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Role of the second transmembrane domain of rat adenosine A_1 receptor in ligand–receptor interaction

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ABSTRACT

Initial mutagenesis studies exploring the ligand recognition model of A_1 adenosine receptor (A_1R) mainly focused on the residues in the 5th–7th transmembrane domains (TMs5–7). Little is known about the role of residues in TM2. To explore the importance of reserved hydrophobic region in TM2 of A_1R, we mutated the hydrophobic residues at positions 65 and 69 to hydrophilic residues (L65T, Leu-65 to Thr-65; I69T, Ile-69 to Thr-69; I69S, Ile-69 to Ser-69) to change the hydrophobicity at the outer end of TM2. Binding assays showed that the affinities of mutant receptors were significantly decreased for ribose group-containing agonists (2-chloro-N^6-cyclopentyladenosine (CCPA) and 5′-*N*-ethyl-carboxamidoadenosine (NECA)) but not for antagonists, N^6-cyclopentyl-9-methyladenine (N-0840), an adenine derivative lacking ribose group, and 8-cyclopentyl-1, 3-dipropylxanthine (DPCPX), a xanthine derivative. This observation suggests that the hydrophobic region at the outer end of TM2 may mediate the recognition of the ribose group of CCPA and NECA.

Abbreviations:
ADA, adenosine deaminase
A_1R, adenosine A_1 receptor
CCPA, 2-chloro-N^6-cyclopentyladenosine
CPA, N^6-cyclopentyladenosine
DMEM, Dulbecco's modified Eagle's medium
DPCPX, 8-cyclopentyl-1,3-dipropylxanthine
FCS, fetal calf serum
GPCR, G protein-coupled receptor
HA, hemagglutinin A
I, isoleucine
L, leucine
N-0840, N^6-cyclopentyl-9-methyladenine
NECA, 5′-*N*-ethyl-carboxamidoadenosine

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PCR, polymerase chain reaction
S, serine
T, threonine
TM, transmembrane domain
V, valine
WT, wild type

1. Introduction

The purine nucleoside adenosine is an important mediator of physiological processes in almost all organ systems. Adenosine is involved in immunological and inflammatory responses, respiratory regulation, the cardiovascular system, the kidney, various CNS-mediated events including sleep and neuroprotection, as well as central and peripheral pain processes. Adenosine exerts these physiological roles mainly by activation cell surface adenosine receptors. Adenosine receptors are members of the superfamily of G protein-coupled receptors (GPCRs). They are single polypeptide chains possessing seven hydrophobic transmembrane domain (TM)-spanning segments that couple to an effector molecule through a trimeric G protein complex. Up to now, four subtypes of adenosine receptors (A_1R, adenosine A_1 receptor; A_{2a}R, adenosine A_{2a} receptor; A_{2b}R, adenosine A_{2b} receptor; A_3R, adenosine A_3 receptor) have been identified [1]. Adenosine exerts the endogenous defensive effect against hypoxia damage and other forms of tissue stress partly through A_1R [2].

A_1R is highly expressed in brain cortex, cerebellum, hippocampus and dorsal horn of spinal cord. Coupling to pertussis toxin-sensitive G protein of G_i and G_o, the receptor activation is linked to the inhibition of adenylyl cyclase [3], modification of phospholipase C activity and/or phosphoinositide turnover [4,5] and hyperpolarisation in a manner independent of cAMP by inducing a potassium current via G protein-gated inwardly rectifying K^+ channels (GIRK) [6]. Similarly, activation of GIRK by A_1R will also block N-type Ca^{2+} channels [7].

