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Thermodynamics of Full Agonist, Partial Agonist, and Antagonist Binding to Wild-Type and Mutant Adenosine A_1 Receptors

Alessandro Dalpiaz, Andrea Townsend-Nicholson,† Margot W. Beukers,* Peter R. Schofield‡ and Adriaan P. IJzerman†§*

*Leiden/Amsterdam Center for Drug Research, Division of Medicinal Chemistry, P.O. Box 9502, 2300RA Leiden, The Netherlands; †Department of Anatomy and Developmental Biology, UCL, Gower St., London, U.K.; and ‡Garvan Institute of Medical Research, 384 Victoria St., Darlinghurst 2010, Australia

ABSTRACT. A thermodynamic analysis of the binding of a full agonist (N^6-cyclopentyladenosine), a partial agonist (8-butylamino-N^6-cyclopentyladenosine) and an antagonist (8-cyclopentyltheophylline) to human wild-type and mutant (mutation of a threonine (Thr) to an alanine (Ala) residue at position 277) adenosine A_1 receptors expressed on Chinese hamster ovary (CHO) cells, and to rat brain adenosine A_1 receptors was undertaken. The thermodynamic parameters $\Delta G°$ (standard free energy), $\Delta H°$ (standard enthalpy) and $\Delta S°$ (standard entropy) of the binding equilibrium to rat brain receptors were determined by means of affinity measurements carried out at four different temperatures (0, 10, 20 and 25°) and van't Hoff plots. Two temperatures (0 and 25°) were considered for human receptors. Affinity constants were obtained from inhibition assays on membrane preparations of rat brain and CHO cells by use of the antagonist [^{3}H]1,3-dipropyl-8-cyclopentylxanthine ([^{3}H]DPCPX) as selective adenosine A_1 receptor radioligand. As for rat brain receptors, full agonist binding was totally entropy driven, whereas antagonist binding was essentially enthalpy driven. Partial agonist binding appeared both enthalpy and entropy driven. As for human receptors, full agonist affinity was highly dependent on the presence of Thr^{277}. Moreover, affinity to both wild-type and mutant receptors was enhanced by temperature increase, suggesting a totally entropy-driven binding. Antagonist binding did not depend on the presence of Thr^{277}. Antagonist affinity decreased with an increase in temperature, suggesting a mainly enthalpy-driven binding. Partial agonist binding was significantly dependent on the presence of Thr^{277} at 25°, whereas such a dependence was not evident at 0°. It is concluded that Thr^{277} contributes only to the binding of adenosine derivatives and that its role changes drastically with the receptor conformation and with the type of agonist (full or partial) interacting with the adenosine A_1 receptors. BIOCHEM PHARMACOL **56**;11: 1437–1445, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. binding thermodynamics; cloned human adenosine A_1 receptor; mutant human adenosine A_1 receptor; high affinity and low affinity receptor states; full and partial agonist

The physiological effects of adenosine are mediated by adenosine receptors which have been classified into four subtypes: A_1, A_{2A}, A_{2B}, and A_3 [1]. All belong to the superfamily of GPCRs¶ and are characterized by a structure with seven α-helical TMs connected by intra- and extracellular loops [2]. GPCRs exist in equilibrium between two different conformations, which correspond to a high and a low affinity binding state, antagonists bind equally well to the two receptor states, and agonists discriminate between them [3, 4].

Adenosine A_1 receptors are ubiquitous throughout the body. Therefore, their activation may result in a plethora of effects, either desired or unwanted. One of the more interesting beneficial effects is the ischemic tolerance and protection which results from activation of adenosine A_1 receptors in neuronal and cardiac tissues [5, 6].

All known adenosine receptor agonists are structurally related to adenosine and are, therefore, characterized by the presence of a ribose and a purine moiety (Fig. 1); N^6-substituted analogs are potent and selective A_1 agonists [7]. Typical adenosine receptor antagonists are xanthine derivatives: DPCPX and CPT (Fig. 1) are both A_1-selective [7]. The recent discovery of partial agonists for the adenosine A_1 receptors [8] may permit the inhibition of some of the

§ Dr. Adriaan P. IJzerman, Leiden Amsterdam Center for Drug Research, Division of Medicinal Chemistry, P.O. Box 9502, Einsteinweg 55, 2300RA Leiden, The Netherlands. Tel. 0031715274651; FAX 0031715274565.

¶*Abbreviations:* ADA, adenosine deaminase; BCA, bicinchoninic acid; CHO, Chinese hamster ovary; CPA, N^6-cyclopentyl-adenosine; NECA, 5′-(*N*-Ethyl)-carboxamidoadenosine; 8BCPA, 8-butylamino-N^6-cyclopentyladenosine; DPCPX, 1,3-dipropyl-8-cyclopentylxanthine; CPT, 8-cyclopentyltheophylline; GDP, guanosine-5′-diphosphate; GPCR, G protein-coupled receptor; TM, transmembrane domain; Thr, threonine.

