THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 274, No. 6, Issue of February 5, pp. 3617–3621, 1999

Printed in U.S.A.

Identification of the Adenine Binding Site of the Human A_1 Adenosine Receptor*

(Received for publication, March 20, 1998, and in revised form September 20, 1998)

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To provide new insights into ligand-A_1 adenosine receptor (A_1AR) interactions, site-directed mutagenesis was used to test the role of several residues in the first four transmembrane domains of the human A_1AR. First, we replaced eight unique A_1AR residues with amino acids present at corresponding transmembrane (TM) positions of A_{2A}ARs. 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 N^6-substituted agonists was reduced, and binding of ligands without N^6 substituents was eliminated. When Leu-88 was converted to Ala-88, the binding of ligands with N^6 substituents was reduced to a greater extent than ligands without N^6 substituents. Following conversion of Pro-86 to Phe-86, the affinity for N^6-substituted agonists was lost, and the affinity for ligands without N^6 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 A_1AR selective compounds. Using computer modeling based on the structure of rhodopsin, a revised model of adenosine-A_1AR interactions is proposed with the N^6-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 A_1 adenosine receptors (A_1ARs)[1] (1–3). Because activation of A_1ARs has considerable therapeutic importance in treating clinical conditions (1–3), there is considerable interest in deciphering how adenosine interacts with A_1ARs.

A_1ARs are G protein-coupled receptors that have seven transmembrane (TM) spanning domains (Fig. 1) (1–3). Initial structure-function studies of A_1ARs 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 270 was found to account for species-related differences in affinity for A_1-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 A_1/A_{2A}ARs have shown that TMs 1–4 of A_1ARs contain the sites that confer the ligand binding characteristics of an A_1AR (7). Because modification of the N^6 adenine position confers A_1AR selectivity of adenosinergic compounds (8), this observation strongly suggests that the N^6-adenine position interacts with sites within TMs 1–4 (7). Within the first four TM domains of the A_1AR, 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 A_1ARs is not at hand.

To provide additional insights into how ligands interact with A_1ARs, 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 A_1ARs with amino acids present at corresponding positions in A_{2A}ARs. 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-A_1AR interactions is proposed.

EXPERIMENTAL PROCEDURES

cDNAs—The cDNA encoding the full-length human A_1AR was provided by Dr. S. M. Reppert (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 A_1AR. Another set of oligonucleotide primer pairs (primers C and D) was designed to generate a 3′ fragment of the A_1AR 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 A_1AR using flanking primers (A and D).

Flanking PCR primers contained *Hin*dIII (A primers) or *Xba*I (D primers) restriction endonuclease sites at the ends. After fusion reactions, PCR products were digested with *Hin*dIII and *Xba*I and were subcloned into the mammalian expression vector pcDNA3 (Invitrogen; San Diego, CA). Mutant receptors were then sequenced.

Acute Transfections—Receptor cDNA expression was characterized

* This work was supported by National Institutes of Health Grant R01 NS326224. 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.

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[1] The abbreviations used are: AR, adenosine receptor; TM, transmembrane; PCR, polymerase chain reaction; CPA, N^6-cyclopentyladenosine; CCPA, 2-chloro-N^6-cyclopentyladenosine; CADO, 2-chloroadenosine; R-PIA, N^6-(phenylisopropyl)adenosine; DPCPX, 1,3-dipropyl-8-cyclopentylxanthine; NECA, 5′-*N*-ethylcarboxamidoadenosine; N-0840, N^6-cyclopentyl-9-methyladenine; WT, wild-type.