The pharmacological and functional characterization of A_1R has been extensively studied, and the development of agonists and antagonists for A_1R has so far been directed by traditional medicinal chemistry. However, the structure–activity relationship of A_1R is not very clear. The availability of genetic information promises to facilitate understanding of the drug–receptor interaction leading to the rational design of a potentially therapeutically important class of drugs. A_1R, like the other GPCRs, is one integral membrane protein. It is not easy to crystallize and, hence, to obtain precise structure elucidation through X-ray diffraction. Molecular modeling may further rationalize observed interactions between the receptor and a ligand. Recently, some mutagenesis studies indicated some amino acids residues as being important for agonist or antagonist binding or both [8,9]. The cavity formed by the seven amphipathic alpha-helices was characterized by a rather distinct partition between hydrophobic and hydrophilic regions. Initial structure–function studies of A_1R mainly focused on amino acids within TM6 and TM7. His-278 in TM7 was identified as a site that interacted with both agonist and antagonist, whereas mutation of His-251 to Leu-251 in TM6 resulted in decreasing of antagonist affinity but not agonist affinity [10]. Within TM7, Ile-270 was found to account for species-related differences in affinity for A_1R selective drugs [11]. All studies reinforced the suggestion of the two distinct sites of interaction for agonist and antagonist. Adjacent to the histidine in TM7, the Thr-277 was found to mediate agonist but not antagonist binding. Moreover, Thr-277 interacted with the 5′ position of the adenosine ribose moiety, and also formed a probable molecular contact site with 5′ substitution found in 5′-*N*-ethyl-carboxamidoadenosine (NECA), an A_1R agonist [12]. Studies of an increasing number of point mutations led to further insight in the ligand binding sites. It was proposed that N^6-adenine position oriented toward the top of TM3 and the ribose group interacted with bottom half of TM3 and TM7 [13].

However, there has been little information about the role of residues in TM2 for the ligand–receptor interaction of A_1R. Based on the X-ray crystal structure of bovine rhodopsin, homology modeling showed these TMs might be spatially close [8,9]. Different species of A_1R showed highly hydrophobic conserved in TM2. To provide new insight into ligand–A_1R interactions, we examined the specificity contribution of two conserved hydrophobic residues at positions 65 and 69 at the outer end of TM2.

2. Materials and methods

2.1. Materials

The cDNA encoding the hemagglutinin A (HA) tagged-wild type (WT) rat A_1R inserted in the pcDNA3 eukaryotic expression vector (pcDNA3-HA-A_1R) was a kind gift from Dr. Christian Nanoff, University of Vienna, Austria. pcDNA3.1$^{(+)}$ was from Invitrogen (San Diego, CA, US). CCPA, NECA, DPCPX and N-0840 were purchased from Sigma (St. Louis, MO, US). Adenosine deaminase (ADA) was from Roche Biochemicals (Mannheim, Germany). [^{3}H]DPCPX (128.00 Ci/mmol) was purchased from Amersham Bioscience (Buckinghamshire, UK).

2.2. Generation of WT and mutant adenosine A_1 receptors

The rat WT A_1R cDNA was amplified from the pcDNA3-HA-A_1R plasmid using the following primers: A_1Rsense, 5′-CCCAAGCTTATGCCGCCCTACATCTCGGCCTTCC-3′; A_1Rantisense, 5′-CCGGAATTCCTAGTCCTCAGCTTTCTCCTCTGGG-3′. Mutant receptors were made by the polymerase chain reaction (PCR) overlap-extension method [14]. Primer pairs were designed to introduce desired mutation (L65T): L65Tsense, 5′-CCCACTGGCCATCCTTACAAACATTGGGCCACAGA-3′;

L65Tantisense, 5′-TCTGTGGCCCAATGTTTGTAAGGATGGC-CAGTGGG-3′. To generate the 5′ fragment of mutant receptor, oligonucleotide primers, A_1Rsense and L65Tantisense were used as pairs in the first PCR cycle. Similarly, A_1Rantisense and L65Tsense were used to generate the 3′ fragment of mutant receptor. L65Tsense and L65Tantisense were complementary primers, so the 5′ and 3′ fragments of the mutant receptor could anneal, and then extended to get the full length of the mutant receptor in the absence of any primers. Finally, the full length mutant receptor was amplified using A_1Rsense and A_1Rantisense as pairs in the second PCR cycle. I69T, I69S, and I69V mutant receptors were constructed using the same method. The primers used were as follows: I69Ssense, 5′-CCCACTGGCCATCCTTTCAAACATTGGGCCACAGA-3′; I69Santisense, 5′-TCTGTGGCCCAATGTTTGAAAGGATGGCCAGTGGG-3′; I69Vsense, 5′-CCCACTGGCCATCCTTGTAAACATTGGGCCA-CAGA-3′; I69Vantisense, 5′-TCTGTGGCCCAATGTTTACAAG-GATGGCCAGTGGG-3′; I69Tsense, 5′-CCCACTGGCCATCCTT-ACAAACATTGGGCCACAGA-3′; I69Tantisense, 5′-TCTGTGGC-CCAATGTTTGTAAGGATGGCCAGTGGG-3′.