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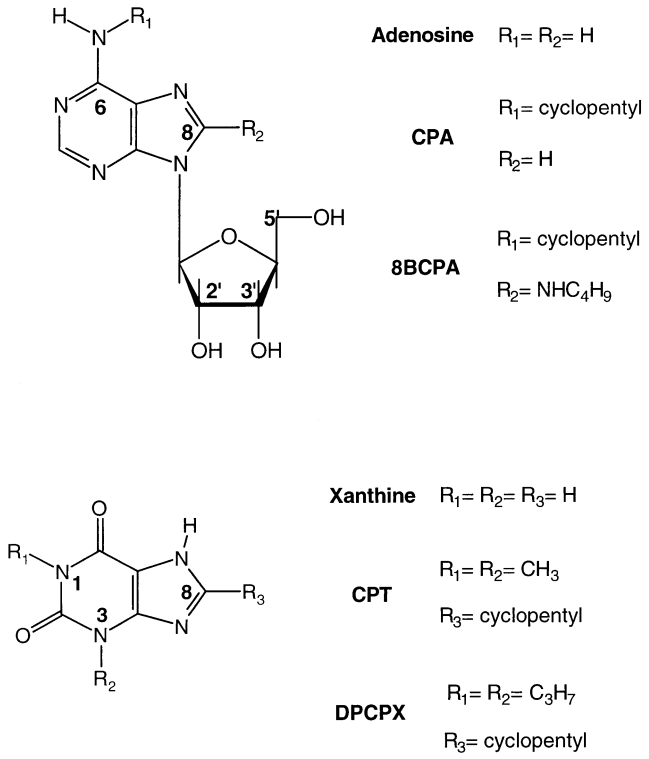

FIG. 1. Chemical formulas of adenosine, xanthine, and A_1 selective ligands: CPA = full agonist; 8BCPA = partial agonist; DPCPX and CPT = antagonists.

side effects that accompany adenosine A_1 receptor activation by exogenous agents [9–11]. It has recently been demonstrated that N^6, C8- di-substituted adenosine derivatives are relatively potent A_1-selective partial agonists [12].

Differences in thermodynamic behavior of agonists and antagonists have suggested that the ribose moiety of agonists interacts in a domain of adenosine A_1 receptor that is more hydrophilic than that involved in the interaction with adenine or xanthine rings [8, 13]. Molecular models of the adenosine A_1 receptor [14], based on its amino acid sequence [15–17], suggested the same conclusions as those obtained in thermodynamic studies.

Two independent studies performed on mutant adenosine A_1 receptors have indicated that Thr^{277} is responsible for the interaction with the 5′ position of the ribose moiety in adenosine derivatives [18, 19]. It has also been demonstrated that this residue does not influence the binding of the xanthine antagonists [18]. Studies of chimeric A_1/A_{2A} receptors have suggested that the important determinants for human A_1 adenosine receptor ligand specificity are present in the first four TMs [20]. Recent mutation analysis of these TMs has led to similar conclusions [21]. TM VII of adenosine A_1 receptors may, therefore, be involved in a direct interaction with the ribose moiety of the agonists, with a special role for Thr^{277}.

This paper reports affinity data, at different temperatures, of ligands selective for both rat and human adenosine A_1 receptors. The studies were performed on membranes of rat brain cortex (0, 10, 20, and 25°) and CHO cells stably transfected with wild-type (CHO A_1) and mutant human receptors (0 and 25°), obtained by mutation of Thr^{277} to Ala (CHO A_1 $T^{277}A$). High and low affinity states of the receptors were analyzed from a thermodynamic point of view. The analysis was quantitative for rat brain receptors and qualitative for human receptors. In particular, we determined the standard free energy ($\Delta G°$), enthalpy ($\Delta H°$), and entropy ($\Delta S°$) for the binding of a full agonist (CPA), a partial agonist (8BCPA) and an antagonist (CPT) (Fig. 1). Comparison of thermodynamic data concerning wild-type and mutant human receptors suggests that Thr^{277} makes different contributions to the low and high affinity states of the adenosine A_1 receptor, respectively.

MATERIALS AND METHODS

Materials

[^{3}H]DPCPX (specific activity 108 Ci/mmol) was purchased from NEN (Du Pont Nemours). CPA, DPCPX, and CPT were obtained from RBI. 8BCPA was prepared as described previously [12]. ADA was obtained from Boehringer Mannheim and BSA from Sigma. BCA and BCA protein assay reagent were purchased from Pierce Chemical Company. Male Wistar rats were obtained from Sylvius Laboratories. Bovine calf serum was obtained from Hyclone Laboratories Inc. and streptomycin/penicillin was obtained from Ducheva. Trypsin solution 2.5% and all other cell culture reagents were obtained from Gibco Laboratories. All other chemicals were obtained from standard sources.

Thermodynamic Data Determination

There are two main experimental methods used to evaluate $\Delta G°$, $\Delta H°$ and $\Delta S°$. In one, the equilibrium constant ($\Delta G° = -RT \ln K_A$) is determined by direct microcalorimetric measurements which give the corresponding $\Delta H°$ values. This method is not practical in receptor binding studies due to the low receptor concentration in most tissues (1–100 fmol/mg of tissue for a typical neurotransmitter [22]). The only practical method of determining $\Delta G°$, $\Delta H°$ and $\Delta S°$ consists in measuring K_A at different temperatures, followed by van't Hoff analysis. Two cases are to be distinguished:

1. The standard specific heat difference of the equilibrium (ΔC_p°) is essentially zero. In this case, the van't Hoff equation $\ln K_A = -\Delta H°/RT + \Delta S°/R$ gives a linear plot $\ln K_A$ versus 1/T. The standard enthalpy can be calculated from the slope, -$\Delta H°/R$ and the standard entropy from the intercept, $\Delta S°/R$ or as $\Delta S° = (\Delta H° - \Delta G°)/T$ with T = 298.15 K and R = 8.314 JK^{-1} mol^{-1}. The linearity of van't Hoff plots is not common in reactions involving biomacromolecules in solution but appears to be typical as far as membrane receptor binding is concerned [23].

