A<sub>1</sub> Adenosine Receptors

TWO AMINO ACIDS ARE RESPONSIBLE FOR SPECIES DIFFERENCES IN LIGAND RECOGNITION*

Amy L. Tucker‡§, Anna S. Robeva†, Heidi E. Taylor‡, Diane Holoten‡, Matthew Bockner‡, Kevin R. Lynch‡‡, and Joel Linden‡‡

From the Departments of †Internal Medicine, Cardiovascular Division, §Pharmacology, and ‡Biochemistry, Health Sciences Center, University of Virginia, Charlottesville, Virginia 22908

The A<sub>1</sub> adenosine receptor has been cloned from five species, dog, cow, rat, rabbit, and human (10–12). Despite >90% receptor identity at the amino acid level, the pharmacology of adenosine A<sub>1</sub> receptors differs markedly between species (15–19). There are differences, not only in the affinities for different ligands, but in rank order potencies as well. Bovine and canine receptors differ the most, with bovine A<sub>1</sub> adenosine receptors having higher affinity for N-ethyl-substituted adenine analogs such as the agonist R-PIA<sup>1</sup> and the antagonist N-0861; bovine A<sub>1</sub> receptors also have higher affinity for C-8-substituted xanthine antagonists such as CPA (11, 15, 20). Rat and human receptors are intermediate in their binding characteristics (15, 17, 18). These binding properties are similar for native brain membrane receptors and for recombinant receptors expressed in COS-1 cells.<sup>2</sup>

The goal of this study was to identify regions of the A<sub>1</sub> adenosine receptors responsible for conferring species specificity in binding. Our approach was to construct chimeric canine/bovine receptors to identify the domain(s) of the receptor responsible for imparting species-dependent characteristics. This was followed by site-directed mutagenesis to target individual amino acids. The results implicate two amino acids in TM7, 270 and 277 (isoleucine/methionine, bovine/canine) as being primarily responsible for species differences in A<sub>1</sub> receptor subtype selectivity.

Species differences in ligand binding to A<sub>1</sub> adenosine receptors were localized to the seventh transmembrane (TM7) region based on the binding of [8,3H]cyclopentyl-1,3-dipropylxanthine and three other ligands to wild type and six bovine/canine interspecies receptor chimeras expressed in COS-1 cells. Subsequent site-directed mutagenesis experiments identified amino acid 270 (isoleucine/methionine, bovine/canine) as being primarily responsible for species differences in the binding of N-ethyl-substituted compounds, R-<sup>2</sup>N<sup>-</sup>-phenylisopropyladenosine (R-PIA) and (S)-<sup>2</sup>N<sup>-</sup>-endoonorbornan-2-yl-9-methyladenosine, and the C-8-substituted xanthine, [3H]cyclopentyl-1,3-dipropylxanthine. These data are consistent with the hypothesis that the N<sup>8</sup> region of adenosine and the C-8 region of xanthine bind to the same region of the receptor. A second TM7 amino acid, 277 (serine/threonine, bovine/canine), selectively influences the binding of the ribose-substituted adenosine analog, 5'-N-ethylcarboxamidoadenosine to a variable extent, depending on the nature of amino acid 270. We hypothesize that amino acid 270 of the A<sub>1</sub> receptor interacts with the N<sup>8</sup> region of adenosine, while amino acid 277 is important, especially in the absence of an N<sup>8</sup> substitution, for interactions with a distinct nucleoside region, possibly on the ribose.

Experimental Procedures

Materials—Restriction enzymes were obtained from Promega (HindIII, KpnI, NcoI) and Life Technologies, Inc. (SspI, HpaI, Sapl). DNA Sequenase II kits were obtained from United States Bio labs. [3H]CPX was from DuPont-NEN. N-0861 was a gift from Whitty Research, Inc. R-PIA, NECA, and buffer reagents were purchased from Sigma. Mutagenesis was performed using the Altered Sites Kit from Promega. Media for bacterial culture were from Life Technologies, Inc. Reagents used in the electrophoresis of sequencing reactions were from Kodak. The canine A<sub>1</sub> adenosine receptor cDNA (RDC7) was a gift from G. Vassart, Brussels, Belgium. The bovine A<sub>1</sub> adenosine receptor was cloned as described previously (20).

