Full-length article

Modulating effect of adenosine deaminase on function of adenosine A_1 receptors[1]

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Key words

adenosine deaminase; adenosine A_1 receptor; radioligand assay; calcium; fluorescence; diagnosis

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Abstract

Aim: To study the modulating effect of adenosine deaminase (ADA) on yhe adenosine A_1 receptor (A_1R) in HEK293 cells stably expressing the human A_1R. **Methods:** cDNA was amplified by RT-PCR using total RNA from human embryo brain tissue as the template. The PCR products were subcloned into the plasmid pcDNA3 and cloned into the plasmid pcDNA3.1. The cloned A_1R cDNA was sequenced and stably expressed in HEK293 cells. The modulating effect of adenosine deaminase on A_1R was studied by using [^{3}H]DPCPX binding assay and an intracellular calcium assay. **Results:** HEK293 cells stably expressing human A_1R were obtained. Saturation studies showed that the K_D value and B_{max} value of [^{3}H]DPCPX were 1.6±0.2 nmol/L and 1.819±0.215 nmol/g of protein respectively, in the absence of ecto-ADA respectively, and 1.3±0.2 nmol/L and 1.992±0.130 nmol/g of protein in the presence of ecto-ADA respectively, suggesting that the K_D value and B_{max} value of [^{3}H]DPCPX were unaffected by ecto-ADA. In the case of [^{3}H]DPCPX competition curves obtained from intact cells or membranes, A_1R agonist CCPA/[^{3}H]DPCPX competition curve could be fitted well to a one-site model in the absence of ecto-ADA and a two-site model in the presence of ecto-ADA with a K_H value of 0.74 (0.11–4.8) nmol/L (intact cells) or 1.8 (0.25–10) nmol/L (membrane) and a K_L value of 0.94 (0.62–1.41) μmol/L (intact cells) or 0.77 (0.29–0.99) μmol/L (membrane). The K_L value is not significantly different from the IC_{50} value of 0.84(0.57–1.23) μmol/L (intact cells) or 0.84 (0.63–1.12) μmol/L (membrane) obtained in the absence of ecto-ADA. Similar results were obtained from the CPA/[^{3}H]DPCPX competition curve in the absence or presence of ecto-ADA on intact cells or membranes. Intracellular calcium assay demonstrated that the EC_{50} value of CPA were 10 (5–29) nmol/L and 94 (38–229) nmol/L in the presence or absence of ecto-ADA, respectively. **Conclusion:** A_1R stably expressed in the HEK293 cells display a low affinity for agonists in the absence of ADA and high and low affinities for agonists in the presence of ADA. The presence of ADA may promote the signaling through the adenosine A_1 receptor in HEK293 cells.

Introduction

Adenosine is a ubiquitous physiological regulator and neuromodulator capable of ultiple physiological actions in various systems[1]. Adenosine receptors are members of the G-protein-coupled receptor superfamily, and comprise A_1, A_{2a}, A_{2b}, and A_3 adenosine receptors, identified by convergent data from molecular, biochemical, and pharmacological studies[2]. A_1R is widely expressed in the brain, adipose tissue, the testis, and the spinal cord[3]. Via A_1R, adenosine reduces heart rate[4], glomerular filtration rate, and renin release in the kidney[5], induces bronchoconstriction[6,7] and inhibits

lipolysis. A_1R can be coupled to different pertussis toxin-sensitive G proteins[8–10], which mediate the inhibition of adenylyl cyclase[11] and regulate Ca^{2+} and K^+ channels and inositol phosphate metabolism[12]. A_1R present two different affinities for agonists, which have classically been attributed to a different coupling to heterotrimeric G proteins[13], coupled receptor-G protein complexes display high affinity for A_1R agonists, whereas uncoupled receptors display low affinity[13,14].

Adenosine deaminase (ADA, E.C.3.5.4.4) is an enzyme which catalyzes the hydrolytic deamination of adenosine to inosine. ADA is located both in the cytosol and on the cell membrane. Recent evidence suggest that ecto-ADA had extra-enzymatic and co-stimulatory functional roles. ADA modulates ligand binding and signaling through A_1R on DDT_1MF-2 cells, a smooth muscle cell line[15]. ADA seems to be necessary for the high affinity binding of agonists to A_1R[16,17]. In the present study, the effect of ADA on the ligand-mediated regulation of A_1R in HEK293 cells stably expressing human A_1R has been studied.