All the WT and mutant A_1R PCR products were digested by *Hind*III and *Eco*RI, and then inserted into the pcDNA3.1$^{(+)}$ vectors digested by the same enzymes. WT and mutant receptors were checked by DNA sequencing.

2.3. Cell culture and transfection procedures

Human embryonic kidney 293 (HEK293) cell lines were cultured at 37 °C and 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS). Cells were seeded into 100 mm dishes and transiently transfected with 15 μg of DNA/dish using a modified calcium phosphate precipitation procedure as described previously [15,16].

2.4. Membrane preparation

Cells were harvested 48 h after transfection, and then washed twice with ice-cold PBS. The cell pellet was resuspended in hyponic buffer (5 mM Tris–Cl, 2 mM EDTA, pH 7.4, leupeptin 5 μg/ml, pepstatin A 5 μg/ml, aprotinin 5 μg/ml, PMSF 1 mM) and sonicated on ice. The homogenate was centrifuged at 960 × *g* for 10 min at 4 °C. The supernatant was recentrifuged twice at 40,000 × *g* for 30 min at 4 °C. Finally, the pellet was resuspended in the same buffer and stored at −80 °C. The protein concentration was determined by the BCA Kit (Pierce) as described previously [17,18].

2.5. [^{3}H]DPCPX binding assay

All binding assays were performed in triplicate using membranes or intact cells treated with 65 nM ADA for 30 min at 4 °C prior to binding [19]. Saturation binding assays were performed with increasing concentrations ranging from 0.05 to 6 nM of antagonist [^{3}H]DPCPX in 50 mM Tris–Cl buffer and 15–25 μg membrane protein in a total volume of 200 μl. Nonspecific binding was determined by adding a final concentration of 10 μM nonlabeled DPCPX. Competition binding assays were performed using 0.5 nM [^{3}H]DPCPX and different concentrations of compounds (CCPA, NECA and N-0840). The mixtures were incubated at 37 °C for 60 min. Binding reactions were terminated by rapid filtration through Whatman GF/B filters under reduced pressure using a MT-24 cell harvester. Filters were washed three times with 9 ml of ice-cold buffer. The radioactivity was counted with Beckman LS6500 liquid scintillation analyzer [17].

2.6. Statistical analysis

Binding parameters were calculated using Prism software (GraphPAD, San Diego, US). IC_{50} values obtained from competition curves were converted to K_i values using the Cheng Prusoff equation [20]. Data were expressed as means ± S.E.M. and analyzed by Student's *t*-test.

3. Results

Sequence alignments for selected transmembrane domains of four rat adenosine receptor subtypes, human and mouse A_1Rs were shown in Fig. 1B. The adenosine receptors exhibited high degree of identity between species in TM2. The mostly conserved residues in this domain especially at the position close to the second extracellular loop were hydrophobic. To determine the contribution of hydrophobic region of A_1R in this position to ligand binding, site-direct mutagenesis was used to change two hydrophobic residues (Leu-65 and Ile-69) to hydrophilic residues (Thr or Ser). Additionally, Ile-69 was changed to Val-69, another hydrophobic residue with less hydrophobic property. The two positions were pointed by arrows (Fig. 1A). WT A_1R, L65T, I69S, I69T and I69V mutant A_1R cDNAs were transiently expressed in HEK293 cells. Membranes or intact cells were used in equilibrium binding assays at 37 °C. The ligands used in our assays (shown in Fig. 2) were the A_1R-specific antagonists, DPCPX and N-0840, and agonists, CPA, CCPA and NECA. DPCPX is a xanthine derivative. CPA, CCPA and NECA are adenine nucleosides with ribose moiety. N-0840 is a N^6-cyclopentyladenine analogue but lacking the ribose moiety.