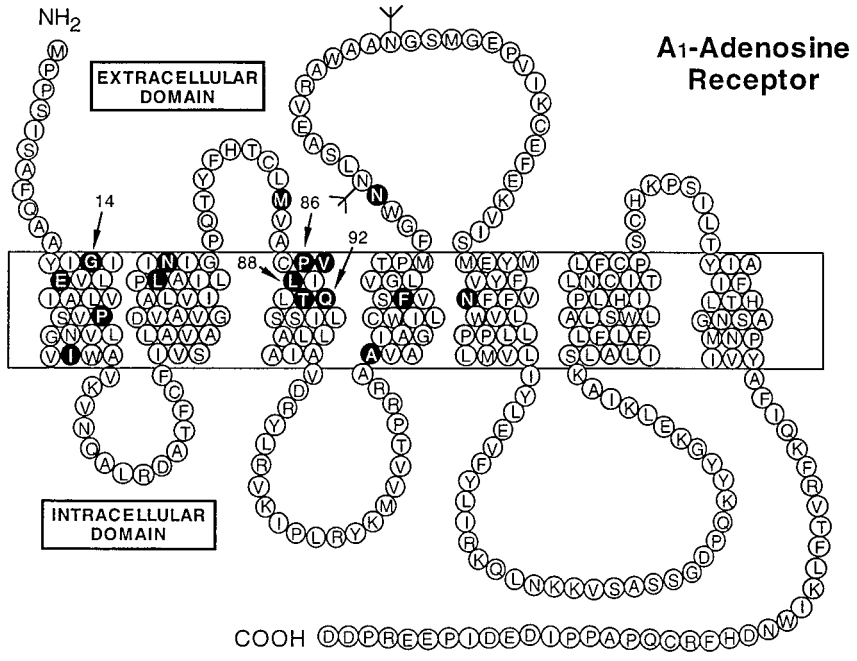

FIG. 1. **Schematic representation of the human A_1AR.** Sites that were mutated in this report are represented by *black circles*.

using COS-7 cells as described (12). COS cells were grown as monolayers in Dulbecco's minimal essential medium (DMEM; Life Technologies, Inc.) supplemented with 10% fetal calf serum, penicillin (100 units/ml), and streptomycin (100 g/ml), in 5% CO_2 at 37 °C. Cells were acutely transfected using the DEAE-dextran method. 10-cm plates were individually transfected with 5–10 μg of DNA, or were sham transfected. At 48–72 h after transfection, cells were tested by radioreceptor assay. Under those conditions, we found that there was very little evidence of receptor-G protein coupling (7), similar to that as reported by others (5).

Radioreceptor Assays—Radioligand binding studies were performed using intact cells as described (7, 11). The radioligands used were [^{3}H]CCPA (NEN Life Science Products, Boston, MA; specific activity, 33 Ci/mmol) and [^{3}H]DPCPX (NEN Life Science Products; specific activity, 100 Ci/mmol). All determinations were done in quadruplicate. When constructs with different levels of expression were compared, we adjusted tissue per tube so that amounts of specific binding per tube were similar among the different constructs.

Molecular Modeling—A model for the human A_{2A}AR deposited in the Protein Data Bank (entry 1mmh) by Van Rhee and co-workers (13) was used for construction of our model of the human A_1AR. First, the helical parts of the two sequences retrieved from the GPCR DataBase Project[2] were aligned as described by IJzerman *et al.* (14) for the canine A_1 and A_{2A} receptors. Subsequently, all amino acid differences between the canine and human A_1ARs were identified and converted to human A_1AR motifs. NECA, the ligand present in the A_{2A} receptor model, was changed to CPA, which is the reference agonist for A_1ARs. Steric clashes between amino acid side chains and CPA were removed by rotation of side chains only. Guided by the results from the mutagenesis studies presented in this report, we included Leu-88, Thr-91, and Gln-92 in a putative N^6-binding region. As a consequence, Ser-94, Thr-277, and His-278 were found to be close to the ribose moiety of CPA. After these manipulations, a short minimization procedure with default parameters was followed in which all side chains within 4 Å of CPA were relaxed. All calculations were performed with the software package QUANTA 96 (MSI, Waltham, MA, USA) running on a Silicon Graphics Indigo O2 workstation.

Statistical Analysis—Saturation and competition binding data were analyzed by computer using an iterative nonlinear regression program (15). Comparisons among multiple groups were performed by one-way analysis of variance, with post-test comparison among groups performed by the Bonferroni method. Comparisons between paired groups were performed by the paired *t* test. The InStat, Vers. 3, statistics program (GraphPad; San Diego, CA) was used for statistical computations.

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

[2] On WWW site: http://swift.embl-heidelberg.de/7tm/.