Materials and methods

Materials High glucose Dulbecco's modified Eagle's medium (DMEM), fetal calf serum, G418, benzylpenicillin and streptomycin were obtained from Gibco. Restricted enzyme (*Hind*III, *EcoR*I, *Xho*I), T4 DNA ligase, reverse transcriptase enzyme and buffer were purchased from Promega (USA). Pyrobest DNA polymerase and PCR buffer were obtained from TaKaRa (Dalian, China). N^6-cyclopentyladenosine (CPA), 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), and 2-Chloro-N^6-cyclopentyladenosine (CCPA) were obtained from RBI (Natick, MA, USA). Adenosine deaminase was obtained from Roche Biochemicals (Mannheim, Germany). Fluo-3/AM, *p*-(dipropylsulfamoyl)benzoic acid, pluronic F-127, HEPES, Triton X-100, bovine serum albumin, leupeptin, pepstatin A, aprotinin and PMSF were purchased from Sigma (USA). [^{3}H]DPCPX was purchased from Amersham (USA). Plasmids pcDNA3, pcDNA3.1(+), and Lipofectamine^{2000} were purchased from Invitrogen (USA). Human embryo brain tissue was donated by Huashan Hospital (Shanghai, China). PCR primers were synthesized by Shenyou (Shanghai, China).

Table 1. Human A_1 receptor PCR primers.

A_1R-1SE	GCGCGAATTCTTGGTGACCTTGGGTGCTTG
A_1R-1AS	GCGCCTCGAGGCTCAGAACACTGTTGCCTCTA
A_1R-2SE	GCGCAAGCTTGCCGCCACCATGGCCCCGCCCT-CCATCTCAGCTTTCC
A_1R-2AS	GCGCGAATTCCTAGTCATCAGGCCTCTCTTCTGGG

Cloning of the human A_1R cDNA Human brain total RNA was obtained from human embryo brain tissue. The total RNA was reverse-transcribed using oligo-dT18 as a primer. With the cDNA, PCR was carried out using the primers A_1R-1SE and A_1R-1AS (Table 1). Gel-purified PCR product was treated with *EcoR*I/*Xho*I and subcloned into the *EcoR*I/*Xho*I site of the mammalian expression vector pcDNA3. With the subcloned A_1R-pcDNA3 plasmid as template, PCR was carried out using the primers A_1R-2SE and A_1R-2AS (Table 2), which resulted in full length codon sequence cDNA. Gel-purified PCR product was treated with *Hind*III/*EcoR*I and cloned into the *Hind*III/*EcoR*I site of the mammalian expression vector pcDNA3.1(+).

Production of HEK293 cell lines stably expressing human A_1R HEK293 Cells were transfected with the A_1R-pcDNA3.1(+) expression vector, using the Lipofectamine2000 reagent. Cells were treated with selection medium containing G418 (1 g/L) for 3 weeks to select stably transfected cells displaying neomycin resistance. Between 2 and 3 weeks in the selection process, resistant cells began to appear. They were sought out by serial dilutions and allowed to grow from single cells. Receptor expression of single cell-derived colonies was tested by radioligand binding assay.

Table 2. Representative competition inhibition experiments of CCPA and CPA versus the A_1R antagonist [^{3}H]DPCPX in intact cells and membrane preparations from A_1R cells. R_H is expressed as a mean. K_H, and K_L/IC_{50} are expressed as a mean with 95% confidence intervals.

		ADA	R_H (%)	K_H (nmol/L)	K_L/IC_{50} (μmol/L)
CCPA	Intact cells	–	0		0.84 (0.57–1.23)
		+	18 (3)	0.74 (0.11–4.8)	0.94 (0.62–1.41)
	Membranes	–	0		0.84 (0.63–1.12)
		+	25 (5.0)	1.8 (0.25–10)	0.77 (0.29–0.99)
CPA	Intact cells	–	0		1.3 (0.9–1.7)
		+	18 (3)	0.62 (0.1–4.9)	2.1 (1.3–3.3)
	Membranes	–	0		2.0 (1.5–2.5)
		+	32 (3)	0.47 (0.16–1.3)	2.7 (1.6–4.6)

Cell culture Human embryonic kidney (HEK293) cells were cultured in DMEM containing streptomycin (100 mg/L), benzylpenicillin (1000 kU/L) and fetal bovine serum (10%). Cells were incubated at 37 °C in 5% CO_2. Stably transfected HEK293 cell lines were cultured in DMEM high glucose medium containing streptomycin (100 mg/L), benzylpenicillin (1000 kU/L), G-418 (200 mg/L), and fetal bovine serum (10%). Cells were incubated at 37 °C in 5% CO_2. For passaging, the cells were detached from the cell culture flask by washing with phosphate-buffered saline (PBS) and brief incubation with trypsin (0.5 g/L)/EDTA (0.2 g/L). The cells were passaged every 3 d.