When the saturation assays in membrane preparations from cells transiently transfected with WT or mutant A_1R cDNAs were made using A_1R selective antagonist [^{3}H]DPCPX, the K_d and B_{max} values of WT and mutant A_1Rs were obtained (Table 1). The B_{max} values showed that the expression levels at the plasmic membrane were comparable for all constructs. The affinity for [^{3}H]DPCPX displayed by the WT A_1R was consistent with values obtained in binding assays of A_1Rs from several different species [21]. These studies revealed similar antagonist DPCPX binding properties for WT and mutant A_1Rs.

Competitive inhibition assays of A_1R agonist CCPA versus the [^{3}H]DPCPX were performed in intact cells. The K_i values of WT and mutant A_1Rs were shown in Table 1. Similarly, the K_i values of WT and mutant A_1Rs for NECA and N-0840 were obtained (Table 1). Figs. 3–5 showed the representative competition binding curves of WT and mutant A_1Rs for CCPA, NECA and N-0840. As shown in Fig. 3, mutations of L65T, I69T or I69S, significantly decreased the affinity for the selective agonist, CCPA, at least 10-fold, especially that of the I69S mutant (24.9-fold). Nevertheless, the I69V mutant resulted in modest (5.66-fold) but significant decrease of the affinity for

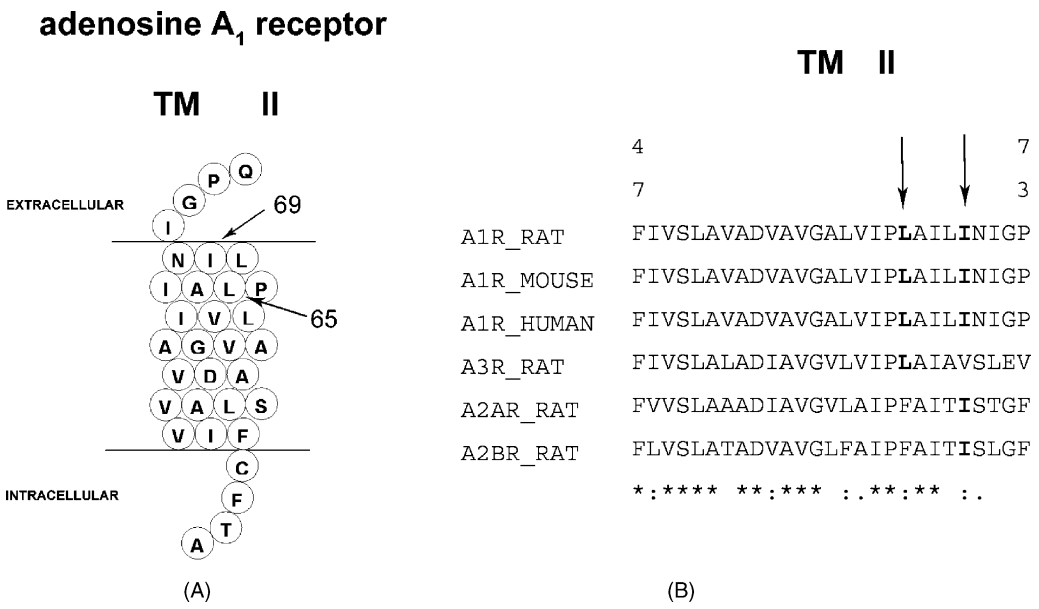

Fig. 1 – Schematic representation of TM2 of rat A_1R (A) and sequence alignments in TM2 of selected adenosine receptors (B). Sites that were mutated in this report are numbered (A) and pointed by arrows (B).