2. ΔC_p° is not equal to zero. In this case, the van't Hoff plot is often parabolic and other mathematical methods are available for the analysis [24].

Preparation of CHO Cells Transfected with Adenosine A_1 Human Receptors

CHO cells stably transfected with the wild-type and mutant $T^{277}A$ human A_1 adenosine receptors were prepared as previously described [17, 18]. Briefly, the cDNA encoding the human A_1 adenosine receptor was subcloned into the pRC/CMV mammalian expression vector. Specific mutation of this cDNA was generated using the oligonucleotide-direct polymerase chain reaction mutagenesis method. The mutation was confirmed by double-stranded dideoxy DNA sequencing. Receptor cDNA constructs were transfected into CHO. K_1 cells [25], and selection for stable transformants was carried out with the neomycin analogue G418 at a concentration of 800 ng/mL.

Cell Culture

Cells were grown in a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's F12 medium containing 10% bovine calf serum, streptomycin (50 μg/mL) and penicillin (50 IU/mL) at 37° in 5% CO_2. Cells were subcultured twice weekly at a ratio of 1:10 and transferred to large 14-cm diameter plates.

Membrane Preparation

Cells were washed with PBS and detached from plates by 5-min incubation at 37° in the presence of 2 mL of trypsol (0.25% trypsin, 4.4 mM EDTA in PBS). Five mL of medium were added to each plate after incubation and the cells were collected and centrifuged for 10 min at 68 *g*. Pellets derived from six plates were pooled and resuspended in 20 mL of ice-cold 50 mM TRIS-HCl buffer, pH 7.4, and homogenized in a Polytron homogenizer (setting 6) for 5 sec. Plasma membranes and the cytosolic fraction were separated by centrifugation at 18,000 *g* in a Beckman L8–50 M/E ultracentrifuge at 10° for 30 min. The pellets were resuspended in the TRIS buffer at approximately 1×10^8 cells/3 mL and 2 IU/mL of ADA were added. After 30-min incubation at 37°, the membranes were stored in 200 μL aliquots at −80°.

Rat cortical brain membranes were prepared according to the method of Lohse *et al.* [26] except that the membranes were incubated with 2 IU/mL of ADA at 37° before storage, as described by Pirovano *et al.* [27]. Membrane protein concentrations were measured with the BCA method [28].

Receptor Binding Assays

Membrane aliquots containing 40 μg of proteins were incubated in 400 μL of 50 mM TRIS-HCl at 25° for 90 min, at 20° for 115 min, at 10° for 180 min, and at 0° for 16 hr, according to previous time–course experiments. All buffer solutions were adjusted to maintain a constant pH of 7.4 at each of these temperatures. Saturation experiments were carried out using twelve different concentrations of [^{3}H]DPCPX ranging from 0.1 to 5 nM. Displacement experiments on rat brain membranes were performed using at least twenty different concentrations of cold drug in the presence of [^{3}H]DPCPX, with concentrations ranging from 0.2 nM (0 and 10°) to 0.4 nM (20 and 25°). Displacement experiments on all CHO cell membranes were carried out with 1 nM of [^{3}H]DPCPX at all temperatures.

On rat brain and CHO A_1 wild-type membranes, nonspecific binding was measured in the presence of 10 μM of CPA, whereas on CHO A_1 T277A membranes nonspecific binding was measured in the presence of 50 μM of CPT. For each single system, it was verified that the same nonspecific binding was measured using 10 μM DPCPX, or 50 μM CPT. For rat brain and CHO A_1 wild-type cell membranes, the same results were obtained with 10 μM of CPA. Separation of bound from free radioligand was performed by rapid filtration through Whatman GF/B filters which were washed eight times with ice-cold buffer. Filter-bound radioactivity was measured by scintillation spectrometry after the addition of 4 mL of Packard Emulsifier Safe. All the values obtained are means of three independent experiments performed in duplicate.

Calculations

Saturation experiment data (K_d and B_{max} values) were obtained by computer analysis of saturation curves. The cold drug concentrations displacing 50% of labeled ligand (IC_{50} values) were obtained by computer analysis of displacement curves. Inhibitory binding constants (K_i values) were derived from the IC_{50} values according to the Cheng and Prusoff equation [29]. In this respect, care was taken that total binding never exceeded 10% of the total amount of radioligand added. All binding data were analyzed using the nonlinear regression curve fitting Graph Pad Prism computer program. The values of thermodynamic terms ($\Delta G°$, $\Delta H°$ and $\Delta S°$) were obtained by linear van't Hoff plot regression, as previously described in "*Thermodynamic Data Determination*" above.

RESULTS

Figure 2 illustrates representative saturation experiments and corresponding Scatchard plots obtained at 25° for the binding equilibrium of [^{3}H]DPCPX to rat brain, CHO A_1 wild-type and CHO A_1 T277A cell membranes, respectively. All Scatchard plots were linear in the concentration range investigated and, analogously, computer analysis of the saturation experiments suggested a one-site, rather than a two-site, binding model.