RESULTS

Experiment 1, A_1ARs/A_{2A}AR 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 A_1ARs, differences in the amino acid sequences of A_1 and A_{2A}ARs were identified. First, the amino acid sequences of all cloned A_1ARs and A_{2A}ARs 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 A_1ARs and A_{2A}ARs were then identified. Using site-directed mutagenesis, human A_1AR residues were replaced by the corresponding amino acids of A_{2A}ARs. Saturation studies were then performed using [^{3}H]CCPA or [^{3}H]DPCPX (Table I). Competition studies were next performed using a fixed dose of [^{3}H]DPCPX and graded doses of DPCPX or CPA and several other compounds (Table II). These studies revealed similar ligand binding properties for the WT-A_1AR and Cys-31, Phe-65, Phe-82, Lys-125, and Leu-144 mutant A_1AR constructs (Tables 1 and 2). In contrast, when the WT-A_1AR 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 1 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 A_1ARs domains failed to identify a site that interacts with the adenine N^6 position (11). Therefore, we examined the role of carboxamide sites within TMs 1–4 (Asn-70, Gln-92). These amino acids contain oxygen and nitrogen atoms that may form hydrogen bonds with similar atoms in adenosine. Ala-70 and Ala-92 constructs were thus generated and tested. Competition studies were then performed using a fixed dose of [^{3}H]DPCPX and graded doses of CPA or DPCPX. When the Ala-70 construct was tested, no differences in affinity for ligands were seen in comparison with studies of the WT-A_1AR. In contrast, the Ala-92 construct had markedly reduced affinity for CPA.

Next, to assess potential regions of the adenosine molecule that could interact with Gln-92, competition studies were performed using compounds with (CPA, R-PIA) or without (NECA, 2 chloroadenosine) N^6 substitutions. In comparison with that

TABLE I

Binding affinities for [^{3}H]CCPA and [^{3}H]DPCPX in A_1/A_{2A} transposition studies

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 A_1AR.

Receptor construct	[^{3}H]CCPA			[^{3}H]DPCPX		
	K_d	B_{max}	Change (from WT)	K_d	B_{max}	Change (from WT)
	nM	*fmol/mg*		*nM*	*fmol/mg*	
WT A_1AR	0.6 ± 0.15	550 ± 62		0.7 ± 0.2	565 ± 75	
$A_1AR \rightarrow A_{2A}AR$						
$Gly^{14} \rightarrow Thr^{14}$	0.007 ± 0.02*	550 ± 62	0.011	0.8 ± 0.3	650 ± 34	1.1
$Pro^{25} \rightarrow Leu^{25}$	1.8 ± 0.2*	230 ± 80	3.0	0.7 ± 0.2	346 ± 76	1.0
$Ile^{31} \rightarrow Cys^{31}$	0.7 ± 0.1	458 ± 34	1.2	0.8 ± 0.2	546 ± 25	1.1
$Leu^{65} \rightarrow Phe^{65}$	0.6 ± 0.1	568 ± 54	1.0	0.7 ± 0.4	498 ± 66	1.0
$Met^{82} \rightarrow Phe^{82}$	0.7 ± 0.2	412 ± 23	1.2	0.8 ± 0.2	426 ± 21	1.1
$Ala^{125} \rightarrow Lys^{125}$	0.7 ± 0.1	569 ± 81	1.2	0.7 ± 0.2	512 ± 85	1.0
$Phe^{144} \rightarrow Leu^{144}$	0.6 ± 0.2	396 ± 43	1.0	0.8 ± 0.2	456 ± 84	1.1

TABLE II

K_I values from competition of [^{3}H]DPCPX binding in A_1/A_{2A} transposition studies

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 A_1AR. *, $p < 0.05$ by analysis of variance with Bonferroni post-test comparison *versus* WT-A_1AR.