Construction of Chimera—Canine adenosine A<sub>1</sub> cDNA in Bluescript was digested with KpnI and HindIII (37°C, 2 h) and the released insert subcloned into the mutagenesis vector pALTER which had been digested with the same two restriction enzymes. The bovine A<sub>1</sub> receptor cDNA PBV13 in Bluescript was digested with SspI (37°C, 2 h) to remove a large portion of the noncoding region that includes a unique HindIII site. The plasmid was religated and the inserted insert subcloned into the mutagenesis vector pALTER using KpnI and XbaI sites (37°C, 2 h). Oligonucleotides complementary to the noncoding strand were designed to introduce unique, silent restriction sites.

*This work was supported by National Institutes of Health Grant R01-HL37942. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

†To whom correspondence should be addressed: Box 158, Dept. Cardiology, Health Sciences Center, University of Virginia, Charlottesville, VA 22908. Tel.: 804-824-5800 Fax: 804-822-3183.

+T. Tucker, unpublished results.

<sup>1</sup>The abbreviations used are: R-PIA, R-<sup>2</sup>N<sup>-</sup>-phenylisopropyladenosine; N-0861, (S)-<sup>2</sup>N<sup>-</sup>-endoonorbornan-2-yl-9-methyladenosine; CPA, 8-cyclopentyl-1,3-dipropylxanthine; NECA, 5'-N-ethylcarboxamidoadenosine; TM, transmembrane.

27900
sites in TM regions 4 and 5, which flank the second exofacial loop. The oligonucleotides 5'-TCTCTGTTGCCGCTTAACCCGGGATCTC-3' and 5'-TCTTTGCTGGTGGCTGCGCCCCTGTCG-3' were used as primers to introduce silent HpaI sites into the fourth transmembrane region of the bovine and canine clones, respectively. NcoI sites were placed into TMS of both receptors using 5'-TCGAGGAGGT-CTCATCTGGGATAGTGGGCTG-3'. The mutated cDNAs were used to construct six bovine adenosine A1 receptor chimeras.
PALT-RDC7 and PALT-BOV13S were digested with restriction endonucleases as defined in Table I and the resulting DNA fragments purified on a low melt agarose gel. The recovered DNA fragments were ligated together in the combinations depicted in Table I. The mutated cDNAs were used to introduce amino acid changes were engineered to contain a silent, restriction enzyme and DNA fragments used for ligating the appropriate fragments summarized in Table I. One bovine receptor consists of three segments and is named according to the species from which each segment was derived. The wild type bovine and canine receptors are referred to as BBD and DDD, respectively. Chimera are named by three-letter codes indicating the species of the corresponding segment of the receptors. Eight receptors were characterized in this study (two wild type and six chimeras). Splice sites for the chimeras occur at Leu-140 in TM4 and Ser-176 in TM5 and flank the second exofacial loop containing a hypervariable region (Fig. 1). With the exception of the BDD chimeric receptor (Bmax 200 fmol/mg of protein), all of the receptors, including wild type, chimeric, and mutant (see below), showed good expression (>1 pmol/mg of protein); however, because of the variability in Bmax among the different receptors, the Scatchard plots are modified such that they are normalized to Bmax. The slopes of these modified Scatchard plots are proportional to ligand affinity. Pharmacologic characterization of the expressed receptors was done using four ligands. The two antagonists chosen were from different classes of compounds, [3H]CPX is a C8-substituted xanthine, while N-0861 contains an N-substituted adenine ring, but does not have a ribose at the N9 position of the adenosine as would adenosine. The ribose is required for agonist activity (26). The two agonists chosen were R-PIA, an N9-substituted ligand, and NECA, a ribose 5'-substituted ligand.

Wild type bovine (BBB) A1 adenosine receptors bind [3H]CPX with a 20-fold higher affinity than canine (DDD) receptors (Fig. 2A, Table II). The N9-substituted ligands, R-PIA and N-0861 also bind to bovine receptors with 54- and 26-fold a higher affinity, respectively; conversely, NECA binds to the bovine receptor with a 20-fold lesser affinity than the canine receptor (Fig. 2B, Table II). Similar patterns of relative binding affinities of these ligands have been noted using brain membranes.