The dissociation binding constants (K_d) and B_{max} values derived from the saturation experiments of [^{3}H]DPCPX to rat brain membrane adenosine A_1 receptors performed at

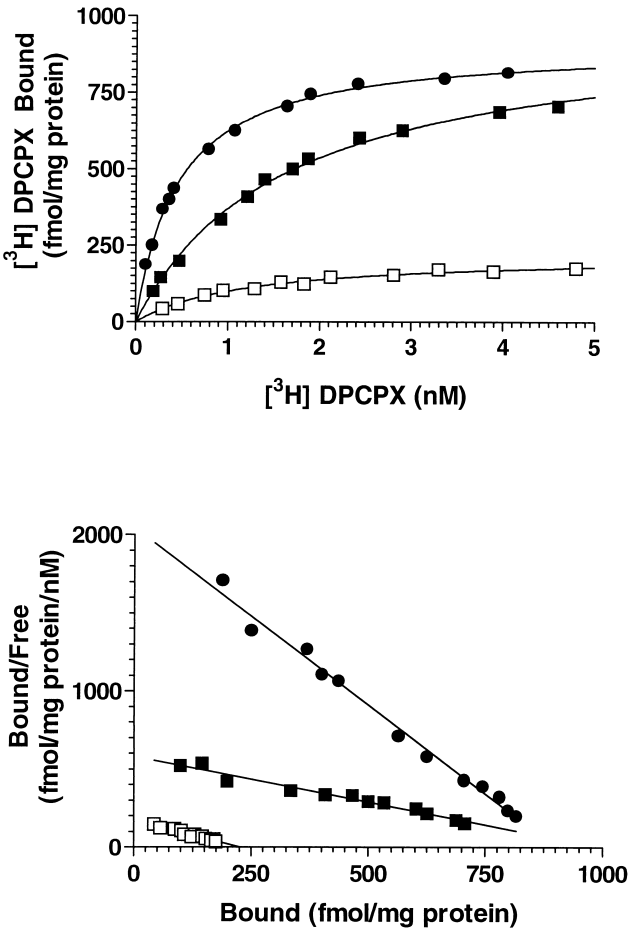

FIG. 2. Saturation curves and their Scatchard plots (bound/free versus bound) obtained at 25° for the binding equilibrium of [^{3}H]DPCPX to rat cortex (●), CHO A_1 cell membranes(■) and CHO A_1 T277A cell membranes (□). The linearity of the Scatchard plots ($r \geq 0.98$) and computer analysis of the saturation plots indicate the presence of a single class of binding sites in each experimental system.

the four chosen temperatures (0, 10, 20, and 25°) are shown in Table 1; inhibitory binding constants (K_i) for the displacement, on the same system, of [^{3}H]DPCPX by the other ligands (CPA, CPT, and 8BCPA) are also shown. The full agonist CPA recognized two affinity binding states at all temperatures investigated, with a high affinity state population of approximately 40%. The antagonist CPT bound to either receptor state with the same affinity, in agreement with the results obtained in the [^{3}H]DPCPX saturation experiments. The partial agonist 8BCPA recognized two affinity binding states at 20 and 25°, with a high affinity state population of approx. 20%, but it seemed to interact with one binding state at 0 and 10°. While K_d and K_i values were largely temperature dependent (see below), B_{max} values appeared to be independent of temperature, suggesting an identical receptor population.

Table 2 reports the values obtained by [^{3}H]DPCPX saturation and CPA, 8BCPA, and CPT displacement experiments with [^{3}H]DPCPX carried out at two temperatures (0 and 25°) on CHO A_1 wild-type and CHO A_1 T277A cell membranes. Three main observations were found for wild-type human adenosine A_1 receptors: 1) the full agonist CPA recognized two affinity binding states at the two temperatures investigated, with a high affinity state population of approximately 75%; 2) the antagonists [^{3}H]DPCPX and CPT bound to either receptor state with the same affinity; and 3) the partial agonist 8BCPA recognized two affinity binding states at 25° (50% of the high affinity population), but it seemed to interact with one binding state at 0°. Moreover, K_d and K_i values appeared to be somewhat higher than those obtained in the rat brain membrane experiments (with the exception of CPA's low affinity data), whereas B_{max} values appeared to be the same.

All adenosine and xanthine derivatives recognized only one affinity binding state on T277A adenosine A_1 receptors at the two temperatures investigated. In comparison with the data on the human adenosine A_1 wild-type receptor, it was observed that the high affinity binding state recognized by CPA (full agonist) and 8BCPA (partial agonist) in the wild-type was lost in the T277A receptor. Moreover, the affinity of CPA (K_i = 8.2 μM at 25° and 33.1 μM at 0°) was dramatically lower (more than 100-fold) than its interaction with the wild-type A_1 receptor in the low affinity binding state (K_i = 76 nM at 25° and 100 nM at 0°). Small differences were instead observed for the corresponding affinity values of 8BCPA at 25° and for the K_i values at 0° (wild-type receptor: K_i = 7.1 μM at 25° and 4.0 μM at 0°; mutated receptor: K_i = 33.2 μM at 25° and 8.3 μM at 0°). Similarly, comparison of CPT affinity data between human receptors also revealed small differences (wild-type receptor: K_i = 31.8 nM at 25° and 10 nM at 0°; mutated receptor: K_i = 70.9 nM at 25° and 35.3 nM at 0°).