Drug	K_I values			
	$Gly \rightarrow Thr^{14}$	$Pro \rightarrow Leu^{25}$	$Pro \rightarrow Phe^{86}$	WT A_1AR
		M		
NECA	7.0 ± 4.3 E-7	1.5 ± 2.2 E-5*	>1 E-5*	5.1 ± 2.1 E-6
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*	5.6 ± 3.2 E-6
Change from WT	.001	3.9	11.1	
R-PIA	2.7 ± 0.9 E-9*	1.3 ± 0.4 E-6	4.4 ± 1.2 E-5*	3.7 ± 0.8 E-7
Change from WT	0.07	3.5	118	
CPA	1.2 ± 1.2 E-9*	7.9 ± 2.6 E-6*	1.27 ± 1.3 E-5*	1.3 ± 1.2 E-7
Change from WT	.009	60	92	
DPCPX	4.3 ± 1.2 E-9	3.3 ± 1.3 E-9	1.6 ± 0.3 E-9	2.2 ± 0.2 E-9
Change from WT	1.9	1.5	0.7	

observed for the WT-A_1AR, 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 as compared with the WT-A_1AR.

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 A_1/A_{2A}AR transposition studies at sites in TM3 (Pro-86 → Phe-86, Leu-96 → Phe-96). Competition studies showed that conversion of Leu-96 to Phe-96 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-A_1AR interactions was performed based on the structure of rhodopsin (16). The results of computer modeling experiments are illustrated in Fig. 2. In Fig. 2*A*, 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. 2*B* represents the same interaction shown from a different angle. Because of the helical nature of TM3, Pro-86 cannot be brought close 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. 2*C*. 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).

DISCUSSION

Studies of A_1AR-ligand interactions have largely focused on the importance of sites in TMs 6–7 and have been used to generate models of adenosine-A_1AR 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-A_1AR 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 adenine position determine whether a ligand will be selective for A_1ARs (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_1ARs (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

TABLE III

K_I values from competition of [^{3}H]DPCPX binding in TM3 site-directed mutagenesis studies

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 A_1AR. *, $p < 0.05$ by analysis of variance with Bonferroni post-test comparison *versus* wild type A_1AR.

Drug	K_I values				
	Val → Ala^{87}	Leu → Ala^{88}	Thr → Ala^{91}	Gln → Ala^{92}	WT A_1AR
			M		
NECA	4.2 ± 4.3 E-6	1.5 ± 0.3 E-4*	7.7 E-4*	>1 E-4*	5.1 ± 2.1 E-6
Change from WT	0.82	29.4	150	>200	
CADO	1.3 ± 2.9 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	
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	500	200	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	
N 0840	2.3 ± 1.2 E-6	>1 E-4*	>1 E-4*	>1 E-4*	7.5 ± 1.2 E-7
Change from WT	3.0	>100	>100	>100	