Membrane preparation The A_1R cells (HEK293 cells stably expressing human A_1R) were lifted from Petri dishes with a cell scraper. Harvested cells were washed twice with ice-cold PBS and centrifuged at 420×*g* for 5 min at 4 °C. The cell pellet was resuspended with hyponic buffer (Tris-HCl 5 mmol/L, EDTA 2 mmol/L, pH 7.4, leupeptin 1 mg/ L, pepstatin A 1 mg/L, aprotinin 1 mg/L, PMSF 1 mmol/ L) and sonicated (18 s) three times on ice. The homogenate was centrifuged at 960×*g* for 10 min at 4 °C. The precipitated nucleic fraction was discarded and the supernatant was centrifuged at 40 000×*g* for 30 min at 4 °C. The pellet was washed with 50 mmol/L Tris-HCl buffer (pH 7.4) and centrifuged again under the same conditions. Finally, the pellet was resuspended in the same buffer, and protein concentration was determined by using the BCA Kit (Pierce) as described previously[18].

[^{3}H]DPCPX binding assays in intact cells and membranes Binding assays in intact cells were performed in a reaction tube at a density of 2.0×10^5 cells per tube, using [^{3}H]DPCPX as a radioligand. Cells were resuspended in 50 mmol/L Tris-HCl (pH 7.4) and treated with ADA 65 nmol/L at 4 °C for 30 min[17]. After this treatment, cells were incubated for 1 h at 37 °C with [^{3}H]DPCPX 0.5 nmol/L for competition assays. Different concentrations of the A_1R agonist CCPA or CPA were used in the competition curves.

Membranes (30–50 μg proteins) from A_1R cells were resuspended in Tris-Cl 50 mmol/L (pH 7.4) and treated with ADA 65 nmol/L at 4 °C for 30 min. Saturation assays were performed at different concentrations of [^{3}H]DPCPX (0.05–10 nmol/L) using unlabeled DPCPX (20 μmol/L) to obtain nonspecific binding. Competition curves were carried out by using [^{3}H]DPCPX 0.5 nmol/L and different concentrations of A_1R agonists CCPA or CPA.

After incubation for 1 h, the binding assays were stopped by rapid filtration through Whatman GF/B filters, and the filters were immediately washed three times with ice-cold buffer. Filters were then transferred to Eppendoff tubes, and scintillation liquid was added to measure the radioactivity.

Intracellular Ca^{2+} measurements Ca^{2+} fluorescence measurements were performed using a NOVOstar plate reader with a pipettor system (BMG labtechnologies, Offenburg, Germany). A_1 cells were harvested with 0.05% trypsin/0.02% EDTA and rinsed with high glucose DMEM containing 10% fetal bovine serum, streptomycin 100 mg/L, and benzylpenicillin 1000 kU/L. Pelleted cells were resuspended in fresh medium and kept under 5% CO_2 at 37 °C for 1 h and vortexed every 15 min. After two washes with Krebs-HEPES buffer, cells were loaded with Fluo-3/AM 5 μmol/L for 30 min containing 1% pluronic F-127 and *p*-(dipropylsulfamoyl)benzoic acid 2.5 mmol/L. Then cells were rinsed 3 times with Krebs-HEPES buffer containing 0.5% bovine serum albumin, the diluted, and evenly plated into 96-well plates at a density of 1×10^4 cells/well. Microplates were kept at 37 °C for 15 min[19, 20]. Buffer alone or different concentrations of CPA were then injected sequentially into separate wells, and fluorescence intensity was measured at 520 nm for 50 s at 0.2 s intervals. The excitation wavelength was 485nm.

$[Ca^{2+}]_i$ was calculated as follows: $[Ca^{2+}]_i=K_d(F-F_{min})/(F_{max}-F)$. F_{max} refers to fluorescence intensity measured after permeabilization of the cells with 1% Triton X-100. Ten mmol/L EDTA was added to chelate Ca^{2+} and minimum fluorescence intensity was obtained (F_{min}). A K_d value of 324 nmol/L was used for Fluo-3.

