Identification of the Adenine Binding Site of the Human A1 Adenosine Receptor*

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To provide new insights into ligand-A1 adenosine receptor (A1AR) interactions, site-directed mutagenesis was used to test the role of several residues in the first four transmembrane domains of the human A1AR. First, we replaced eight unique A1AR residues with amino acids present at corresponding transmembrane (TM) positions of A2AARs. We also tested the role of carboxamide amino acids in TMs 1–4, and the roles of Val-87, Leu-88, and Thr-91 in TM3. Following conversion of Gly-14 in TM1 to Thr-14, the affinity for adenosine agonists increased 100-fold, and after Pro-25 in TM1 was converted to Leu-25, the affinity for agonists fell. After conversion of TM3 sites Thr-91 to Ala-91, and Gln-92 to Ala-92, the affinity for N6-substituted agonists was reduced, and binding of ligands without N6 substituents was eliminated. When Leu-88 was converted to Ala-88, the binding of ligands with N6 substituents was reduced to a greater extent than ligands without N6 substituents. Following conversion of Pro-86 to Phe-86, the affinity for N6-substituted agonists was lost, and the affinity for ligands without N6 substitution was reduced. These observations strongly suggest that Thr-91 and Gln-92 in TM3 interact with the adenosine adenine moiety, and Leu-88 and Pro-86 play roles in conferring specificity for A1AR selective compounds. Using computer modeling based on the structure of rhodopsin, a revised model of adenosine-A1AR interactions is proposed with the N6-adenine position oriented toward the top of TM3 and the ribose group interacting with the bottom half of TMs 3 and 7.

Adenosine exerts potent biological effects in many tissues via specific receptors that include A1 adenosine receptors (A1ARs)1 (1–3). Because activation of A1ARs has considerable therapeutic importance in treating clinical conditions (1–3), there is considerable interest in deciphering how adenosine interacts with A1ARs.

A1ARs are G protein-coupled receptors that have seven transmembrane (TM) spanning domains (Fig. 1) (1–3). Initial structure-function studies of A1ARs focused on amino acids within TMs 5–7 (4). His-256 in TM6 was identified as a site that interacts with antagonists (4). Within TM7, the amino acid at position 277 was found to account for species-related differences in affinity for A1-selective drugs (5). The amino acid at position 277 was shown to interact with the 5′ position of the adenosine ribose moiety (6). It was also suggested that His-278 in TM7 is important for ligand binding (4).

More recently, studies of chimeric A1/A2AARs have shown that TMs 1–4 of A1ARs contain the sites that confer the ligand binding characteristics of an A1AR (7). Because modification of the N6-adenine position confers A1AR selectivity of adenosinergic compounds (8), this observation strongly suggests that the N6-adenine position interacts with sites within TMs 1–4 (7). Within the first four TM domains of the A1AR, mutation of Glu-16 in TM1 results in broad decreases in agonist affinity, and mutation of Ser-94 in TM3 results in a complete loss in ligand binding (7). Yet, despite these observations, a clear understanding of how adenosine interacts with A1ARs is not at hand.

To provide additional insights into how ligands interact with A1ARs, we have tested the potential roles of several amino acids in TMs 1–4 in ligand binding. First, we have replaced amino acids within TMs 1–4 of A1ARs with amino acids present at corresponding positions in A2AARs. We have also examined the potential roles of carboxamide and several other amino acids in TM3. Using these approaches, we now identify putative binding sites in TM3 that interact with the adenosine adenine group and a revised model of ligand-A1AR interactions is proposed.

EXPERIMENTAL PROCEDURES

cDNAs—The cDNA encoding the full-length human A1AR was provided by Dr. S. M. Repperd (Boston, MA). This cDNA has been extensively characterized (9).

Generation of Mutant Receptors—Mutant receptors were made by the polymerase chain reaction (PCR) overlap-extension method (10). Primer pairs were designed to introduce mutations as described (11). Oligonucleotides were synthesized using an Applied Biosystems Oligonucleotide Synthesizer (Foster City, CA). To generate the front part of mutant receptors, oligonucleotide primer pairs (primers A and B) were designed to generate a 5′ fragment of the A1AR. Another set of oligonucleotide primer pairs (primers C and D) was designed to generate a 3′ fragment of the A1AR receptor. B and C primers contained sequences that encoded for the desired mutations.