Figure 3 illustrates the [^{3}H]DPCPX displacement experiments carried out at 0 and 25° using CHO A_1 and CHO A_1 T277A cell membranes. The mutation in the human adenosine A_1 receptor of Thr^{277} by alanine hardly affected antagonist binding, whereas it had dramatic effects on agonist binding (Table 2). Such a substitution only marginally affected the partial agonist interaction with the receptor at 0°, whereas it caused the high affinity binding state to disappear (25°, Table 2).

Figure 2 and data from Table 2 show that [^{3}H]DPCPX K_d values were independent of the human receptor mutation, whereas B_{max} values were dependent on the cell lines. Relatively low levels of expression of the mutant A_1 receptor were observed.

The temperature dependence of the affinity constants $K_A = 1/K_i$ is illustrated by van't Hoff plots (ln K_A versus 1/T). In Fig. 4, data for experiments on rat brain membranes (A), CHO A_1 (B), and CHO A_1 T277A cell membranes (C), respectively are summarized. For the rat brain adenosine A_1 receptors, all van't Hoff plots were linear in the range of temperature investigated and those of CPA (full agonist) displayed a negative slope (i.e. the affinity was enhanced by an increase in temperature) for both high and low affinity binding states. Van't Hoff plots of CPT (antagonist) displayed a positive slope (i.e. the

TABLE 1. [^{3}H]DPCPX binding parameters of rat brain membranes at 0–25°

Ligand		Rat brain adenosine A_1 receptors			
		0°	10°	20°	25°
[^{3}H]DPCPX	K_D (nM)	0.15 ± 0.01	0.18 ± 0.01	0.25 ± 0.02	0.41 ± 0.03
	B_{MAX}	1002 ± 13	968 ± 14	1003 ± 15	918 ± 13
CPA	K_i high (nM)	7.2 ± 1.5	6.6 ± 1.7	2.7 ± 0.5	1.7 ± 0.5
	K_i low (nM)	164 ± 26	127 ± 42	102 ± 10	78 ± 7
	f high	0.34 ± 0.01	0.30 ± 0.02	0.47 ± 0.04	0.44 ± 0.03
8BCPA	K_i high (nM)	—	—	213 ± 18	133 ± 8
	K_i low (nM)	1040 ± 40	1080 ± 70	1600 ± 100	1800 ± 100
	f high	—	—	0.26 ± 0.02	0.19 ± 0.01
CPT	K_i (nM)	2.6 ± 0.2	3.5 ± 0.1	8.5 ± 0.5	9.6 ± 0.3

The parameters are expressed as 1) dissociation constants, K_D (nM) and B_{MAX} (fmol/mg protein) for [^{3}H]DPCPX derived from saturation experiments to adenosine A_1 receptors and 2) inhibitory constants, K_i (nM) for CPA (full agonist), 8BCPA (partial agonist), and CPT (antagonist) obtained on the same system by displacement of [^{3}H]DPCPX. Fractions (*f*) of the high affinity receptor population are reported when computer analysis suggested a two-site rather than a one-site binding model. Values ± SEM are from three independent experiments performed in duplicate.

affinity was enhanced by a decrease in temperature). As far as the partial agonist was concerned, it was necessary to define the meaning of the K_i values corresponding to low temperatures (0 and 10°). In fact, the monophasic inhibition curves indicated that 8BCPA bound to high and low affinity states of the receptor with similar affinity values. On the other hand, it has been reported that adenosine A_1 partial agonists are able to stimulate G protein activation at high temperatures (20 and 25°), but are not able to do so at lower temperatures [30]. Such behavior suggests that at low temperatures adenosine A_1 receptors are in the low affinity state for partial agonists [30]. Therefore, we evaluated the slope of the van't Hoff plot of 8BCPA for the high affinity state with respect to the K_i values at 20 and 25° only. The final thermodynamic parameters for rat brain binding are reported in Table 3. Equilibrium standard enthalpy, $\Delta H°$, and entropy, $\Delta S°$, values show that: 1) the binding of the agonist (CPA) was endothermic and consequently totally entropy driven at both high and low affinity states ($\Delta H° = 39$ kJ mol^{-1}, $\Delta S° = 297$ J mol^{-1} K^{-1} for the high affinity binding state; $\Delta H° = 19$ kJ mol^{-1}, $\Delta S° = 198$ J mol^{-1} K^{-1} for the low affinity binding state); 2) the binding of the antagonist (CPT) was exothermic and essentially enthalpy driven ($\Delta H° = -44$ kJ mol^{-1}, $\Delta S° = 5$ J mol^{-1} K^{-1}); and 3) the binding of the partial agonist (8BCPA) at the low affinity binding state was exothermic and enthalpy–entropy driven ($\Delta H° = -17$ kJ mol^{-1}, $\Delta S^o = 53$ J mol^{-1} K^{-1}). This last behavior could be weakly influenced by a potential presence of a low percentage of the high affinity binding site at low temperatures. Thermodynamic data on partial agonist binding at the high affinity state are not reported since the two temperatures at which a high affinity binding state was discerned (20 and 25°) are too close to permit such an evaluation. Nevertheless, the affinity seemed to be enhanced by an increase in temperature, as already observed for other partial agonists at adenosine receptors [8, 9] under similar experimental conditions (binding experiments performed in the presence of 50 mM of TRIS buffer and in the absence of guanine nucleotides).

