobically interact with Trp247$^{6.48}$ via the alkyl substituent in the N1 position of PSB-603. Because of this interaction, a 4-fold reduction in affinity for PSB-603 at the W247A mutant compared to the wt receptor could be observed. Because of the somewhat reduced affinity of PSB-603, the K_i values determined for the W247A mutant are not really comparable to those at the wt receptor. Despite the pronounced potency reduction of NECA at the W247A mutant in cAMP accumulation assays, NECA was able to displace the radioligand more potently than expected. An explanation for this could be that the radioligand, which was used at the same concentration in both experiments, showed somewhat reduced affinity for the mutant compared to the wt receptor. To conclude, Trp247$^{6.48}$ is especially important for the binding of nucleosidic agonists and plays a less important role in the binding of non-nucleosidic agonists and antagonists. Interestingly, for the W243A mutant of the A_3 receptor, contrary results were observed by another group.[73] For the W243A mutant of the A_3 receptor, the affinity of antagonists but not that of agonists was severely reduced, and the receptor's activity was abolished, implying the involvement of Trp6.48 in the activation of the receptor.[73] In the crystal structures of the A_{2A} receptor, van der Waals interactions were observed between Trp247$^{6.48}$ and NECA (2YDV), Ado (2YDO), and ZM241385 (3EML), as well as the xanthines caffeine (3RFM) and XAC (3REY), while nonpolar interactions were found between Trp6.48 and the ribose moiety of UK-432097 (3OAK).[52,53,56,57] Although Trp6.48 is conserved in all adenosine receptors, the homologous tryptophan residues appear to contribute differently to ligand binding and activation. It can clearly be demonstrated that Trp247$^{6.48}$ is not required for receptor activation by non-nucleosidic agonists, like BAY60−6583, thus providing further evidence that the toggle switch hhypothesis does not generally apply to explain activation of class A GPCRs.

Like Leu81$^{3.28}$ and Asn186$^{5.42}$, Val250$^{6.51}$ also appeared to be essential for xanthine antagonist binding, which is indicated by the radioligand's affinity loss at the V250A mutant. Additionally, Val250$^{6.51}$ appears to be involved in binding of both investigated agonists, although the effect on NECA binding was much more pronounced. With a 2-fold rightward shift of the curve for BAY60−6583 with the V250A mutant, Val250$^{6.51}$ was found to play a moderate role in the binding of this ligand. However, there was a drastic decrease in the mutant's potency for NECA by a factor of 166, indicating that Val250$^{6.51}$ is not only important for antagonist binding but also an essential mediator for binding of nucleosidic agonists such as the adenosine derivative NECA. Probably because this mutant showed a higher expression level than the wt receptor, both agonists exhibited an increased efficacy. Val250$^{6.51}$ was predicted to hydrophobically interact with each ligands' aromatic core structure: with the adenine moiety of NECA, the heteroaromatic ring of BAY60−6583, and the xanthine moiety of PSB-603. Accordingly, data from crystal structures of the human A_{2A} receptor indicated that the Val250$^{6.51}$ homologue of the A_{2A} receptor, Leu249$^{6.51}$, hydrophobically interacts with the bicyclic ring of the antagonist ZM241385 (3EML) and the ribose moiety of UK-432097 (3QAK), NECA (2YDV), and adenosine (2YDO).[52,53,56,57] The findings of Jaakola et al. for the alanine mutant of the Val250$^{6.51}$ homologue of the A_{2A} receptor (L249A) are in agreement with our results: specific binding of the antagonist radioligand [^{3}H]ZM241385 at the L249A A_{2A} receptor mutant was lost, and the agonist CGS21680 showed a significantly decreased potency in cAMP assays.[74] The interaction of Val250$^{6.51}$ with the ligands is especially interesting for the design of A_{2B} receptor selective ligands, because this valine residue is unique for the A_{2B} receptor, while all other adenosine receptors possess a leucine residue at this position.