Data analysis Experiments were performed in triplicate. All data were expressed as mean±SD and data were analyzed with the GraphPad Prism 4.0 program (GraphPad Software, San Diego, CA, USA). Student's *t*-test was used for statistical analysis.

Results

Cloning of the human adenosine A_1R cDNA To obtain the cDNA with the full length codon sequence of human A_1R, a reverse transcription reaction was carried out using the total RNA extracted from human embryo brain tissue. Using cDNA with full length codon sequence of A_1R, a PCR product (1332 bp) was obtained using primers described previously. The PCR product subcloned into a pcDNA3 plasmid and cDNA (981 bp) containing the full A_1R codon sequence was obtained using this subcloned vector as a template. The cDNA was then cloned into the mammalian expression vector pcDNA3.1. Sequence analysis demonstrated that the sequence of the constructed A_1R-pcDNA3.1 expression vector was identical to that of human A_1R cDNA in the gene bank.

Selection of cell clones Colonies of the selected stable

integrants were initially analyzed for human A_1R expression by using a receptor binding assay using [^{3}H]DPCPX. From the transfected HEK293 cell clones, one cell line was obtained with specific [^{3}H]DPCPX binding.

Saturation experiments with the adenosine A_1 receptor antagonist [^{3}H]DPCPX As Figure 1 shown, no significant difference was found between the A_1R cells in the absence and presence of ADA with respect to the B_{max} and K_D values for the A_1R binding sites labeled with [^{3}H]DPCPX. The B_{max} values for the A_1R cells were 1.819±0.215 and 1.992±0.130 nmol/g of protein (n=3) in the absence and presence of ADA, respectively. The K_D values for the A_1R cells were 1.6±0.2 and 1.3±0.2 nmol/L (n=3) in the absence and presence of ADA, respectively. The non-specific binding was <5% of the total binding.

Competition experiments of A_1R agonist CCPA or CPA versus the A_1R antagonist [^{3}H]DPCPX Competition experiments with CCPA versus the A_1R antagonist [^{3}H]DPCPX in intact A_1R cells or membrane preparations from A_1R cells incubated previously with ADA showed a significantly better fit with the 2 binding sites model than that with the 1 binding site model (F test, P<0.05). Similar K_H and K_L values were obtained in intact cells or membrane preparations from A_1R cells incubated previously with ADA, and the proportions of the A_1R in the high affinity state (R_H values) were 18%±3% and 20%±5% , respectively (Figure 2 and Table 2). In intact A_1R cells or membrane preparations from A_1R cells in the absence of ADA, a significant better fit for one binding site (R_H=0) was obtained, with the IC_{50} values very similar to the K_L value obtained in intact cells or membrane preparations previously treated with ADA. Similar results were obtained from competition experiments with CPA versus the adenosine A_1R antagonist [^{3}H]DPCPX in intact A_1R cells or membrane preparations from A_1 cells in the absence of ADA (Figure 3, Table 2).

Effects of A_1R agonist CPA on intracellular calcium level in the presence or absence of ADA An increase in intracellular Ca^{2+} appears to be a universal second messenger signal for a majority of recombinant GPCRs[20]. A_1R agonists evoked a concentration-dependent and reproducible Ca^{2+} signal at A_1R[22]. To assess the effect of the presence of ADA on A_1R signal transduction, we performed an intracellular calcium assay. A significant difference was found between the A_1R cells in the absence and presence of ADA with respect to the EC_{50} values of A_1R agonist CPA. A_1R agonist CPA induced an intracellular [Ca^{2+}] increase. The EC_{50} values for the A_1R cells in the absence and presence of ADA were 94 (38–229) and 10 (5–29) nmol/L, respectively. CPA appears more potent at the A_1 cells in the presence of ADA compared with the A_1R cells in the absence of ADA (Figure 4).