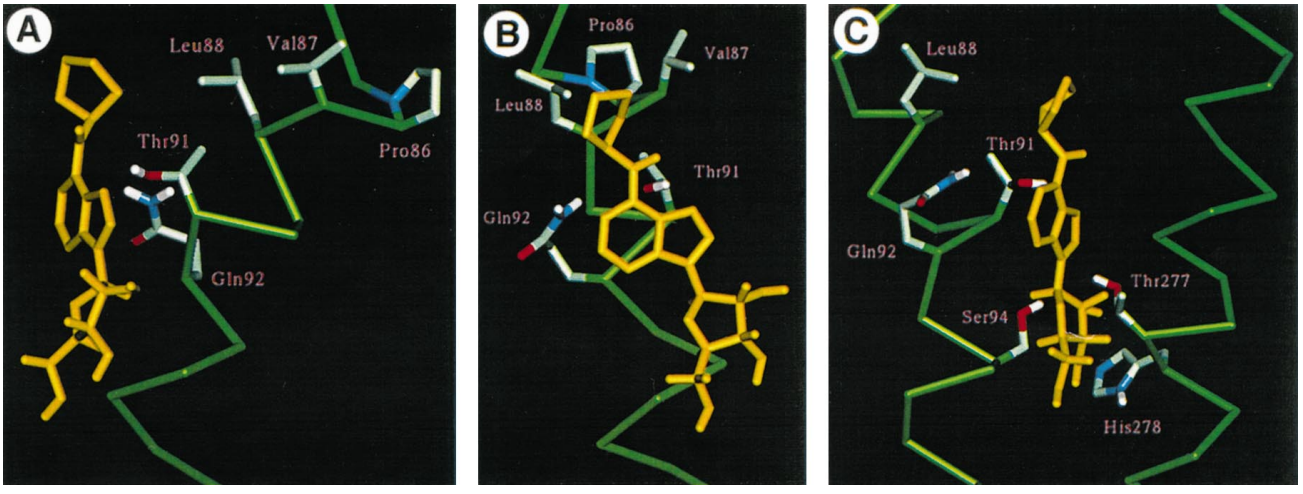

FIG. 2. **Computer modeling of CPA-A1AR interactions.** *A* and *B*, CPA interactions with TM3 shown from two different perspectives. *C*, CPA-ribose interactions with TM3 (*left helix*) and TM7 (*right helix*) residues.

reduction in the affinity of N^6-substituted and non-substituted ligands, suggesting that Leu-88, Thr-91, and Gln-92 interact with the N^6 substituents. Other investigators have also observed differential reduction in the affinity of N^6-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 N^6 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 A_1ARs and Glu-11 in A_{2A}ARs 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 (N^1, N^3, N^6, N^7, and N^9) 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 N^6, N^7, and N^9 nitrogen atoms results in more than a 1000-fold loss in affinity for A_1ARs (8). Removal of the N^1 and N^3 nitrogen molecules results in 10- and 100-fold reductions in affinity for A_1ARs, 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 A_1ARs (8).

Previous models of adenosine-A_1AR interactions have been guided by site-directed mutagenesis studies of sites in TMs 5–7 (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 N^6 position interacts with His-250 in TM6. However, the primary amino acid sequence is very similar between A_1 and A_{2A}ARs in this putative N^6 binding region (14), making it difficult for this model to account for the considerably different binding properties of A_1AR and A_{2A}ARs.

In the past, models for the adenosine A_1, A_{2A}, and A_3ARs 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 (16). Considering the importance of sites in TM3 and TM7 on ligand-A_1AR interactions shown in these and other studies (4–6), we therefore decided to generate a rhodopsin-based model for the human A_1AR. As shown in Fig. 2*C*, TMs 3 and 7 are in close proximity in this A_1AR 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 A_{2A}AR sites that are equivalent to Thr-91 and Gln-92 have been shown to affect ligand binding (13). Second, photoaffinity labeling studies us-

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 N^6-substituted adenines like N-0848 are quite similar to those of N^6-substituted adenosines (23), indicating that the N^6-substituents of both adenosine agonists and adenine antagonists coincide and occupy the same binding site. The compound N-0861, the norbornanyl variant of N-0840, also is very selective for A_1, supporting the notion that the N^6-substituents of N-0840 and CPA coincide (24).

Based on our model, the two aliphatic, lipophilic residues Val-87 (the equivalent of the aspartate residue important for binding in many biogenic amine receptors) and Leu-88 could have a favorable interaction with the N^6-cyclopentyl substituent in CPA. However, mutation studies showed that only Leu-88 influences the binding of N^6-substituted agonists, with the affinity for R-RIA reduced by the greatest extent. Because R-PIA has the longest and most hydrophobic N^6-side chains of the ligands tested (8), these observations support the notion that Leu-88 interacts with hydrophobic N^6-substituents. Additional support for this possibility comes from observations that mutations at Thr-91 and Gln-92 affected CPA binding less than NECA or CADO binding. Interaction of N^6-substituents with Leu-88 may thus facilitate agonist in the absence of sites that interact with the nitrogen ring, possibly at the N^6 nitrogen.

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 A_1ARs to favor the binding of N^6-substituents to A_1ARs.

We recognize that our model does not yet accommodate the role of other sites that may influence the conformational state of A_1ARs and indirectly influence adenosine-A_1AR interactions. As mentioned above, modification of sites in TM1 of A_1ARs (Thr-14, Glu-16) and A_{2A}ARs (Glu-13) induces broad changes in the affinity for agonists, whereas Asp-55 in TM2 of A_1ARs 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-A_1AR interactions, now provides a conceptual framework for explaining the role of TM3 in ligand binding and A_1AR ligand selectivity.