Receptor fragments were generated using 1 μg of DNA as the substrate for PCR reactions, and PCR reactions were performed using the Gene Amp Kit reagents (Perkin Elmer). PCR was generally performed using 30 cycles at 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 2 min. PCR products were then separated on a 1% agarose gel and eluted. Receptor fragments (A-B and C-D) were then combined in a third PCR reaction to generate a full-length A1AR using flanking primers (A and D). Flanking PCR primers contained HindIII (A primers) or XhoI (D primers) restriction endonuclease sites at the ends. After fusion reactions, PCR products were digested with HindIII and XhoI and were subcloned into the mammalian expression vector pcDNAs (Invitrogen; San Diego, CA). Mutant receptors were then sequenced.

Acute Transfections—Receptor cDNA expression was characterized...
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**Fig. 1.** Schematic representation of the human A₁AR. Sites that were mutated in this report are represented by black circles.

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**RESULTS**

**Experiment 1, A₁ARs/A₂AAR Amino Acid Transposition Studies in TMs 1, 2, and 4**—To identify potential sites within TMs 1–4 that may play a role in conferring binding properties of A₁ARs, differences in the amino acid sequences of A₁ and A₂AARs were identified. First, the amino acid sequences of all cloned A₁ARs and A₂AARs within TMs 1–4 of different species present in the GenBank™ data base were compared to identify common amino acids among the different species. Universal differences among all A₁ARs and A₂AARs were then identified.

Using site-directed mutagenesis, human A₁AR residues were replaced by the corresponding amino acids of A₂AARs. Saturating studies were then performed using [³H]CCPA or [³H]DPCPX (Table I). Competition studies were next performed using a fixed dose of [³H]DPCPX and graded doses of DPCPX or CPA and several other compounds (Table II). These studies revealed similar ligand binding properties for the WT-A₁AR and the Gly-14 → Thr-14 constructs were compared, markedly increased affinity for agonists was seen for the mutant receptor, and when the Pro-25 → Leu-25 construct was examined, the affinity for agonists fell (Tables I and 2).

**Experiment 2, Mutations of Carboxamide Amino Acids**—Previous attempts aimed at modifying several hydroxyl or polar amino acids within TMs 1–4 that are unique to A₁ARs domains failed to identify a site that interacts with the adenine N₆ position (11). Therefore, we examined the role of carboxamide amino acids in the WT-A₁AR and Cys-31, Phe-65, Phe-82, Lys-125, and Leu-144 mutant constructs were thus generated and tested. Competition studies were then performed using [³H]CCPA or [³H]DPCPX (Table I). Competition studies were next performed using a fixed dose of [³H]DPCPX and graded doses of DPCPX or CPA and several other compounds (Table II). These studies revealed similar ligand binding properties for the WT-A₁AR and the Gly-14 → Thr-14 constructs were compared, markedly increased affinity for agonists was seen for the mutant receptor, and when the Pro-25 → Leu-25 construct was examined, the affinity for agonists fell (Tables I and 2).

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

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2 On WWW site: http://swift.embl-heidelberg.de/7tm/.
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All values are means of three to six separate studies per construct. S.E. values are given when there are three or more studies per construct, *, p < 0.05 by analysis of variance with Bonferroni post-test comparison versus wild type A1AR.

| Receptor construct | \[^{[H]}]CCPA | \[^{[H]}]DPCPX |
|--------------------|---------------|---------------|
|                    | \(K_d\) | \(B_{max}\) | Change (from WT) | \(K_d\) | \(B_{max}\) | Change (from WT) |
| WT A1AR            | 0.6 ± 0.15  | 550 ± 62     |               | 0.7 ± 0.2 | 565 ± 75  |               |
| A1AR → Ala-AR      | 0.6 ± 0.15  | 550 ± 62     | 0.011         | 0.8 ± 0.3 | 650 ± 34  | 1.1           |
| Gly14 → Thr14      | 0.007 ± 0.02* | 550 ± 62     | 0.011         | 0.8 ± 0.3 | 650 ± 34  | 1.1           |
| Pro25 → Leu25      | 1.8 ± 0.2*  | 230 ± 80     | 3.0           | 0.7 ± 0.2 | 346 ± 76  | 1.0           |
| Ile31 → Cys31      | 0.7 ± 0.1   | 458 ± 34     | 1.2           | 0.8 ± 0.2 | 546 ± 25  | 1.1           |
| Leu88 → Phe88      | 0.6 ± 0.1   | 568 ± 54     | 1.0           | 0.7 ± 0.4 | 498 ± 66  | 1.0           |
| Met87 → Phe87      | 0.7 ± 0.2   | 412 ± 23     | 1.2           | 0.8 ± 0.2 | 426 ± 21  | 1.1           |
| Ala125 → Lys125    | 0.7 ± 0.1   | 568 ± 81     | 1.2           | 0.7 ± 0.2 | 512 ± 85  | 1.0           |
| Phe144 → Leu144    | 0.6 ± 0.2   | 396 ± 43     | 1.0           | 0.8 ± 0.2 | 456 ± 84  | 1.1           |