### RESULTS

To identify regions of the adenosine A1 receptor responsible for conferring differences in ligand binding between bovine and canine adenosine A1 receptors, canine/bovine chimeric receptors were constructed by ligating the appropriate fragments described in Table I. One bovine receptor consists of three segments and is named according to the species from which each segment was derived. The wild type bovine and canine receptors are referred to as BBD and DDD, respectively. Chimera are named by three-letter codes indicating the species of the corresponding segment of the receptors. Eight receptors were characterized in this study (two wild type and six chimeras). Splice sites for the chimeras occur at Leu-140 in TM4 and Ser-176 in TM5 and flank the second exofacial loop containing a hypervariable region (Fig. 1). With the exception of the BDD chimeric receptor (Bmax 200 fmol/mg of protein), all of the receptors, including wild type, chimeric, and mutant (see below), showed good expression (>1 pmol/mg of protein); however, because of the variability in Bmax among the different receptors, the Scatchard plots are modified such that they are normalized to Bmax. The slopes of these modified Scatchard plots are proportional to ligand affinity. Pharmacologic characterization of the expressed receptors was done using four ligands. The two antagonists chosen were from different classes of compounds, [3H]CPX is a C8-substituted xanthine, while N-0861 contains an N-substituted adenine ring, but does not have a ribose at the N9 position of the adenosine as would adenosine. The ribose is required for agonist activity (26). The two agonists chosen were R-PIA, an N9-substituted ligand, and NECA, a ribose 5'-substituted ligand.

Wild type bovine (BBB) A1 adenosine receptors bind [3H]CPX with a 20-fold higher affinity than canine (DDD) receptors (Fig. 2A, Table II). The N9-substituted ligands, R-PIA and N-0861 also bind to bovine receptors with 54- and 26-fold a higher affinity, respectively; conversely, NECA binds to the bovine receptor with a 20-fold lesser affinity than the canine receptor (Fig. 2B, Table II). Similar patterns of relative binding affinities of these ligands have been noted using brain membranes.

**Ligand Binding to A1 Receptor Species Chimeras**—Representative experiments showing equilibrium binding of [3H]CPX to the six bovine/canine chimeric receptors are shown in Fig. 3 and data from multiple experiments are summarized in Table II. All of the receptor constructs ending in bovine sequence containing TMs 5-7 bind [3H]CPX with relatively high affinity, with Kd values between 0.56 to 1.06 nM, while all of those ending in canine sequence have lower affinity, with Kd values between 11 and 15 nM. A similar pattern is seen in competition binding assays with the other ligands. Table II shows the Kd values of R-PIA, N-0861, and NECA for wild type and chimeric receptors. The binding affinities of these ligands depend predominantly on the carboxyl third of the chimeric receptors. This point is illustrated graphically in Fig. 4. Thus, a consistent
finding for all of the ligands tested, whether they bind preferentially to bovine or canine receptors, is that species dependent binding properties are conferred primarily by the carboxyl-terminal third of the receptors.

**Ligand Binding to Transmembrane 7 Mutants of Adenosine A<sub>1</sub> Receptors**—Prior studies on G-protein linked receptors for ligands with low molecular masses have implicated the transmembrane regions as forming the ligand binding pocket (27–29). There are two amino acid differences between bovine and canine adenosine A<sub>1</sub> receptors in putative TMs 5–7, amino acids 270 and 277, both in TM7. These were targeted for site-directed mutagenesis. Mutant receptors were constructed in which amino acids 270 and 277 were switched, individually or together, from bovine to canine or vice versa. Table III shows the \( K_d (\text{{[^{3}H]CPX}}) \) and \( K \) values (R-PIA, NECA, N-0861) of various ligands for wild type and mutant receptors. Both amino acids contribute to species differences in ligand binding to an extent that depends on the nature of the ligand. This point is illustrated graphically in Fig. 5. For the C-8-substituted xanthine, CPX, and the N<sup>6</sup>-substituted adenine compounds, R-PIA and N-0861, amino acid 270 is the major determinant of species differences in receptor affinity. Of the eight receptors examined (two wild type and six mutant), the four receptors containing Ile in position 270 invariably bound these three ligands with higher affinity than the four receptors with Met in position 270. For example, the affinities of \( [^{3}H]\)CPX for receptors with Ile-270 are on average 5.9-fold higher than receptors with Met-270. In the cases of R-PIA and N-0861 this factor is 7.3-fold, respectively. By comparison, changing the amino acid in position 277 from Thr to Ser always reduces NECA binding affinity, but the reduction in affinity is small (1.4–1.9 fold) when amino acid 270 is Met, and large (4.8–10.9 fold) when amino acid 270 is Ile. Thus, the relatively low affinity of the bovine receptor can be attributed to both Ile-270 and Ser-277, and changing either of these amino acids to the corresponding canine sequence substantially increases NECA binding affinity.