Acknowledgment—We thank David Danraj for assistance in some of these studies.

REFERENCES

1. Shryock, J. C., and Belardinelli, L. (1997) *Am. J. Cardiol.* **79,** 2–10
2. Brundege, J. M., and Dunwiddie, T. V. (1997) *Adv. Pharmacol.* **39,** 353–391
3. Palmer, T. M., and Stiles, G. L. (1995) *Neuropharmacology* **34,** 683–694
4. Olah, M. E., Ren, H., Ostrowski, J., Jacobson, K. A., and Stiles, G. L. (1992) *J. Biol. Chem.* **267,** 10764–10770
5. Tucker, A. L., Robeva, A. S., Taylor, H. E., Holeton, D., Bockner, M., Lynch, K. R., and Linden, J. (1994) *J. Biol. Chem.* **269,** 27900–27906
6. Townsend-Nicholson, A., and Schofield, P. R. (1994) *J. Biol. Chem.* **269,** 2373–2376
7. Rivkees, S. A., Lasbury, M. E., and Barbhaiya, H. (1995) *J. Biol. Chem.* **270,** 20485–20490
8. Tivedi, B. K., Bridges, A. J., and Bruns, R. F. (1990) in *Adenosine and Adenosine Receptors* (Williams, M., ed) pp. 57–103, Humana Press, Clifton, NJ
9. Rivkees, S. A., Lasbury, M. E., Stiles, G. L., Henergariu, O., and Vance, G. (1995) *Endocrine* **3,** 623–629
10. Ho, S. N., Hunt, H. D., Horton, R. M., Pullen, J. K., and Pease, L. R. (1989) *Gene* (*Amst.*) **77,** 51–59
11. Barbhaiya, H., McClain, R., Ijzerman, A., and Rivkees, S. A. (1996) *Mol. Pharmacol.* **50,** 1635–1642
12. Cullen, B. R. (1987) *Methods Enzymol.* **152,** 684–704
13. Jiang, Q., van Rhee, A. M., Kim, J., Yehle, S., Wess, J., and Jacobson, K. A. (1996) *Mol. Pharmacol.* **50,** 512–521
14. IJzerman, A. P., van Galen, P. J., and Jacobson, K. A. (1992) *Drug Des. Discov.* **9,** 49–67
15. McPherson, G. A. (1985) *J. Pharmacol. Methods* **14,** 213–228
16. Unger, V. M., Hargrave, P. A., Baldwin, J. M., and Schertler, G. F. (1997) *Nature* **389,** 203–206
17. IJzerman, A. P., von Frijtag Drabbe Kunzel, J. K., Kim, J., Jiang, Q., and Jacobson, K. A. (1996) *Eur. J. Pharmacol.* **310,** 269–272
18. Baldwin, J. M. (1993) *EMBO J.* **12,** 1693–1703
19. Baldwin, J. M. (1994) *Curr. Opin. Cell Biol.* **6,** 180–190
20. IJzerman, A. P., van der Wenden, E. M., van Galen, P. J., and Jacobson, K. A. (1994) *Eur. J. Pharmacol.* **268,** 95–104
21. van Galen, P. J., van Bergen, A. H., Gallo-Rodriguez, C., Melman, N., Olah, M. E., IJzerman, A. P., Stiles, G. L., and Jacobson, K. A. (1994) *Mol. Pharmacol.* **45,** 1101–1111
22. Kennedy, A. P., Mangum, K. C., Linden, J., and Wells, J. N. (1996) *Mol. Pharmacol.* **50,** 789–798
23. Ukena, D., Padgett, W. L., Hong, O., Daly, J. W., Daly, D. T., and Olsson, R. A. (1987) *FEBS Lett.* **215,** 203–208
24. Shryock, J. C., Travagli, H. C., and Belardinelli, L. (1992) *J. Pharmacol. Exp. Ther.* **260,** 1292–1299
25. Olah, M. E., Jacobson, K. A., and Stiles, G. L. (1994) *J. Biol. Chem.* **269,** 24692–24698

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J. Biol. Chem. 1999, 274:3617-3621.
doi: 10.1074/jbc.274.6.3617