Role of Mutated Amino Acids Localized in TM7. The potencies at the S279A mutant for both tested nucleoside agonists, adenosine and NECA, were significantly reduced compared to those at the wt receptor as determined in functional assays. This was confirmed in radioligand binding studies with NECA. Adenosine could not been employed in the binding studies as explained above, but it can be expected that the same was true for the nucleoside adenosine. In contrast to both nucleosidic agonists, BAY60−6583 even gained potency at the A_{2B} receptor when Ser279$^{7.42}$ was exchanged for alanine. In cAMP assays, BAY60−6583 showed a 5-fold increase in potency, while its K_i value in radioligand binding assays for labeling the low-affinity conformation for agonists was similar to that at the wt receptor. Possibly Ser279$^{7.42}$ contributes to the regulation of receptor density. In fact, the level of cell surface expression of the A_{2B} receptor was higher when Ser279$^{7.42}$ was exchanged for alanine (187% vs 100% for wt). The computer-generated receptor model predicted no direct interation of Ser279$^{7.42}$

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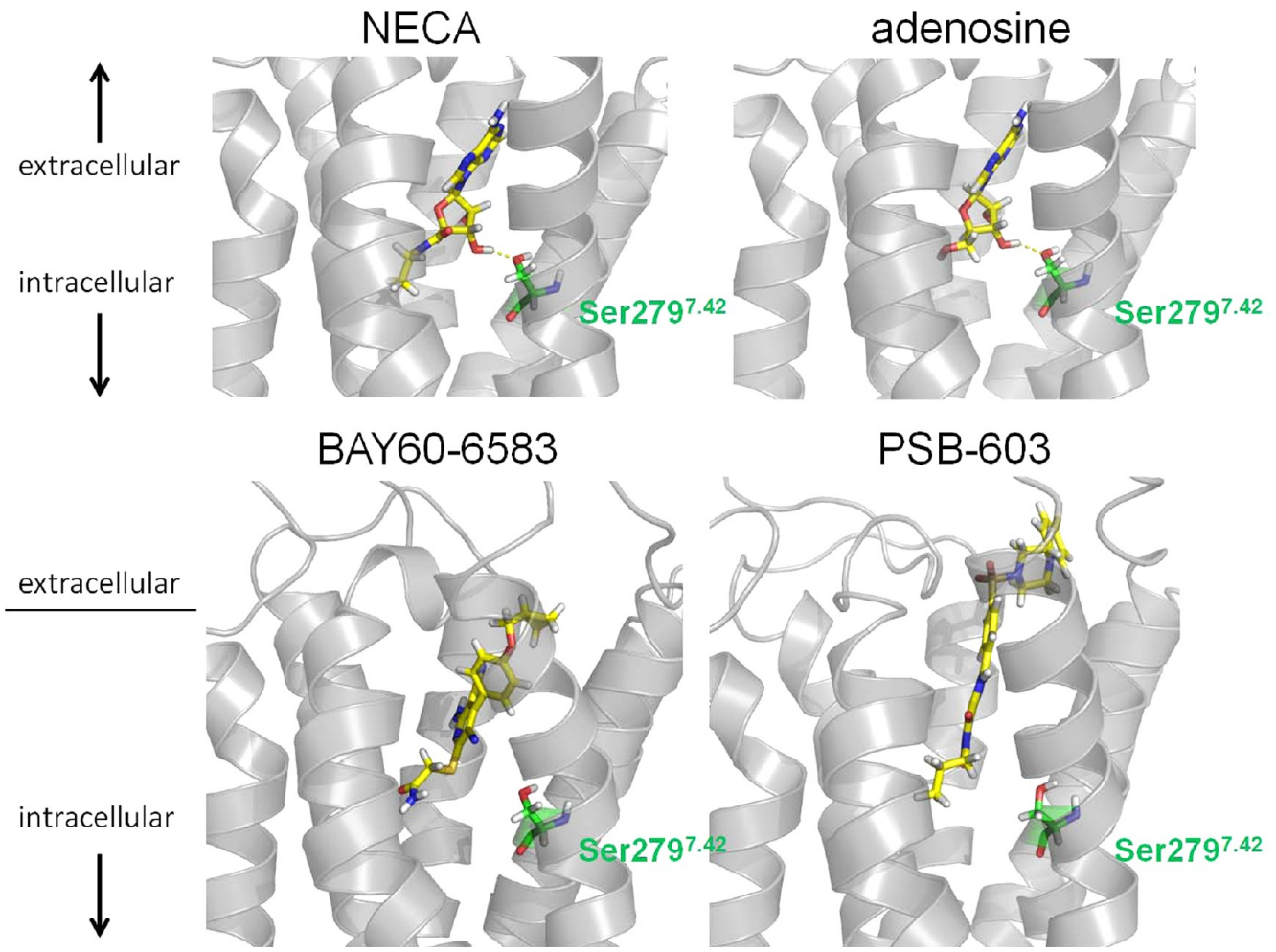