**DISCUSSION**

This study identifies amino acids in TM7 responsible for conferring species selectivity in ligand binding to A<sub>1</sub> adenosine receptors. The influence of these two amino acids on binding varies depending on the nature of the ligand. Adenine and xanthine compounds substituted with aryl or cycloalkyl substituents at the N<sup>6</sup> or the C-8 positions, respectively, bind preferentially to bovine as opposed to canine receptors, primarily due to amino acid 270, isoleucine in the bovine receptor and methionine in the canine receptor. This is entirely consistent with the data of Peet et al. (30) which suggests that the N<sup>6</sup> position of adenines and the C-8 position of xanthines occupy the same position in the binding pocket of the A<sub>1</sub> adenosine receptor. This N<sup>6</sup>/C-8 model also is supported by an analysis of steric and electrostatic properties of ligands (31) and by binding studies with various ligands to membranes expressing recombinant dog, rat, and bovine A<sub>1</sub> adenosine receptors. The N<sup>6</sup>/C-8 orientation appears also to hold for A<sub>1</sub> adenosine receptors (9, 32).

NECA, which like adenosine is unsubstituted in the N<sup>6</sup> position, binds preferentially to receptors that contain Thr-277 (canine) as opposed to Ser-277 (bovine). Changing amino acid 277 from threonine to serine consistently reduces the affinity of receptors for NECA. It is striking that this effect is relatively large if amino acid 270 is isoleucine rather than methionine. Consistent with these results is the recent finding that mutation of threonine 277 in the human A<sub>1</sub> adenosine receptor to

---

**Fig. 1. Diagrammatic representation of the structure of the A<sub>1</sub> adenosine receptor of the dog and cow.** The canine receptor forms the backbone of the diagram with each amino acid represented by its single-letter code. Where the bovine sequence differs, its amino acid sequence is designated with black circles. The putative transmembrane regions are embedded in the grey banner representing the cell membrane. Splice sites for chimera, Leu-140 and Ser-176, are indicated with **bold circles**. The initial methionine is orientated extracellularly; the terminal aspartate, intracellularly. The **inset** denotes the amino acids in TM7 targeted for mutagenesis.
A, Adenosine Receptor Chimeras and Mutants

panels

symbols

Points

of other compounds that bind to addition of data and the structure of CPX. Nonspecific binding is defined by the three experiments. A, error bars are not visible because they are smaller than the size of the series of two to six experiments. S.E. smaller than the

FIG. 2. Radioligand binding to transfected COS-1 cells. A, Top panels show specific (circles) and nonspecific (squares) equilibrium binding of [3H]CPX to membranes of cells expressing bovine and canine A1 adenosine receptors, respectively. Points are means ± S.E., n = 3, the error bars are not visible because they are smaller than the size of the symbols. The bottom panel shows Scatchard transformations of these data and the structure of CPX. Nonspecific binding is defined by the addition of 1 μM CPX. Shown are representative curves from a series of three experiments. B, competition for [3H]CPX binding and structures of other compounds that bind to A1 adenosine receptors. Solid and open symbols depict binding to bovine and canine receptors, respectively. Points are means ± S.E., n = 3. Shown are representative curves from a series of two to six experiments. S.E. smaller than the symbols are not shown.