Values are means of three or more separate studies per drug in which samples were tested in quadruplicate in each study in side-by-side studies with the wild-type human A1AR. *, p < 0.05 by analysis of variance with Bonferroni post-test comparison versus WT-A1AR.

| Drug         | Gly → Thr14 | Pro → Leu25 | Pro → Phe86 |
|--------------|-------------|-------------|-------------|
| NECA         | 7.0 ± 4.3 E-7 | 1.5 ± 2.2 E-5* | >1 E-5*     |
| Change from WT | 0.13        | 2.9         | >10         |
| CADO         | 6.0 ± 2.7 E-9* | 2.2 ± 3.3 E-5 | 6.2 ± 2.3 E-5* |
| Change from WT | .001       | 3.9         | 111         |
| R-PIA        | 2.7 ± 0.9 E-9* | 1.3 ± 0.4 E-6 | 4.4 ± 1.2 E-5* |
| Change from WT | 0.07        | 0.37        | 118         |
| CPA          | 1.2 ± 1.2 E-9* | 7.9 ± 2.6 E-6* | 1.27 ± 1.3 E-5* |
| Change from WT | .009       | 92          | 1.3 ± 1.2 E-7 |
| DPCPX        | 4.3 ± 1.2 E-9 | 3.3 ± 1.3 E-9 | 1.6 ± 0.3 E-9 |
| Change from WT | 1.5         | 1.5         | 0.7         |

DISCUSSION

Studies of A1AR-ligand interactions have largely focused on the importance of sites in TMs 6–7 and have been used to generate models of adenosine-A1AR interactions (4–6). In these models, it is suggested that the ribose group interacts with TM7 and the adenine group interacts with TMs 6 and 7 (4–6). Based on the results of the site-directed mutagenesis studies presented in this report, a revised model of ligand-A1AR interactions is proposed in which the adenine group interacts with TM3, and the ribose group interacts with TMs 3 and 7.

Modifications present on the \(N^6\) adenosine position determine whether a ligand will be selective for A1ARs (8). Foremost in identifying potential residues that can interact with the \(N^6\) position is consideration of chimeric receptor studies showing that TMs 1–4 confer the ligand binding properties of \(A_2\)ARs (7). Thus, it is very likely that the \(N^6\) binding site will be located within TMs 1–4. Of the sites that we have tested, only mutations of Leu-88, Thr-91, or Gln-92 resulted in the differential observed for the WT-A1AR, the affinity of each compound for the Ala-92 construct was markedly reduced. However, reductions in affinity for NECA and CADO were greater than reductions in affinity for CPA or R-PIA. When competition studies were performed using the compound N-0840, which is structurally similar to CPA but lacks a ribose group, the Ala-92 construct had markedly reduced affinity for the ligand compared with the WT-A1AR.

**Experiment 3, Additional Site-directed Mutagenesis Studies in TM3**—Because the above studies suggest that Gln-92 interacts with the adenine group, we next examined the role of other amino acids within TM3. First, we performed additional A1/A2A transposition studies of Leu-88, Thr-91, or Gln-92. Conversion of Val-87 to Ala-87, more distant from the cyclopentyl group than Leu-88, did not alter ligand binding properties. However, after conversion of Pro-86 to Phe-86, the binding of \(N^6\)-substituted ligands (CPA, R-PIA) to the mutant construct was reduced more than 10-fold (Table II).