Figure 7. Molecular interaction of the human adenosine A_{2B} receptor with ligands. Homology models of the human adenosine A_{2B} receptor with NECA, adenosine, BAY60−6583, and PSB-603 docked. For docking the antagonist PSB-603, the 3EML-based[52] homology model was used, and for the docking of the agonists NECA, adenosine, and BAY60−6583, the 3QAK-based[56] model of the A_{2B}AR receptor was used. Ligands are shown as sticks in CPK colors with carbons colored yellow. Ser279$^{7.42}$ is shown as sticks with carbons colored green. Predicted hydrogen bonds are shown as yellow dots.

with BAY60−6583 (see Figure 7). Thus, the increased level of cell surface expression of the mutant might contribute to the increased potency of BAY60−6583. Adenosine and NECA showed a severe reduction in potency at the S279A mutant, indicated by their drastically increased EC_{50} values (6200- and 1500-fold, respectively). In radioligand binding assays, NECA, even in the highest applicable concentration (1 mM), was not able to significantly displace the radioligand from its specific binding site. Thus, Ser279$^{7.42}$ plays an absolutely essential role in the binding of nucleosidic agonists. The strong binding of these agonists has been predicted to be mediated by a hydrogen bond with the 3′-hydroxyl group of the ribose moiety (see Figure 7), as it has been found in the crystal structures of the A_{2A} receptor bound to the nucleosidic agonists UK-432097, NECA, and adenosine.[56,57] Such an interaction with nucleosidic agonists has also been suggested for Thr277$^{7.42}$, the homologous amino acid of the A_1 receptor.[75] PSB-603 showed an only 2-fold reduction in affinity at the S279A mutant. Therefore, Ser279$^{7.42}$ does not appear to be of great importance for binding of this antagonist. Accordingly, there was no direct interaction predicted between Ser279$^{7.42}$ and PSB-603 as shown in Figure 7. Other groups reported comparable results for mutants of homologous amino acids in other adenosine receptor subtypes: the T277A mutant of the A_1 receptor showed drastically reduced affinities for the nucleoside-derived agonists *R*-PIA, *S*-PIA, and NECA but affinities for the antagonist DPCPX [**5** (Figure 1)] comparable to that of the wt receptor,[75,76] while for the T277S mutant, the effect on the agonist potencies was far less pronounced.[75] The S277A mutant of the A_{2A} receptor was unable to bind the agonists CGS21680, NECA, and CADO but showed no significant difference in antagonist binding (XAC, CPX, and CGS15943) compared to that of the wt.[69] When Ser277$^{7.42}$ was exchanged for threonine or asparagine instead, only very moderate changes in agonist affinities were observed.[69] Together with our results, all these findings indicate an essential role for the conserved hydrogen bond donor function (of serine or threonine) in this position for all adenosine receptor subtypes. This is essential for nucleoside agonist binding but not for non-nucleoside agonist or antagonist binding. Interestingly, for all mutants with a severe loss of affinity for the nucleosidic agonist NECA (W247A, V250A, and S279A), an increase in receptor expression levels could be observed. This might be due to the fact that agonist (adenosine)-induced desensitization of the receptor was abolished or due to an enhanced thermostability as it had been observed for the S277A mutant of the A_{2A} receptor.[53]

The H280A mutant was lacking both agonist-induced cAMP accumulation and specific radioligand binding despite its expression at the cell surface. These results led to the assumption