serine or alanine causes a relatively selective decrease in NECA binding affinity (33). Moreover, interaction of A1 adenosine receptor ligands with amino acid 277 is predicted in the binding model of Dudley et al. (34). Our data indicate some interplay between amino acids in positions 270 and 277 in determining affinities for ligands without bulky N6 or C-8 substituents. One possibility is that the relatively small isoleucine side chain promotes docking of aryl or cycloalkyl N6/C-8 substituents, while the slightly larger methionine group repels these, but interacts favorably with the N6 region of unsubstituted agonists. In the absence of the methionine at 270 (such as in the bovine receptor which has an isoleucine, or the human receptor containing a threonine), the amino acid at position 277, via interactions with a different region of the ligand, has a larger influence on ligand affinity for 5′ or C2-substituted ligands without N6 substitutions. A threonine at position 277 favors high affinity binding to such ligands. The rat adenosine A1 receptor shows a ligand binding profile that is consistent with this theory; this receptor displays high affinity for N6- and C-8-substituted ligands and for ligands with 5′- or C2-substitutions. As would be predicted, it has an isoleucine at position 270 as does the bovine A1 receptor, but a threonine at position 277 like the canine A1 receptor.

It is possible that mutations in receptor amino acids alter binding affinity either because ligands interact directly with mutated amino acids, or because the mutations change receptor conformation to indirectly influence binding to remote domains. We have attempted to minimize the latter possibility in this study by making conservative interspecies mutations that are not likely to produce changes in receptor structure. The mutations we have introduced apparently do not produce major changes in receptor structure or expression. Thus, we hypothesize that amino acid 270 is directly involved in the docking of the N6 portion of adenines and the C-8 portion of xanthines. However, in the absence of structural data it is not possible to exclude the possibility of indirect effects of changing amino acids 270 and 277 on a remote ligand recognition domain.

Our data support a model for ligand binding in which the N6 or C-8 substituents of the ligand interact with a region of the receptor containing amino acid 270, while a different region of the ligand, perhaps on the ribose, interacts with a hydroxyl on amino acid 277. Because the antagonists, CPX and N-0861, do not have ribose moieties and their binding is not significantly affected by the T/S mutations at position 277, the interaction of the ribose at this position is an attractive hypothesis. Either the 5′-N-ethylcarboxyl substituent or some other portion of the ribose moiety of NECA may interact at position 277. Binding studies on mutant receptors using ligands substituted at other positions, such as 2-chloroadenosine, which has a chlorine at the C2 position on the adenine ring, will help to clarify this.

This study exploits unique species pharmacology in order to better understand ligand orientation in the adenosine A1 receptor binding site(s). While there are computer-generated models for the A1 binding domain, supportive structure-function analysis of adenosine receptors has been limited to date. Ijzerman et al. (35) have proposed interaction of histidines 251 and 278 with the N6 region of the adenine and ribose hydroxyl groups, respectively, in part based on early chemical modification studies of the adenosine A1, A2a, and A2b receptors using the histidine-selective agent diethylpyrocarbonate. These studies showed that alkylation histidine residues modified ligand binding characteristics (36-38). Examination of the sequences for the putative transmembrane amino acids of the adenosine A1 receptor reveals two histidines, one in each of transmembranes 6 and 7. Data from mutational analyses of the two transmembrane histidines in the bovine A1 receptor have been difficult to interpret.
### Summary of binding affinities of the antagonists [3H]CPX and N-0861 and the agonists R-PIA and NECA for wild type and chimeric A<sub>a</sub> adenosine receptors

Affinities are expressed as the means ± S.E. of two to six independent experiments performed in triplicate. Also indicated for each ligand tested is the fold change from the wild type canine affinity.

| Receptor | CPX       | R-PIA | N-0861 | NECA |
|----------|-----------|-------|--------|------|
|          | $K_a$ ± S.E. | Change from DDD | $K_a$ ± S.E. | Change | $K_a$ ± S.E. | Change |
| DDD      | 11.05 ± 0.46 | 1     | 4.76 ± 0.27 | 1     | 3.18 ± 0.72 | 1     |
| BDD      | 11.56 ± 1.15 | 0.96  | 8.58 ± 0.45 | 0.55  | 1.93 ± 0.7 | 1.65  |
| BBB      | 12.15 ± 2.80 | 0.91  | 1.57 ± 0.20 | 3.03  | 1.96 ± 1.01 | 1.62  |
| BDB      | 18.0 ± 1.89  | 0.74  | 0.97 ± 0.13 | 4.51  | 2.97 ± 0.45 | 1.07  |
| DBB      | 0.56 ± 0.08  | 19.63 | 0.09 ± 0.02 | 52.9  | 0.12 ± 0.03 | 28.5  |
| DBD      | 0.89 ± 0.13  | 12.4  | 0.21 ± 0.01 | 22.7  | 0.30 ± 0.01 | 10.6  |
| BDB      | 0.75 ± 0.15  | 14.7  | 0.07 ± 0.01 | 68.6  | 0.08 ± 0.02 | 39.8  |
| DDB      | 1.05 ± 0.18  | 10.5  | 0.36 ± 0.04 | 13.2  | 0.21 ± 0.10 | 15.1  |