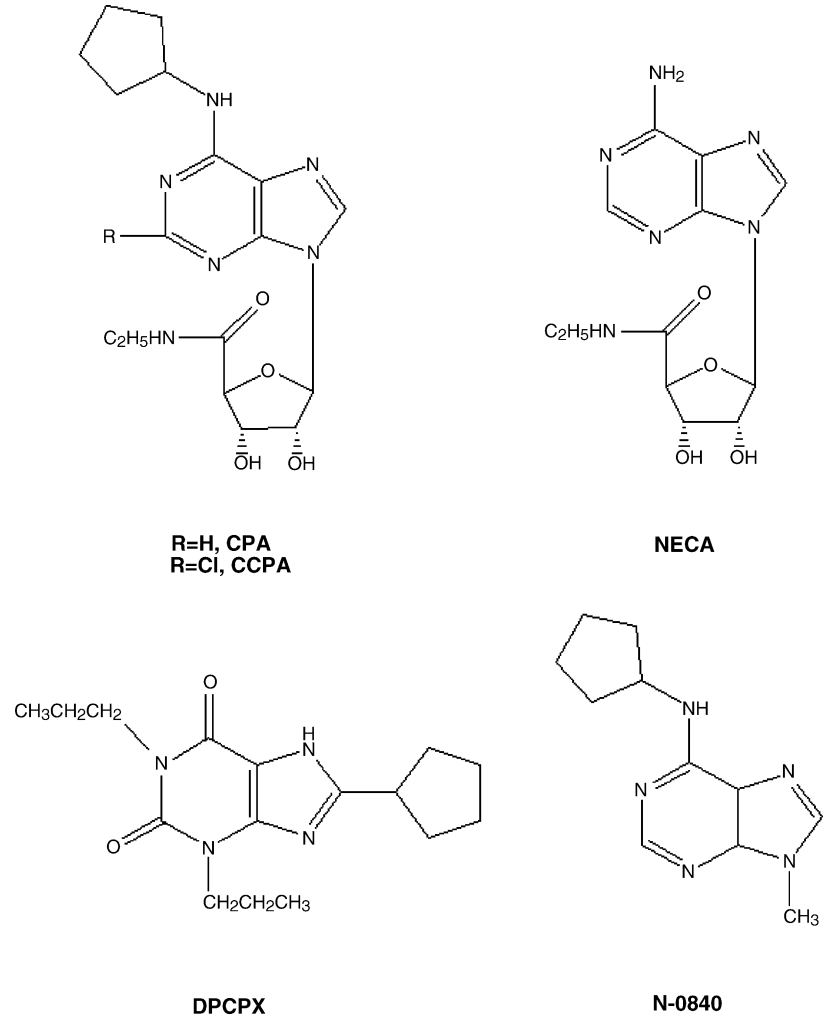

Fig. 2 – The structures of CPA, CCPA, NECA, DPCPX, and N-0840. CPA, CCPA and NECA are adenine nucleosides with ribose moiety. N-0840 is a N^6-cyclopentyladenine analogue but lacking the ribose moiety. DPCPX is a xanthine derivative.

Table 1 – Binding properties of [^{3}H]DPCPX, CCPA, NECA and N-0840 to WT and mutant A_1Rs

Receptor constructs	Antagonist [^{3}H]DPCPX		K_i values (M)		
	K_d values (nM)	B_{max} (pmol/mg prot)	Agonist CCPA	Agonist NECA	Antagonist N-0840
WT A_1R	1.24 ± 0.07	2.23 ± 0.06	2.65 ± 0.28E−7	3.81 ± 0.29E−6	9.65 ± 0.15E−7
L65T A_1R	0.91 ± 0.08	1.98 ± 0.08	2.95 ± 0.76E−6*	3.83 ± 0.21E−5*	1.83 ± 0.20E−6*
Change from WT	0.73	0.89	11.1	10	1.9
I69T A_1R	1.05 ± 0.06	1.90 ± 0.05	3.44 ± 0.07E−6*	3.43 ± 0.15E−5*	5.16 ± 0.08E−7*
Change from WT	0.85	0.85	13	9	0.53
I69S A_1R	1.18 ± 0.07	2.16 ± 0.07	6.6 ± 0.35E−6*	7.23 ± 0.38E−5*	1.39 ± 0.10E−6*
Change from WT	0.95	0.97	24.9	19	1.44
I69V A_1R	0.98 ± 0.08	2.39 ± 0.09	1.50 ± 0.03E−6*	1.17 ± 0.11E−5*	1.10 ± 0.05E−6
Change from WT	0.79	1.1	5.66	3.1	1.14

K_d and B_{max} values were obtained by saturation binding assays in corresponding membrane preparations. K_i values were obtained from competition binding assays using corresponding intact cells. All data are expressed as mean ± S.E.M. of at least three independent experiments. Also indicated for each compounds tested on the mutant receptors is the fold change from WT in the affinity observed.
* Signify $p < 0.05$ by analysis of variance with t-test comparison vs. WT A_1R.