The affinity data obtained at 0 and 25° for human adenosine A_1 receptors can be used as input to evaluate whether the slopes of the ln K_A versus 1/T plots of the compounds investigated were positive (indication of an exothermic binding) or negative (indication of an endothermic binding), since linearity of van't Hoff plots appears typical for membrane receptor binding [23]. As reported in Fig. 4B, the binding of CPA at both high and low affinity states of the human adenosine A_1 receptor seemed endothermic (negative slope of van't Hoff plot) and, as a consequence, totally entropy driven. The binding of CPT

TABLE 2. [^{3}H]DPCPX binding parameters of CHO A_1 wild-type and of CHO A_1 T277A cell membranes at 0 and 25°

Ligand		CHO A_1 cells		CHO A_1 T277A cells	
		0°	25°	0°	25°
[^{3}H]DPCPX	K_D (nM)	0.69 ± 0.05	1.59 ± 0.08	0.75 ± 0.07	1.33 ± 0.08
	B_{MAX}	1041 ± 13	968 ± 14	197 ± 11	219 ± 12
CPA	K_i high (nM)	8.6 ± 0.3	2.8 ± 0.2	—	—
	K_i low (nM)	100 ± 8	76 ± 6	33100 ± 1700	8200 ± 700
	f high	0.76 ± 0.02	0.74 ± 0.02	—	—
8BCPA	K_i high (nM)	—	599 ± 45	—	—
	K_i low (nM)	4000 ± 100	7100 ± 200	8300 ± 400	33200 ± 1700
	f high	—	0.49 ± 0.02	—	—
CPT	K_i (nM)	10.0 ± 0.5	31.8 ± 0.6	35.3 ± 1.5	70.9 ± 3.3

The parameters are expressed and obtained as described in Table 1. Values ± SEM are from three independent experiments performed in duplicate.

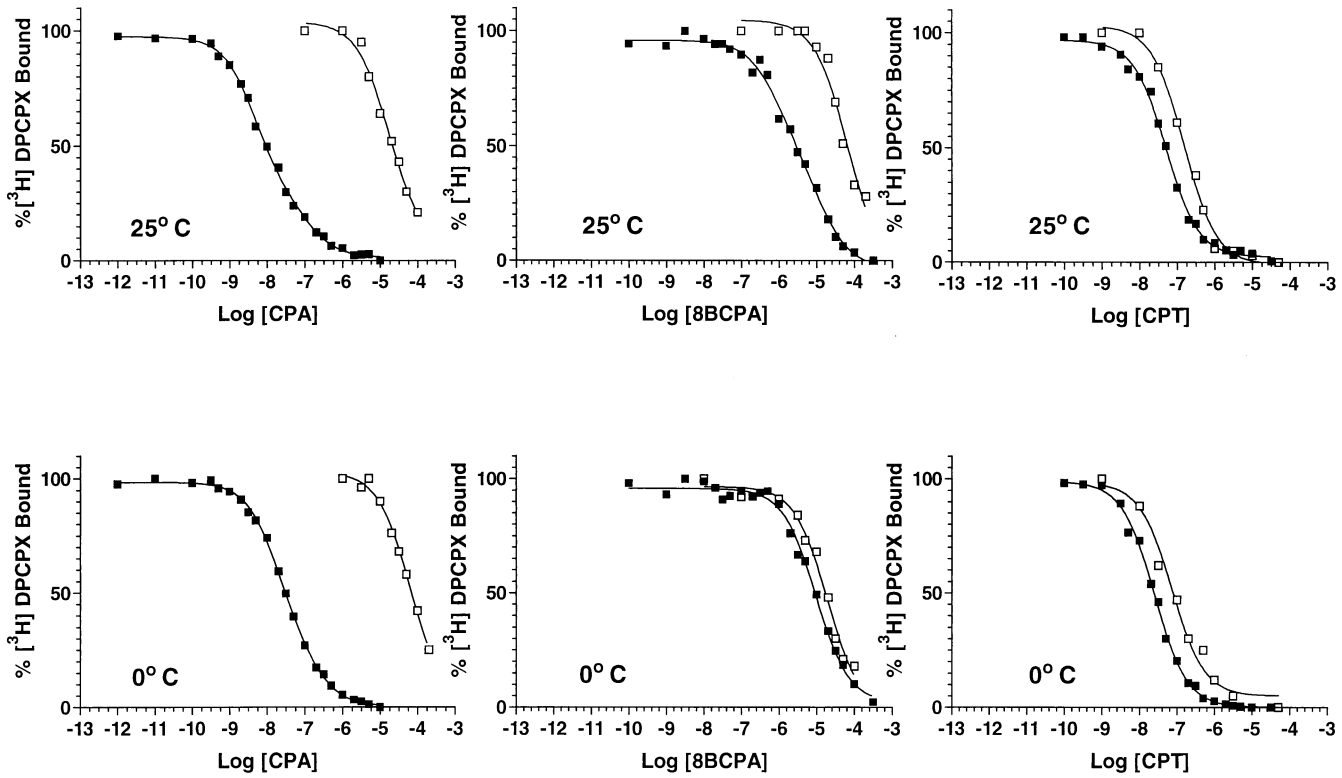

FIG. 3. Competition experiments of CPA (full agonist), 8BCPA (partial agonist), and CPT (antagonist) for specific [^{3}H]DPCPX binding carried out at 0 and 25° on CHO A_1 cell membranes (■) and CHO A_1 T277A cell membranes (□). According to computer analysis of human adenosine A_1 wild-type receptor plots, 1) CPA recognized two affinity binding states at 0 and 25°; 2) 8BCPA recognized two affinity binding states at 25° and one low affinity binding state at 0°; and 3) CPT bound either state with the same affinity at 0 and 25°. Computer analysis of human adenosine A_1 mutated (T277A) receptor plots identified the presence of one affinity binding state for all compounds under investigation.