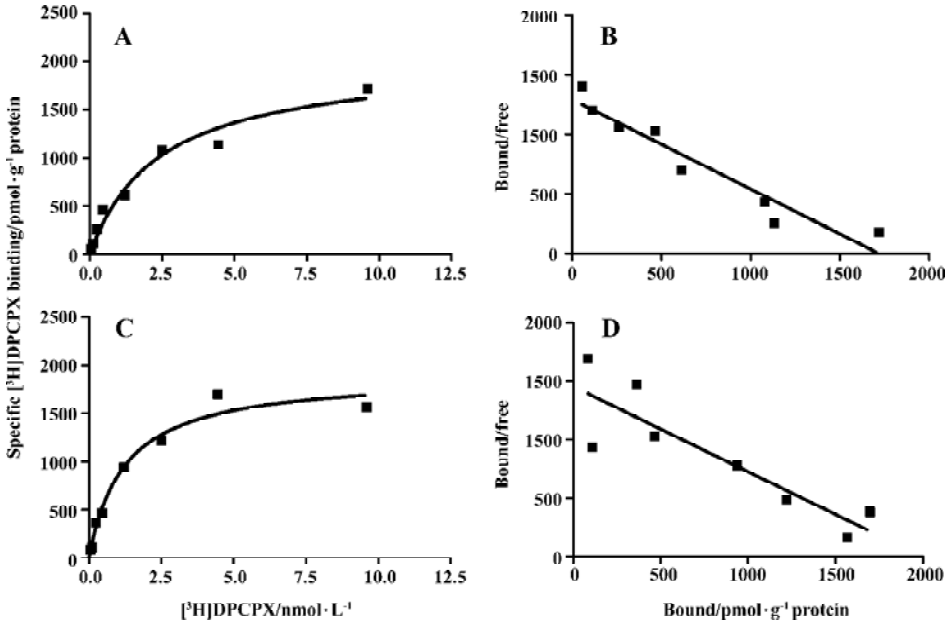

Figure 1. Saturation curve of [^{3}H]DPCPX binding to adenosine A_1R in plasma membrane isolated from A_1R cells untreated (A, B) and treated (C, D) with ADA. Binding assays were performed as described in Methods, using A_1R antagonist [^{3}H]DPCPX as radioligand in a concentration range from 0.05 to 10 nmol/L. A and C: Saturation curve of [^{3}H]DPCPX binding to adenosine A_1 receptors. B and D: Scatchard plot of these data and analysis gave the K_D and B_{max} values indicated in Results. Data are mean±SD and were obtained from three independent experiments performed in triplicate.

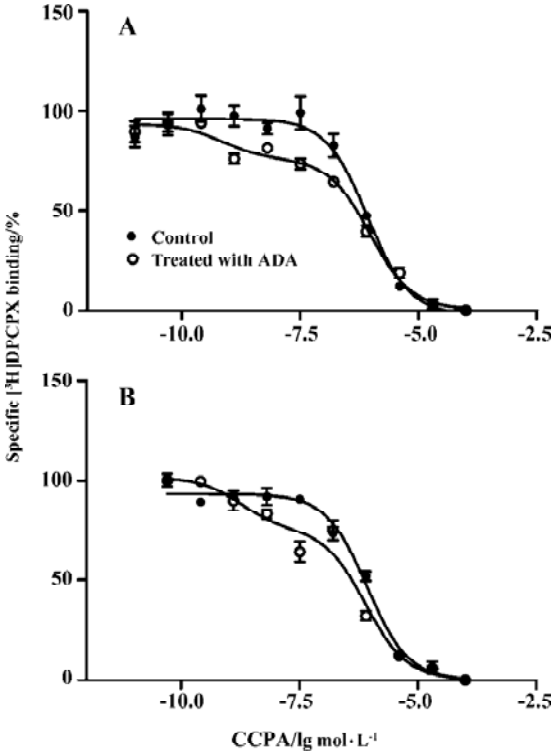

Figure 2. Representative competitive inhibition curve of A_1R agonist CCPA versus the A_1R antagonist [^{3}H]DPCPX in intact cells (A) and membrane preparations (B) from A_1R cells untreated and treated with ADA. Competition assays were done at 0.5 nmol/L [^{3}H]DPCPX and increasing concentrations of CCPA. IC_{50} values are means (95% confidence intervals) obtained from three independent experiments performed in triplicate.