CCPA. The similar results were obtained for the affinities of WT and mutant A_1Rs for another A_1R agonist NECA (Fig. 4). There was only moderate change in the affinity for A_1R selective antagonist N-0840 (Fig. 5, 0.53–1.44-fold).

As shown in Fig. 2, agonists CPA, CCPA and NECA are adenine nucleosides with ribose moiety; antagonist N-0840 is a N^6-cyclopentyladenine analogue but lacking the ribose moiety and antagonist DPCPX is a xanthine derivative. After mutations of hydrophobic residues (Leu-65 and Ile-69) in TM2 to hydrophilic residues (Thr-65, Thr-69 or Ser-69), the affinities for agonists CCPA and NECA were significantly decreased (at least 10-fold decrease compared to WT receptor). However, the affinities for antagonists DPCPX and N-0860 were unchanged or only mildly changed. Moreover, after mutation of Ile-69 to Val-69, the changes of affinities for CCPA, NECA and N-0840 were much less than the other mutant receptors. This was probably because of the less hydrophobic property of valine. These observations strongly suggested that the two hydrophobic residues in TM2 mainly mediated the recognition of agonists CCPA and NECA rather than antagonists DPCPX and N-0840, and more specifically, these residues may be involved in the recognition of the ribose group of CCPA and NECA.

4. Discussions

Some ligand–receptor interaction models of A_1R proposed a cavity formed by the seven amphipathic alpha-helices [8,9]. Initial mutagenesis studies of A_1R–ligand interactions have largely focused on the importance of sites in TM6 and TM7, and have been used to generate models of adenosine–A_1R interactions [10–12]. In these models, it was suggested that the ribose group interacted with TM7 and the adenine group interacted with TM6 and TM7 [10–12]. Additionally, it was

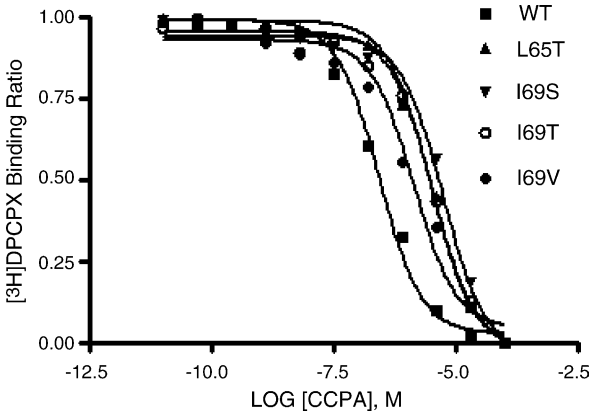

Fig. 3 – Effects of mutations in TM2 on the binding of A_1R agonist CCPA. Representative competitive inhibition curves of A_1R agonist CCPA vs. the A_1R antagonist [^{3}H]DPCPX in intact cells transiently transfected with WT (■), L65T (▲), I69S (▼), I69T (○) or I69V (●) A_1R. Competition assays were performed as described in Section 2. The resulting K_i values of WT, L65T, I69S, I69T and I69V A_1Rs are 0.27, 3.04, 5.36, 3.32 and 1.37 μM, respectively.