**FIG. 3.** Modified Scatchard transformations of [3H]CPX binding to wild type and chimeric A<sub>a</sub> adenosine receptors. The solid and dashed lines show binding to wild type bovine and canine receptors, respectively. The dotted lines show binding to the indicated chimeric receptors. Filled and open squares depict binding to receptor chimeras ending with bovine and canine sequence, respectively. Each plot is representative of three experiments.

Mutation of the histidine residue in TM7 (His-278) to leucine dramatically decreased both agonist and antagonist binding by 90% (39); however, this may have been due to poor receptor expression rather than to the loss of a selective receptor-ligand interaction. Mutation of the histidine in TM6 elicited no change in agonist affinity, but caused a 3.8-fold decrease in antagonist affinity. Again, there was a large (74%) decrease in receptor number. Interestingly, histidine-278 is adjacent to the threonine-277 mutated in this study. Given the dramatic decrease in receptor number and the nonspecific effects on ligand binding seen with mutation of histidines 251 and 278, it is possible that these histidines are important for receptor processing or configuration of the ligand binding domain without directly interacting with ligands. In support of this, Garritaen et al. (40) have shown that the degree of protonation of the histidine residues does not alter ligand affinity; this would be difficult to explain if this amino acid were directly involved in binding. Expression of high numbers of mutated receptors in this study, and ligand-selective effects of these mutations support roles for amino acids 270 and 277 in direct ligand interactions.

Despite their different binding profiles, the amino acid sequences of the canine and bovine adenosine receptors are very similar over most of their length, especially in the transmembrane regions, which typically are thought to form the ligand binding domain. Exceptions to this are seen among the glycoprotein hormone receptors, including the lutropin (LH), folliculin (FSH), and thyrotropin (TSH) receptors, which have large extracellular domains that bind ligands with high affinity (41, 42). The A<sub>a</sub> adenosine receptors differ most between species in a hypervariable region of the second exofacial loop. We have speculated previously (20) that this region is variable either because it does not have an important role in receptor structure-function, so it accommodates frequent mutations, or that this region is responsible for species differences in ligand binding. The results of this study support the former possibility.

**FIG. 4.** Graphical comparison of binding constants of compounds for chimeric A<sub>a</sub> adenosine receptors. The composition of the chimeras is indicated by the three-letter codes shown on the left (see Fig. 3). Solid and striped bars show binding to receptors ending with bovine and canine sequence, respectively. Average $K_a$ values are derived from competition for [3H]CPX binding and are listed in Table 2. Error bars show the –log of the means ± S.E. of binding constants.
All affinities are expressed as the mean ± S.E. of two to six independent experiments performed in triplicate. Also indicated for each ligand tested is the fold change from the wild type canine affinity.

| Receptor          | Amino acid 270/277 | GPX  K_i  PM  fold | R-PIA  K_i  PM  fold | NECA  K_i  PM  fold | NECA  K_i  PM  fold |
|-------------------|---------------------|--------------------|----------------------|---------------------|---------------------|
| Dog (DDD)         | M/T                 | 11.05 ± 0.46       | 1                    | 4.76 ± 0.27         | 1                   | 2.18 ± 1.72         |
| Dog T277S         | M/S                 | 4.72 ± 0.55        | 2.34                 | 3.02 ± 0.03         | 1.58                 | 4.97 ± 0.03         | 0.65                 | 3.08 ± 1.51         | 0.71                 |
| Dog M270I         | I/T                 | 1.58 ± 0.22        | 7                    | 0.20 ± 0.08         | 23.8                 | 0.26 ± 0.02         | 12.2                 | 6.56 ± 0.34         | 0.33                 |
| Dog M270I, T277S  | I/S                 | 0.82 ± 0.06        | 13.5                 | 0.72 ± 0.01         | 6.61                 | 0.36 ± 0.01         | 12.2                 | 3.14 ± 1.50         | 0.07                 |
| Bovine (BBB)      | M/S                 | 0.56 ± 0.08        | 19.6                 | 0.09 ± 0.02         | 32.9                 | 0.12 ± 0.03         | 26.5                 | 42.8 ± 17.11        | 0.05                 |
| Bov 1707M         | M/T                 | 2.76 ± 0.27        | 4                    | 0.91 ± 0.07         | 5.23                 | 0.61 ± 0.28         | 5.21                 | 6.92 ± 0.26         | 0.51                 |
| Bov S277T         | I/T                 | 1.1 ± 0.41         | 10.1                 | 0.44 ± 0.29         | 10.8                 | 0.04 ± 0.002        | 79.5                 | 3.9 ± 0.36          | 0.56                 |
| Bov 1707M, S277T  | M/T                 | 5.17 ± 0.13        | 2.13                 | 1.84 ± 1.56         | 2.58                 | 1.46 ± 1.28         | 2.18                 | 3.75 ± 0.87         | 0.58                 |