Next, we tested the roles of Val-87, Leu-88, and Thr-91 in TM3 by converting these sites to alanine residues. Following conversion of Val-87 to Ala-87, no changes in ligand binding characteristics were seen (Table III). However, after Leu-88 or Thr-91 was converted to Ala, marked reductions in the affinity for agonists were observed (Table III). Competition studies were next performed using the compound N-0840. Suggesting that Thr-91 interacts with the adenine group, this construct had nearly 100-fold reduced affinity for N-0840.

**Experiment 4, Computer Modeling**—Considering the above results suggesting that Thr-91 and Gln-92 influence adenine binding, molecular modeling of CPA-A1AR interactions was performed based on the structure of rhodopsin (16). The results of computer modeling experiments are illustrated in Fig. 2. In Fig. 2A, the upper part of the purine ring and the \(N^6\)-substituent of CPA are shown interacting with residues on TM3 that were mutated in the present study (Thr-91 and Gln-92). Fig. 2B represents the same interaction shown from a different angle. Because of the helical nature of TM3, Pro-86 cannot be brought to CPA if Thr-91 and Gln-92 interact with CPA in a direct way (see also Fig. 2). Leu-88, however, is close to the cyclopentyl group of CPA, in line with its more prominent influence on the binding of \(N^6\)-substituted agonists (CPA and R-PIA) than of NECA and CADO, both agonists without \(N^6\)-substituents. Val-87, more distant from the cyclopentyl group than Leu-88, does not influence binding.

Positioned in the manner shown, CPA will also interact with TM7, which is highlighted in Fig. 2C. The ribose moiety is close to Thr-277 and His-278 and also to Ser-94 (TM3), which are all amino acids shown to influence ligand binding (5, 6).
reduction in the affinity of N6-substituted and non-substituted ligands, suggesting that Leu-88, Thr-91, and Gln-92 interact with the N6 substituents. Other investigators have also observed differential reduction in the affinity of N6-substituted (R-PIA) and non-substituted ligands (NECA) when the binding characteristics were compared for Thr-277 mutations (6). However, this reflects differences in binding of ribose substituents, not N6 substituents (6).

When we modified sites in TM1, we found that conversion of Gly-14 to Thr-14 resulted in increased affinity for agonists. In contrast, modification of Glu-16 in A1ARs and Glu-11 in A2AARs has been shown to result in decreased agonist affinity (11, 17). To date, direct interactions between small molecule ligands and sites in TM1 have yet to be demonstrated (18, 19). However, because molecular modeling studies suggest that TM1 is juxtaposed with TM7 (16, 18, 19), it is possible that TM1 mutations indirectly influence ribose-TM7 interactions. Although less likely, we also recognize the possibility that ribose-hydroxy groups may interact with polar TM1 sites.

Adenosine has several sites that can potentially interact with receptor amino acids (8). The adenine group contains five nitrogen atoms (N1, N2, N3, N7, and N9) that can interact with receptor sites, whereas the ribose moiety contains three hydroxyl groups (2', 3', 5') (8). Within the adenine group, removal of either of the N6, N7, and N9 nitrogen atoms results in more than a 1000-fold loss in affinity for A1ARs (8). Removal of the N7 and N9 nitrogen molecules results in 10- and 100-fold reductions in affinity for A1ARs, respectively (8). The three ribose-hydroxyl groups also are very important for binding, as removal of these groups results in significant reduction in the affinity for A1ARs (8).

Table III

| Drug     | Val → Ala67 | Leu → Ala68 | Thr → Ala69 | Gln → Ala72 | WT A1AR M |
|----------|-------------|-------------|-------------|-------------|-----------|
| NECA     | 4.2 ± 4.3 E-6 | 1.5 ± 0.3 E-4* | 7.7 E-4* | >1 E-4* | >1 E-4* |
| Change from WT | 0.82 | 29.4 | 150 | >200 | 5.1 ± 2.1 E-6 |
| CADO     | 1.3 ± 29 E-6 | 1.1 ± 0.4 E-6 | >1 E-4* | >1 E-4* | 1.0 ± 2.2 E-6 |
| Change from WT | 1.3 | 41 | >100 | >180 | 200 |
| R-PIA    | 2.6 ± 1.1 E-7 | 1.8 ± 0.4 E-4* | 7.2 ± 1.2 E-5* | 2.6 ± 0.7 E-5* | 3.6 ± 2.4 E-7 |
| Change from WT | 0.72 | 50 | 72 | 72 | 72 |
| CPA      | 5.5 ± 1.0 E-7 | 4.2 ± 2.6 E-5* | 9.7 ± 3.3 E-6* | 1.3 ± 1.2 E-5* | 5.6 ± 3.2 E-7 |
| Change from WT | 1.0 | 75 | 17 | 23 | 23 |
| N 0840   | 2.3 ± 1.2 E-6 | >1 E-4* | >100 | >100 | 7.5 ± 1.2 E-7 |
| Change from WT | 3.0 | >100 | >100 | >100 | >100 |