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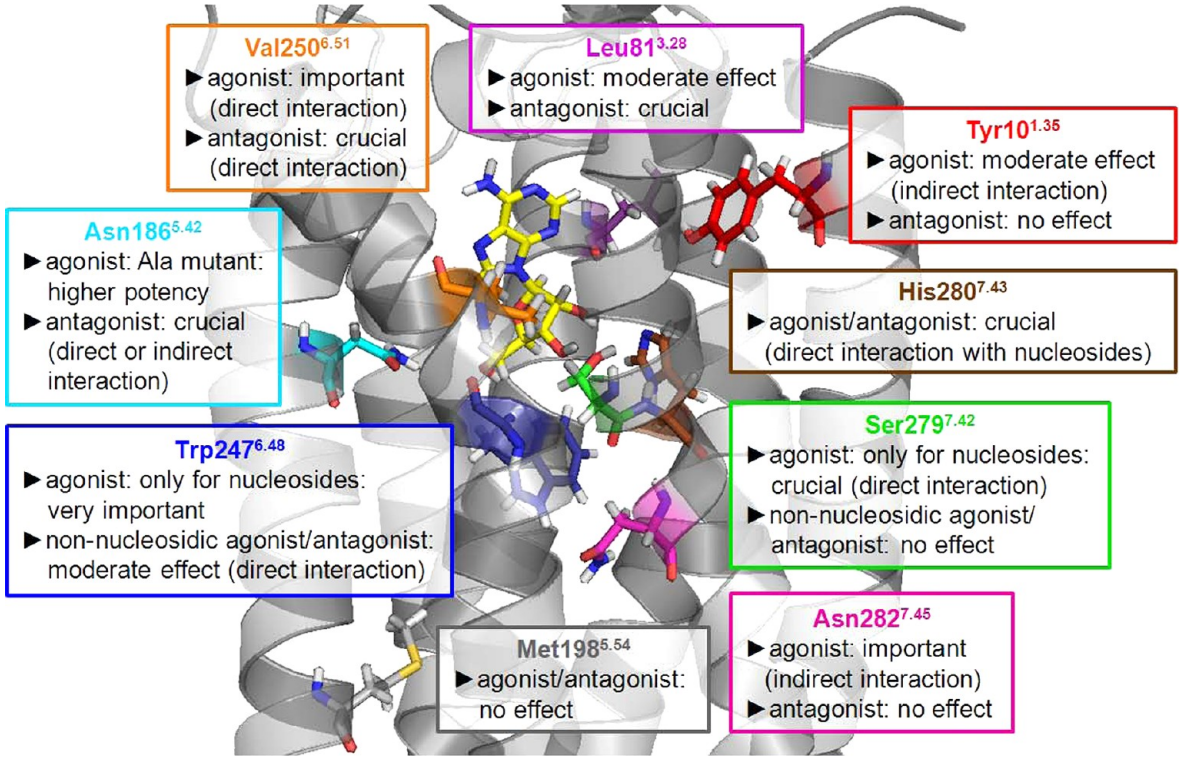

Figure 8. Summary of effects of selected amino acids in the human A_{2B}AR receptor. Exchanged amino acids are highlighted as sticks and colored as follows: Tyr10$^{1.35}$ in red, Leu81$^{3.28}$ in purple, Asn186$^{5.42}$ in cyan, Met198$^{5.54}$ in gray, Val250$^{6.51}$ in orange, Trp247$^{6.48}$ in blue, Ser279$^{7.42}$ in green, His280$^{7.43}$ in brown, and Asn282$^{7.45}$ in magenta. All amino acids were exchanged for alanine, except for Asn282$^{7.45}$, which was exchanged for aspartate.

that His280$^{7.43}$ is essential for both agonist and antagonist binding. This would be in agreement with results obtained for mutants of histidine residues in homologous positions of other adenosine receptor subtypes. For example, the H278A mutant of the A_1 receptor showed a reduction in affinity for nucleosidic agonists (e.g., NECA) as well as xanthine-derived antagonists.[75] On the other hand, it has been reported that the H278C mutant showed a 200-fold reduction in affinity for NECA (**2**), but not for the xanthine-derived antagonist DPCPX (**5**).[77] Furthermore, at the H272E mutant of the A_3 receptor, adenosine activity was completely abolished,[78] while the potencies of NECA as well as various antagonists were significantly reduced.[79] The corresponding histidine residue of the closely related A_{2A} receptor had also been previously exchanged for alanine: the resulting H278A mutant of the A_{2A} receptor showed a loss of affinity for the agonist CGS21680 as well as for the antagonist XAC.[69] Like Ser277, His278$^{7.43}$ is involved in polar interactions with the ribose moiety of nucleosidic agonists as it had been found in the X-ray structures of the A_{2A} receptor in complex with NECA, adenosine, and UK-432097 where it interacts with both the 2′- and 3′-hydroxyl groups of the ribose.[56,57] In addition, His278 of the A_{2A} receptor has been shown to interact with the xanthine antagonist caffeine as well.[53] On the basis of all of these results, it is clear that His280$^{7.43}$ plays an essential role in ligand binding by the A_{2B} receptor. The cell surface expression level of the receptor was reduced by ∼3-fold, which might be explained by His280$^{7.43}$ being important for maintaining the structural integrity of the A_{2B} receptor by contributing to intramolecular bonds. In agreement with this hypothesis, the H278C mutant of the A_1 receptor was described to exhibit a reduced level of cell surface expression as well.[77] However, the same was not found for the H278A mutant of the A_{2A} receptor.[69] From the A_{2A} receptor crystal structures, it can be seen that His278$^{7.43}$ is involved in the activation of the receptor.[53,56,57] His278$^{7.43}$ undergoes a rotameric change that results in a movement of TM7, eventually leading to the constriction of the binding pocket.[53] A similar mechanism could be hypothesized for His280$^{7.43}$ in the A_{2B} receptor. As mentioned above, His280$^{7.43}$ might also be involved in polar interactions with Glu14$^{1.39}$ and Tyr10$^{1.35}$, further stabilizing the active conformation of the receptor.