3. Olson, R. A., and Pease, J. D. (1990) Physiol. Rev. 70, 761–845
4. Cronstein, B. N., Dagunas, L., Nichols, D., Hutchison, A. J., and Williams, M. (1995) J. Clin. Invest. 95, 1150–1157
5. Ramkumar, V., Stiles, G. L., Beaver, M. A., and Ali, H. (1993) J. Biol. Chem. 268, 16887–16890
6. Tierney, J. A., and Duh, M. K. (1994) in Basic Neurochemistry: Molecular, Cellular, and Medical Aspects (Siegel, G. L., ed) pp. 401–416, Raven Press, New York.
7. Yakel, J. L., Warren, R. A., Reppert, S. M., and North, R. A. (1990) Mol. Pharmacol. 37, 277–280
8. Linden, J. (1991) FASEB J. 5, 2986–2976
9. Kloet, K. N., Vogt, H., and Tawfik-Schlieper, H. (1991) Naunyn-Schmiedeberg Arch. Pharmacol. 343, 196–201
10. Ukena, D., Jacobson, K. A., Fagert, W. L., Arora, C., Shamim, M., Kirk, K. L., Olson, R. O., and Daly, J. W. (1986) FEBS Lett. 209, 122–128
11. Murphy, K. M., and Snyder, S. H. (1982) Mol. Pharmacol. 23, 250–257
12. Fortgang, J. W., Valentine, H. L., Stone, A. G., and Williams, M. (1986) Drug Dev. Res. 9, 83–95
13. Brus, R. F., Daly, J. W., and Snyder, S. H. (1980) Proc. Natl. Acad. Sci. U. S. A. 77, 5547–5551
14. Jacobson, K. A., and Jaffe, A. S., D’Anjou, D. L., and Lynch, K. R. (1992) FEBS Lett. 297, 107–111
15. Cullen, B. R. (1987) Methods Enzymol. 132, 684–705
16. Sinnamon, L. S., and Kladnich, S. G., and Hoppe, C. A. (1986) Biochem. Anal. Biochem. 572, 578–580
17. Prater, M. R., Taylor, H. E., Munshi, R., and Linden, J. (1992) Mol. Pharmacol. 42, 74–81
18. Marquardt, D. M. (1963) J. Soc. Industr. Appl. Math. 11, 431–441
19. Linden, J. (1985) J. Cyclic Nucleotide Res. 10, 163–172
20. Taylor, M. D., Moos, W. H., Hamilton, W. H., Stokoe, D. S., Patt, W. C., Badger, G. R., Kristol, J. A., and Heffner, T. F., and Mertz, T. F. (1986) J. Med. Chem. 29, 546–553
21. Dixon, R. A. F., Sigal, I. S., Sando, E., Register, R. B., Candeleria, M. R., Blake, A. D., and Struder, C. D. (1987) Nature 326, 73–77
22. Barrington, W. W., Jacobson, K. A., and Stiles, G. L. (1989) J. Biol. Chem. 264, 13157–13164
23. Lifson, B. J., Aton, B., and Hartley, J. B. (1982) Vision Res. 22, 1499–1542
24. Peter, N. P., Lente, N. M., Meng, E. C., Dudley, M. W., Ogden, A. M., and Berne, D. A., Weintaub, H. J., and Boy, P (1990) J. Med. Chem. 33, 327–3310
25. van der Wenden, E. M., Ijzerman, A. P., and Soudijn, W. (1992) J. Med. Chem. 35, 629–635
26. Linden, J., Taylor, H. E., Bova, A. S., Tucker, A. L., Stickle, J. H., Rieks, S. A., Fink, J. S., and Reppert, S. M. (1986) Mol. Pharmacol. 24, 324–332
27. Townsend-Nicholson, A., and Scheidler, P. F. (1994) J. Biol. Chem. 268, 2373–2376
28. Dudley, M. W., Peter, N. P., Demeter, D. A., Weintaub, H. J., Ijzerman, A. P., Nordvall, P., Galen, P. J., and Jacobson, K. A. (1993) Drug Dev. Res. 33, 287–293
29. Ijzerman, A. P., Van Galen, P. J., and Jacobson, K. A. (1992) Drug Design Discovery 8, 43–67
30. Klitz, K.-N., Mohle, M., and Schwabe, U. (1988) J. Biol. Chem. 263, 17522–17526
31. Garrett, A., Ijzerman, A. P., Beukers, M. W., and Soudijn, W. (1990) Biochem. Pharmacol. 40, 835–842
32. Jacobson, K. A., Stiles, G. L., and J. D. (1992) Mol. Pharmacol. 43, 125–133
33. Obah, M. E., Reh, H., Ottev, J., Jacobson, K. A., and Stiles, G. L. (1992) J. Biol. Chem. 267, 10764–10770
34. Garrett, A., Ijzerman, A. P., Beukers, M. W., Crage, E. J., and Soudijn, W. (1990) Biochem. Pharmacol. 40, 927–934
35. Sengaloff, D. L., and Ascoli, M. (1992) Off. Reprod. Biol. 14, 141–188