appeared exothermic (positive slope van't Hoff plots) and therefore essentially enthalpy driven. The binding of 8BCPT at the low affinity state, according to the previous considerations, seemed exothermic as well.

The data in Fig. 4C indicate that the T277A substitution in the human adenosine A_1 receptor hardly affected 8BCPA and CPT binding thermodynamics. The same mutation, however, caused a substantial drop in CPA's affinity for the low affinity binding state, while it did not change CPA's thermodynamic behavior, which remained entropy driven.

DISCUSSION

Data reported in Tables 1 and 2 indicate that the affinities of ligand binding to rat brain and wild-type human adenosine A_1 receptors were very similar. In general, rat brain receptors appeared to interact with affinities that are 1–5 times higher than those observed for the human receptor. The only exception was that of CPA binding at the low affinity state of the receptor, where rat brain K_i values appeared to be slightly higher than those for the human wild-type receptor. These results corroborate and extend earlier findings that ligand affinities are similar on both human and rat brain adenosine A_1 receptors [31].

Thr^{277} appears to play an important role in the interaction of agonists with human wild-type adenosine A_1 receptors. The mutation of Thr^{277} to Ala caused the disappearance of the high affinity receptor state for both CPA (0 and 25°) and 8BCPA (25°). Such a mutation was, moreover, responsible for a drastic increase in the K_i value (more than 100-fold) of the full agonist for the low affinity binding state, as reported in Table 2. The same mutation, however, does not appear to influence the binding of either the antagonist (0 and 25°) or the partial agonist at 0°. Binding experiments previously performed on intact CHO cells stably transfected with wild-type or mutant T277A human adenosine A_1 receptors, showed that the interaction of [^{3}H]DPCPX and of N^6-(R)- phenylisopropyladenosine (an N^6-substituted full agonist) with the receptor was virtually independent of the presence of Thr^{277} (K_i for wild-type receptor 1–4 times lower than K_i for mutant receptor). The same interaction of NECA (a 5′-substituted full agonist), however, was strongly dependent on the presence of Thr^{277} (K_i for wild-type receptor 320 times lower than K_i for mutant receptor) [18]. It was concluded that Thr^{277} forms a molecular contact site with the 5′-substituent of NECA [18]. The CPA data reported in Table 2, however, are not consistent with these conclusions. The presence of Thr^{277} in both high and low affinity conformations of the human adenosine A_1 receptor appears indispensable for binding with the unmodified ribose moiety. These differing conclu-