Fig. 2. Computer modeling of CPA-A1AR interactions. A and B, CPA interactions with TM3 shown from two different perspectives. C, CPA-ribose interactions with TM3 (left helix) and TM7 (right helix) residues.

Previous models of adenosine-A1AR interactions have been guided by site-directed mutagenesis studies of sites in TM3–7 (14). Considering the possible importance of His-250 in TM6 and His-278 in TM7, IJzerman and co-workers (14) proposed that the 2' and 3'-hydroxyl groups of CPA interact with His-278, and the N6 position interacts with His-250 in TM6. However, the primary amino acid sequence is very similar between A1 and A2AARs in this putative N6 binding region (14), making it difficult for this model to account for the considerably different binding properties of A1AR and A2AARs.

In the past, models for the adenosine A1, A2A, and A3AR receptor have been based on the structural template of bacteriorhodopsin (14, 20, 21). Since those studies, the structure of mammalian rhodopsin has been studied in greater detail (16), revealing similarity to the structure of bacteriorhodopsin. The relative positions of the TMs 3 and 7 in rhodopsin, however, are closer to each other than in bacteriorhodopsin (14, 20, 21). Since those studies, the structure of mammalian rhodopsin has been studied in greater detail (16), revealing similarity to the structure of bacteriorhodopsin. The relative positions of the TMs 3 and 7 in rhodopsin, however, are closer to each other than in bacteriorhodopsin (16). Considering the importance of sites in TM3 and TM7 on ligand-A1AR interactions shown in these and other studies (4–6), we therefore decided to generate a rhodopsin-based model for the human A1AR. As shown in Fig. 2C, TMs 3 and 7 are in close proximity in this A1 AR model, particularly where the ribose moiety of CPA is suggested to bind to Ser-94, Thr-277, and His-278, which are residues that are essential for agonist binding (6, 11).

Our model also suggests that the adenine group interacts with TM3. There is considerable support for this notion. First, mutation of residues in the human adenosine A1AR sites that are equivalent to Thr-91 and Gln-92 have been shown to affect ligand binding (13). Second, photoaffinity labeling studies us-
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ing an antagonist compound show that adenosinergic compounds interact with TM3 (22). Third, mutation of sites in TM3 alter the binding of the antagonist N-0840, which can be regarded as CPA without the ribose moiety (8). Structure-activity relationships for N6-substituted adenines like N-0848 are quite similar to those of N6-substituted adenosines (23), indicating that the N6-substituents of both adenosine agonists and adenosine antagonists coincide and occupy the same binding site. The compound N-0861, the norbornanyl variant of N-0840, also is very selective for A1, supporting the notion that the N6-substituents of both adenosine agonists and adenosine antagonists coincide and occupy the same binding site. The Pro-86 → Phe-86 mutation also induced broad decreases in the affinities of all compounds studied. However, our model suggests that this is an indirect effect, as Pro-86 is quite distant from CPA. Thus, it is possible that Pro-86 alters the conformation of TM1 in A1ARs to favor the binding of N6-substituents to A1ARs.

We recognize that our model does not yet accommodate the role of other sites that may influence the conformational state of A1ARs and indirectly influence adenosine-A1AR interactions. As mentioned above, modification of sites in TM1 of A1ARs (Thr-14, Glu-16) and A2AARs (Glu-13) induces broad changes in the affinity for agonists, whereas Asp-55 in TM2 of A1ARs mediates allosteric effects of sodium ions on ligand binding (11). Sites in the second extracellular loop also may influence adenosine-AR interactions (25). Considering the large number of potential interaction sites in the adenosine molecule (8), it is therefore likely that adenosine ligand-receptor interactions will be quite complex. For the present, our revised model of CPA-A1AR interactions, now provides a conceptual framework for explaining the role of TM3 in ligand binding and A1AR ligand selectivity.

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