REFERENCES
1. Fredholm, B. B., and Dunwiddie, T. V. (1988) Trends Pharmacol. Sci. 9, 130–134
2. Bellardinelli, L., Linden, J., and Berne, R. M. (1989) Circ. Res. 62, 73–97

FIG. 5. Graphical comparison of binding constants of compounds for binding to mutated A₁ adenosine receptors. Mutant receptors containing Met-270 and Ile-270 are depicted with solid bars and striped bars, respectively. Mutations are identified by wild type and mutated single letter codes placed before and after, respectively, the number of the mutated receptor amino acid(s). Average K_i values are derived from competition for [3H]GPX binding and are listed in Table III. Error bars show the log of the mean ± S.E. of binding constants.
42. Salesse, R., Remy, J. J., Levin, J. M., Jallal, B., and Garnier, J. (1991) 
Biochimie (Paris) 73, 109-120
43. Kobilka, B. K., Kobilka, T. S., Daniel, K., Regan, J. W., Caron, M. G., and 
Winlow, E. J. (1988) Science 240, 1310-1316
44. Wess, J., Maggio, R., Palmer, J. R., and Vogel, Z. (1992) J. Biol. Chem. 267, 
19313-19319
45. Suryanarayana, S., Daunt, D. A., Von Zastrow, M., and Kobilka, B. K. (1991) J. 
Biol. Chem. 266, 15488-15492
46. Guan, X.-M., Peroutka, S. J., and Kobilka, B. K. (1992) Mol. Pharmacol. 41, 
695-698
47. Beinborn, M., Lee, Y.-M., McBride, E. W., Quinn, S. M., and Kopin, A. S. (1993) 
Nature 362, 348-353
48. Kao, H.-T., Adham, N., Olsen, M. A., Weinshank, R. L., Branchek, T. A., and 
Hartig, P. R. (1992) FEBS Lett. 307, 324-328
49. Link, R., Daunt, D., Barab, G., Chrusciniski, A., and Kobilka, B. K. (1992) Mol. 
Pharmacol. 42, 16-27
50. Okazawa, D., Maretta, S. A., O'Dowd, B. P., Jin, H., Havlik, S., Peroutka, S. 
J., and Ashkenazi, A. (1992) Nature 360, 161-163
51. Strader, C. D., Sigal, I. S., Candelore, M. R., Randa, E., Hill, W. S., and Dixon, 
R. A. F. (1988) J. Biol. Chem. 263, 10267-10271