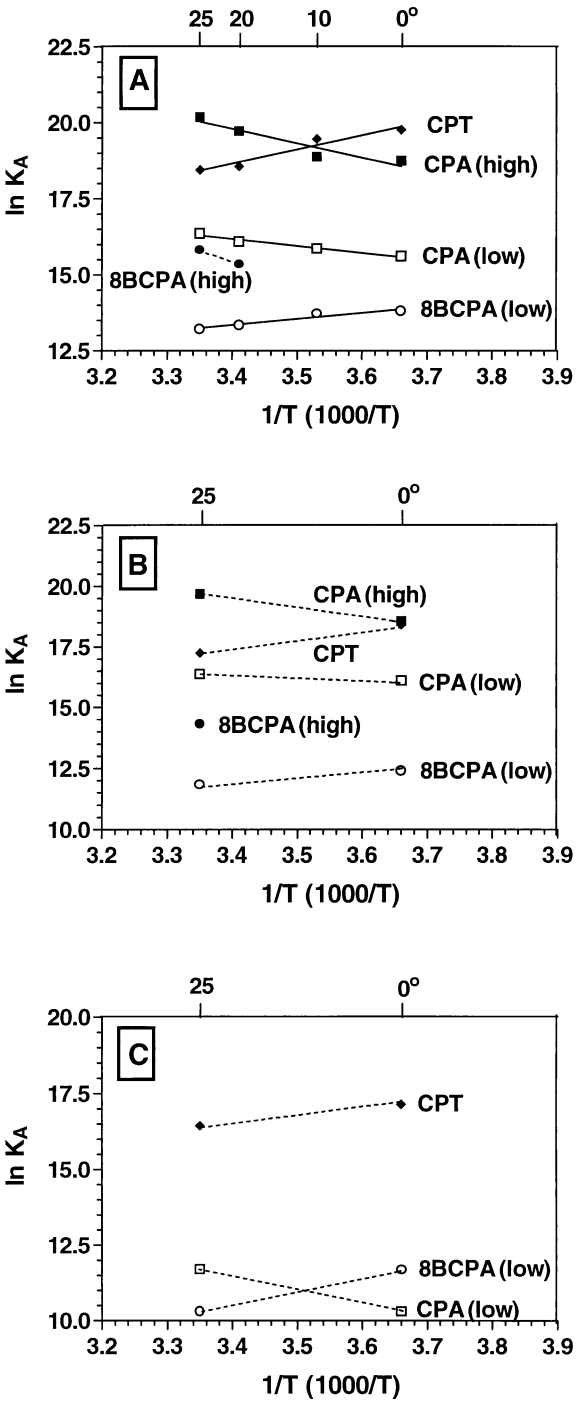

FIG. 4. Van't Hoff plots showing the effect of temperature on the equilibrium binding association constants, K_A, for CPA (full agonist), 8BCPA (partial agonist) and CPT (antagonist) interaction with rat brain (A), CHO A_1 wild-type (B), and CHO A_1 T277A cell (C) membranes, respectively. $K_A = 1/K_i$ values were obtained from [^{3}H]DPCPX competition curves. Linear interpolation over the points connected by the continuous line gives correlation coefficients $r \geq 0.96$. Dashed lines indicate that effect analysis of temperature on K_A permitted a qualitative (slope >0 or <0) rather than a quantitative evaluation of the van't Hoff plot parameters. A positive slope indicates that the binding could be essentially enthalpy driven or enthalpy—entropy driven, whereas a negative slope indicates that the binding was totally entropy driven.

TABLE 3. Thermodynamic parameters for ligand binding to rat brain adenosine A_1 receptor

Drug	ΔG° (kJ mol^{-1})	ΔH° (kJ mol^{-1})	ΔS° (J mol^{-1} K^{-1})
CPA (high)	−49.3 ± 0.2	39 ± 8	297 ± 30
CPA (low)	−40.2 ± 0.3	19 ± 2	198 ± 8
8BCPA (low)	−32.7 ± 0.1	−17 ± 3	53 ± 12
CPT	−45.2 ± 0.1	−44 ± 6	5 ± 22

ΔG°, ΔH° and ΔS° values are given at 298.15 K.

sions may be attributed to dissimilar experimental conditions. Data reported in Table 2 were obtained on membrane preparations, while data for N^6-(R)- phenylisopropyladenosine and NECA were obtained on intact cells.

The thermodynamic data for the rat brain adenosine A_1 receptor reported in Table 3 indicate the binding of CPT (antagonist) to be essentially enthalpy driven, whereas the binding of CPA (full agonist) was completely entropy driven at both high and low affinity receptor states. The thermodynamic discrimination between full agonists and antagonists, revealed under similar experimental conditions (absence of guanine nucleotides) for the high affinity state of the rat brain adenosine A_1 receptor [13, 32, 33] was, therefore, also maintained for the low affinity state. Comparison of Figs. 4A and 4B gives rise to similar conclusions for human adenosine receptors. In this case, it is necessary to assume that the corresponding van't Hoff plots are linear in the range of 0–25°, according to typical behavior of membrane receptors [23].

Both totally entropy-driven agonist binding and essentially enthalpy-driven antagonist binding have been found on other receptors, for example adenosine A_{2A} receptors (GPCRs, [32, 34]), 5-HT_3, and $GABA_A$ receptors (channels, [35, 36]). Agonists and antagonists for the β-adrenergic receptors (a GPCR) also appear to be discriminated from a thermodynamic point of view [37]. Full agonist binding is, in this case, totally enthalpy driven for both high and low affinity binding states, while antagonist binding appears to be both enthalpy- and entropy driven.

Partial agonist (8BCPA) data for the high affinity binding state, as reported in Tables 1 and 2, do not allow an evaluation of thermodynamic parameters. This is due to the potential presence of a low percentage of high affinity states at 0 and 10° for 8BCPA in rat brain and human wild-type receptor, as previously reported in the RESULTS section. It can, nevertheless, be observed that the decrease in affinity with temperature (rat brain receptor) appears to be in accordance with the totally entropy-driven binding found for typical A_1 partial agonists by displacement of [^{3}H]N^6-cyclohexyladenosine [8, 9, 32] under the same experimental conditions (absence of guanine nucleotides).

The binding of 8BCPA at the low affinity receptor state is both enthalpy and entropy driven as reported in Table 3. Similar conclusions, from a qualitative point of view, can be obtained for the human wild-type receptor according to a comparison of Figs. 4A and 4B.

To our knowledge, this is the first report describing a thermodynamic analysis of the contribution of a single amino acid residue to drug-receptor interactions. Thr^{277} does contribute to human wild-type adenosine A_1 receptor binding but only, although not always, in the case of an interaction with ligands which include a ribose moiety. If the presence of Thr^{277} is necessary for this type of interaction, the binding of the wild-type adenosine A_1 receptor seems entirely entropy driven. This is the case for CPA's interaction with either receptor state. If the presence of Thr^{277} is not necessary, the binding seems essentially enthalpy driven. This is the case for CPT's interaction (absence of ribose moiety) with the receptor. Moreover, partial agonist binding (presence of the ribose moiety) does not always appear to depend on the presence of Thr^{277}.

In conclusion, the presence of Thr^{277} in adenosine A_1 receptors contributes to interactions with the ribose moiety of adenosine derivatives. The role of this residue, however, dramatically varies as a function of both the conformational state the receptor and the type of agonist (full or partial) to which the ribose fragment belongs.

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