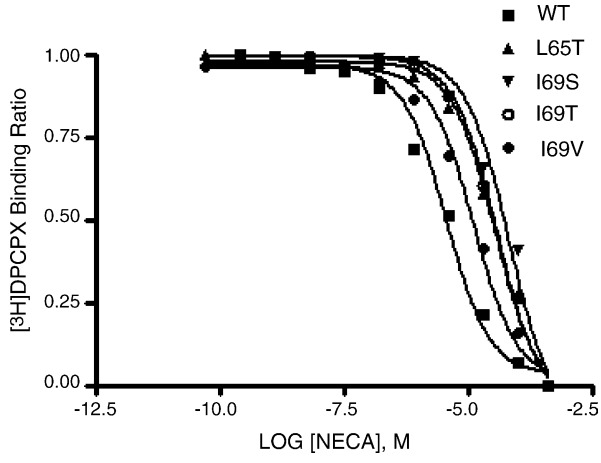

Fig. 4 – Effects of mutations in TM2 on the binding of A_1R agonist NECA. Representative competitive inhibition curves of A_1R agonist NECA vs. the A_1R antagonist [^{3}H]DPCPX in intact cells transiently transfected with WT (■), L65T (▲), I69S (▼), I69T (○) or I69V (●) A_1R. Competition assays were performed as described in Section 2. The resulting K_i values of WT, L65T, I69S, I69T and I69V A_1Rs are 3.59, 34.48, 66.05, 37.63 and 12.75 μM, respectively.

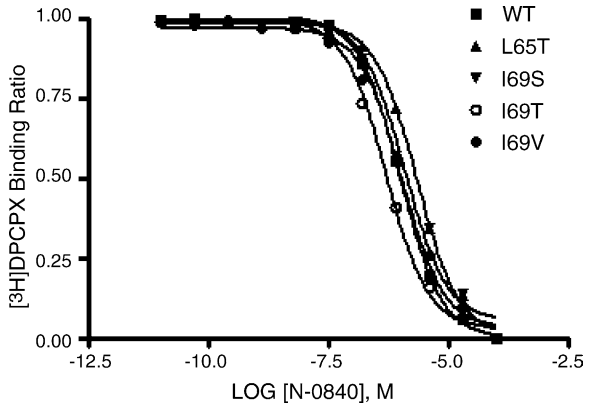

Fig. 5 – Effects of mutations in TM2 on the binding of A_1R antagonist N-0840. Representative competitive inhibition curves of A_1R antagonist N-0840 vs. the A_1R antagonist [^{3}H]DPCPX in intact cells transiently transfected with WT (■), L65T (▲), I69S (▼), I69T (○) or I69V (●) A_1R. Competition assays were performed as described in Section 2. The resulting K_i values of WT, L65T, I69S, I69T and I69V A_1Rs are 0.98, 2.17, 1.23, 0.50 and 1.05 μM, respectively.

suggested previously that both acidic residues (Glu16 in TM1 and Asp55 in TM2) played important roles in agonist/human A_1R interactions [22]. Furthermore, Rivkees et al. proposed a revised model of ligand–A_1R interactions in which the adenine group interacts with TM3, and the ribose group interacts with TM3 and TM7 [13]. Nevertheless, homology modeling showed that TM2, TM3 and TM7 might be in close proximity [8,9]. The conserved residues (L65 and I69) replaced at the outer end of TM2 were hydrophobic, and would not be likely to coordinate the ribose moiety. However, when the hydrophobic region was changed to hydrophilic, the mutations significant decreased their affinities for the ribose-containing agonists but not antagonists without ribose moiety. Based on the results of the site-directed mutagenesis studies, we further elucidated the model of ligand–A_1R interaction in which TM2 also mediated agonist ribose group recognition in an indirect way besides TM3 and TM7, probably by altering the larger structure of the receptor.

In the present studies, mutation of two hydrophobic amino acids (L65 and I69) to hydrophilic ones in TM2 of rat A_1R showed a decrease in agonists CCPA/NECA binding affinities whereas the A_1R mutants showed the same binding affinities in antagonists N-0840 and DPCPX as the WT. The ligands used differed by the presence of a ribose group. Agonists CCPA/NECA, containing a ribose group, showed reduced binding to the A_1 receptor mutants, whereas antagonists DPCPX/N-0840, lacking the ribose part, did not display substantially reduced affinities to the A_1R mutants. It is suggested that the hydrophobic region at the outer of TM2 of A_1R may be important in the recognition of the ribose group of A_1R agonists. There is also considerable support for this notion. L65F mutation in A_1R has been already reported [13]. The L65F mutation has no change in the affinities for both agonist CCPA and antagonist DPCPX. Combining with our result, we could propose that when L65 is replaced by a hydrophobic residue like phenylalanine, the ribose moiety seems to bind well. However, when L65 is replaced by a hydrophilic residue like threonine, the change in hydrophobicity or in conformation of TM2 seems to be the most probable cause of affinity decrease. A similar argument, which strengthens this hypothesis is that I69V mutation gives less dramatic effect on agonist affinity than I69S/T, because the hydrophobic property of valine is less than isoleucine. Rivkees et al. have also reported that the mutation of N70A (asparagines-70 to alanine-70), the next residue of Ile-69, has no change in the affinities for both agonist and antagonist [13]. The reason of that is, first, the polarities of both Asn and Ala are very poor, so the conversion of Asn to Ala at position 70 has only little change in the hydrophobicity in TM2; second, it is possible that the polar moiety of residue at position 70 orientates to the reverse side of the cavity which the agonist binds to. So the N70A mutation might has no change in the affinities for both agonist and antagonist.