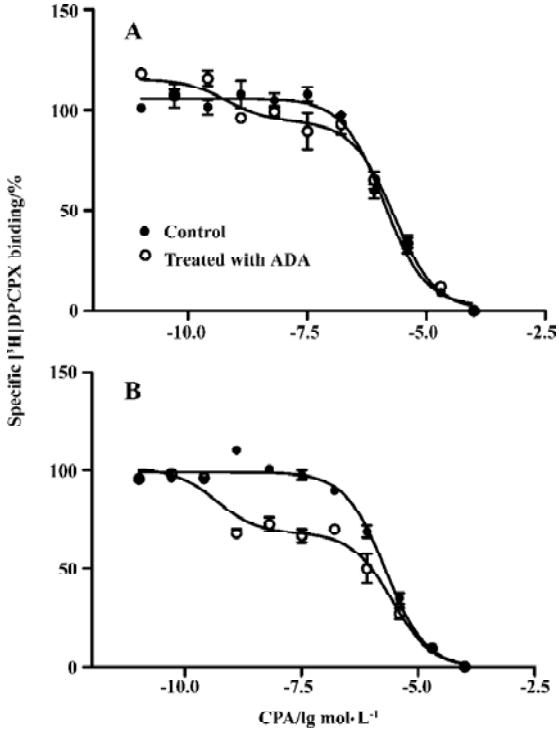

Figure 3. Representative competitive inhibition curve of A_1R agonist CPA versus the A_1R antagonist [^{3}H]DPCPX in intact cells (A) and membrane preparations (B) from A_1R cells untreated and treated with ADA. Competition assays were done at 0.5 nmol/L [^{3}H]DPCPX and increasing concentrations of CPA. IC_{50} values are means (95% confidence intervals) obtained from three independent experiments performed in triplicate.

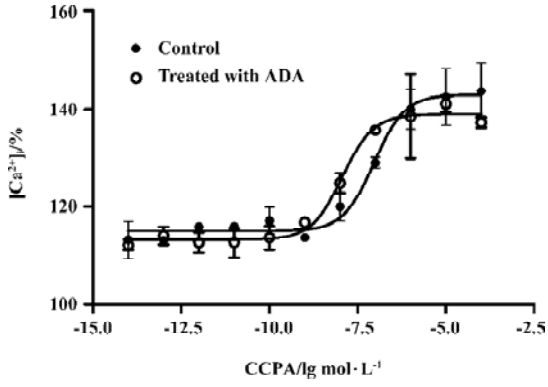

Figure 4. Concentration-response curves for the effect of A_1R agonist CPA on intracellular [Ca^{2+}] in A_1R cells untreated and treated with ADA. Mean±SD. n=3. EC_{50} values is mean (95% confidence intervals) obtained from three independent experiments performed in triplicate.

Discussion

Recently, several evidences demonstrated that cell-surface ADA interacted with A_1 receptors in brain cortex and DDT_1MF-2 cells and that the enzyme was able to modulate ligand binding and signaling through A_1R[16,21–23]. By immunoprecipitation and affinity chromatography, it was found that ADA and A_1R interacted in pig brain cortical membranes. By means of this interaction ADA led to the appearance of the high-affinity site of the receptor. Thus, it seems that ADA is necessary for coupling A_1R to heterotrimeric G proteins[16]. In Chinese hamster ovary (CHO) cells, stably transfected with the human adenosine A_1R, it was found that in the presence of ADA, the [^{3}H]DPCPX/cyclohexyladenoine competition curve could be analysed by a two-site model with 93% of the sites having high affinity and the remainder having low affinity. In the absence of ADA, the [^{3}H]DPCPX/cyclohexyladenosine competition curve was well described by a two-site model. Under these conditions, 70% of the binding of [^{3}H]DPCPX was associated with a high-affinity site with the remaining sites having low affinity. Thus there is a clear difference in the potency of cyclohexyladenosine

at the high-affinity state[24]. In the present study, human adenosine A_1R were cloned and stably expressed in the HEK293 cells and the effects of ecto-ADA on adenosine A_1R were studied. By using competitive inhibition assay of adenosine A_1R agonists CCPA or CPA against [^{3}H]DPCPX, we found that A_1R displayed two different affinities for agonists in the presence of ADA. However, A_1R only displayed a low affinity for agonists in the absence of ADA, suggesting that most of the binding of [^{3}H]DPCPX was associated with the low-affinity site under these conditions and that the interaction of ADA with A_1 adenosine receptors led to the appearance of the high-affinity site of the receptor.

To further demonstrate possible interaction between A_1R and ADA, intracellular calcium assay was carried out in the present studies. The results demonstrated that the EC_{50} value of CPA for inducing increase of intracellular calcium level was lower in the presence of ADA than that in the absence of ADA, suggesting that the presence of ADA may promote signaling through A_1 receptors in the stably transfected HEK293 cells. Our results further support the notion that ADA may promote the signaling through A_1 receptors.

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