At the N282D mutant, both potency determined in functional assays and affinities measured by radioligand binding were reduced for BAY60−6583 and even more so for NECA. On the other hand, this mutation appeared not to affect the binding of PSB-603. Our model predicted no direct interaction of Asn282$^{7.45}$ with any of the tested ligands, but a hydrogen bond between the side chain amino group of Asn282$^{7.45}$ and the side chain carbonyl oxygen atom of Asp53$^{2.50}$ resulted in helix−helix packing, which is also conserved in the A_{2A} receptor. This putative intramolecular bond, which is interrupted in the N282D mutant, appears to be important for agonist binding. Possibly, this is one of the interactions responsible for maintaining the proper conformation of the agonist binding pocket.

■ CONCLUSION

Currently, there is no crystal structure available for the adenosine A_{2B} receptor, only for the adenosine A_{2A} receptor subtype. However, even closely related receptor subtypes like the A_{2A} and A_{2B} receptors (58% identical and 73% similar) can show large differences in structural features, e.g., the number of disulfide bonds[45] or ligand potencies, e.g., for adenosine.[67] Thus, there is a need for additional mutagenesis studies to improve our understanding of the interactions between receptors and ligands. Even with several crystal structures available for various GPCRs (some in multiple conformations), it is still not easy to predict

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the exact binding behavior of specific ligands, and it remains difficult to produce crystal structures for certain ligands in complex with their receptors. Therefore, the interdisciplinary approach, presented in this study using both experimental data and computational predictions, provides valuable information for the rational design of desired highly potent and selective ligands, which are required to validate and exploit their therapeutic potential and to further elucidate the A_{2B} receptor's (patho)-physiological role. Except for mutagenesis studies at the ECL1,[47] ECL2,[45,46] and one single amino acid residue in TM5, Tyr5.58,[46] of the A_{2B} receptor, BAY60−6583 has not been used yet to thoroughly investigate the binding of this non-nucleosidic agonist in comparison to that of the nucleosidic agonists adenosine and NECA.

We could identify amino acids that mediate ligand binding either by directly interacting with the ligands (Trp247$^{6.48}$, Val250$^{6.51}$, and Ser279$^{7.42}$) or by forming intramolecular bonds and, thus, maintaining the conformational stability of the receptor binding pockets (Leu81$^{3.28}$, Asn186$^{5.42}$, His280$^{7.43}$, and Asn282$^{7.45}$). Moreover, we could show that there can be both similarities and differences between the roles of selected amino acid residues and their homologues in the other adenosine receptor subtypes (for a summary, see Figure 8). Structurally distinct classes of agonists can show very different interactions. For example, amino acids like Ser279$^{7.42}$ are exclusively important for the binding of nucleosidic agonists, but not for non-nucleosidic agonists and antagonists. Other amino acids, like Asn186$^{5.42}$, are crucial for the binding of the xanthine antagonist PSB-603, but not for both classes of agonists. Using these data, our A_{2B} receptor homology model could be refined to guide future rational drug design.

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ABBREVIATIONS

ABTS, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); ADA, adenosine deaminase; AR, adenosine receptor; BAY60−6583, 2-[6-amino-3,5-dicyano-4-[4-(cyclopropylmethoxy)-phenyl]pyridin-2-ylsulfanyl]acetamide; BSA, bovine serum albumin; CHO, Chinese hamster ovary; COPD, chronic obstructive pulmonary disease; DMEM, Dulbecco's modified Eagle medium; DMSO, dimethyl sulfoxide; ECL, extracellular loop; ELISA, enzyme-linked immunosorbent assay; HA, hemagglutinin; HBSS, Hank's balanced salt solution; HIF, hypoxia-inducible factor; IL, interleukin; NAD, nicotinamide adenine dinucleotide; NECA, 5′-*N*-ethylcarboxamidoadenosine; NTP, nucleotide triphosphate; PBS, phosphate-buffered saline; PSB-603, 8-[4-[4-(4-chlorophenyl)piperazine-1-sulfonyl)-phenyl]]-1-propylxanthine; SEM, standard error of the mean; TGF, tumor growth factor; TM, transmembrane; VEGF, vascular endothelial growth factor; VSV-G, vesicular stomatitis virus G protein.

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