The interaction of hydrophobic residues at the outer end of TM2 with the ribose part of the agonists NECA and CCPA would possibly orient the agonist ligands so that the adenine moiety is facing into the receptor. It seems controversial with the model proposed by Rivkees et al. in which adenine position orientes toward the top of TM3 and the ribose group interacts with the bottom half of TM3 and TM7 [13]. We propose that, the hydrophobic region formed by the residues at the outer end of TM2 is important for the ribose group recognition at the entrance of cavity formed by seven amphipathic alpha-helices to enable the agonist to go into the cavity. Once the agonist enters the cavity, the hydrogen-bond formed by the ribose moiety of agonist and the hydroxyl moiety of the residues in the bottom half of TM3 and TM7 [13] mainly determines the orientation of agonist in the ligand–receptor interaction. Therefore, when the hydrophobic region at the outer of TM2 is changed to hydrophilic, the introduction of polar hydroxyl moiety in the side chain may form hydrogen-bond with ribose moiety, and then indirectly influences the agonist binding by interfering the agonist entering the cavity. Our study indicates somewhat the dynamic manner of ligand binding to A_1R in an indirect way.

Previous studies showed that almost all the A_1R agonists contained ribose group. The ribose group contains three hydroxyl groups (2′, 3′, 5′) [23]. The three ribose-hydroxyl groups are very important for binding, as removal of these groups results in significant reduction in the affinity of A_1R [23]. In the model proposed by Rivkess et al., the ribose group interacted with TM3 and TM7, particularly the ribose moiety was suggested to bind to Ser-94, Thr-277 and His-278 [13]. It is the hydrogen-bonds formed by the ribose-hydroxyl groups with the hydroxyl groups of these residues, that determines the binding of A_1R agonist. We have investigated the role of hydrophobic region at the outer end of TM2, at the entrance of the cavity which agonist binds to. The result has showed that the hydrophobic region here is important for the recognition of agonist ribose group. It is more likely that when the hydrophobic residues are converted to hydrophilic ones, the hydrophilic moieties of amino acid side chain interact with the hydroxyl groups of ribose moiety to interfere the agonist entering the binding cavity. This is probably the main cause of the significant decrease of ribose-containing agonist binding

affinity. Thus, we speculate that the relatively larger decrease is mainly ribose-based.

The ribose moiety in the ligand has previously been shown to be of great importance for the agonistic effects of the compound [23]. However, IJzerman and his colleagues recently presented a series of nonadenosine agonists selective for the A_1R with an extraordinary pharmacological profile [24]. It will be very interesting and crucial to use these non-nucleoside agonists and nucleoside antagonists in the further studies to elucidate whether this relatively larger decrease is agonist-based or ribose-based precisely.

This study has provided evidence that the hydrophobic residues at positions 65 and 69 in TM2 of rat A_1R are important for agonist but not antagonist recognition. Furthermore, it also shows that mutations of these residues to hydrophilic ones have a moderate effect upon the binding of the N-0840, one adenine nucleoside derivative without the ribose group. All above mentioned indicates that the hydrophobic region mediates the recognition of the ribose group of CCPA and NECA in an indirect way. This identification of the element of the ligand binding domain of the A_1R will contribute to a better understanding of the manner in which adenosine receptor ligands bind to their receptors and effect physiological responses. It will provide us the better information for drug discovery based on adenosine receptors, especially A_1R.

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