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# BINDING THERMODYNAMICS OF ADENOSINE $A_{2a}$ RECEPTOR LIGANDS

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**Abstract**—The thermodynamic parameters $\Delta G°$, $\Delta H°$, and $\Delta S°$ of the binding equilibrium of seven adenosine agonists and five xanthine antagonists binding specifically to adenosine $A_{2a}$ receptors were determined by means of affinity measurements at six different temperatures (0, 10, 20, 25, 30 and 35°) and van't Hoff plots. Affinity constants were measured on rat striatum membranes by saturation experiments for the selective $A_{2a}$ agonist 2-[*p*-(carboxy-ethyl)-phenethylamino]-5′-(*N*-ethyl)carboxamidoadenosine ([$^3$H]CGS 21680) and by inhibition assays of [$^3$H]CGS 21680 binding for all other compounds. Scatchard plots were monophasic in the full range of temperatures, indicating a single class of high affinity binding sites whose receptor density, $B_{MAX}$, is essentially temperature independent. Van't Hoff plots were linear in the temperature range 0–30° for agonists and 0–35° for antagonists; their thermodynamic parameters fall, respectively, in the ranges $7 \leq \Delta H° \leq 50$ kJ/mol and $177 \leq \Delta S° \leq 278$ J K$^{-1}$ mol$^{-1}$ and $-36 \leq \Delta H° \leq -7$ kJ/mol and $-33 \leq \Delta S° \leq 94$ J K$^{-1}$ mol$^{-1}$, showing that agonist binding is *entropy-driven* while antagonist binding is *enthalpy-driven*. The results are compared with those already reported for the binding of the same compounds to rat brain *minus* striatum adenosine $A_1$ receptors obtained by displacing [$^3$H]CHA as $A_1$ selective radioligand (Borea PA *et al.*, *Mol Neuropharmacol* **2**: 273–281, 1992). The comparison suggests that the two receptors are very similar as far as their binding sites are concerned and possibly philogenetically related. The analysis of thermodynamical data makes it possible to propose an analogical model of drug–receptor interaction which may account for both affinity and intrinsic activity properties.

*Key words:* binding thermodynamics; adenosine $A_2$ receptor; thermodynamic compensation; drug–receptor interaction modeling

Thermodynamic parameters of the binding equilibrium of drugs to their receptors have aroused increasing interest in recent years due to the information they may provide on binding mechanisms. Because of the very low receptor concentration (1–100 fmol/mg of tissue for a typical neurotransmitter) direct microcalorimetric measurements cannot be performed, at variance with similar studies on DNA [1] or enzyme binding [2]. The only method practicable for receptor studies is that based on binding constant measurements at different temperatures, followed by van't Hoff analysis. Until now, such studies have been carried out on several receptor systems such as β-adrenoceptor [3–7], opiate [8, 9], benzodiazepine [10, 11] and dopamine [12, 13] receptors. Critical appraisals of the method also exist in the literature [14–16].

Adenosine is an endogenous modulator that mediates a variety of effects, such as general depression of CNS (central nervous system) activity, vasodilation, and inhibition of platelet aggregation. These effects appear to be mainly mediated by specific membrane receptors, called $A_1$ and $A_2$ (of which two further subtypes $A_{2a}$ and $A_{2b}$ exist). Upon activation, these receptors cause inhibition ($A_1$) or stimulation ($A_2$) of adenylyl cyclase activity [17, 18].

The thermodynamics of specific binding to rat brain adenosine $A_1$ receptors has already been studied by different authors [19–21], who agree that this is one of the very few receptorial systems for which pharmacological activity is thermodinamically discriminated, in the sense that agonist binding is entropy-driven while xanthine antagonist binding is mainly enthalpy-driven. Based on these thermodynamic data, a novel functional model of adenosine $A_1$ receptor [21, 22] has been proposed. The only other receptor where such thermodynamic discrimination has been ascertained is the β-adrenoceptor, although in this last case the discrimination is reversed, the binding of agonists being enthalpic and that of antagonists mainly entropic [3].

The present paper is the first to deal with the binding thermodynamics of adenosine agonists and xanthine antagonists (Scheme 1) to the adenosine $A_{2a}$ receptor from a thermodynamic point of view. The results are compared with those obtained for the related $A_1$ receptor [21], providing useful information on the binding mechanisms of both receptor subtypes and their possible philogenetic relationships. In addition it will be shown that the use of thermodynamic binding data can be represented in the form of $\Delta H°$ *versus* $-T\Delta S°$ scatter

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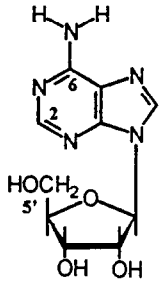
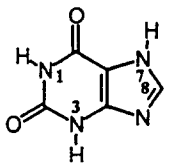


ADENOSINE



XANTHINE

**ANTAGONISTS**

DPCPX= 1,3-dipropyl-8-cyclopenthylxanthine
8-PT= 8-phenyltheophylline
IBMX= 3-isobutyl-1-methylxanthine
Caffeine= 1,3,7-trimethylxanthine
Theophylline= 1,3-dimethylxanthine

**AGONISTS**

CPA= $N^6$-cyclopentyladenosine
CHA= $N^6$-cyclohexyladenosine
R-PIA, S-PIA= R(-), S(+)-$N^6$-(2-phenylisopropyl)adenosine
NECA= 5'-(N-ethyl-carboxamido)adenosine
HE-NECA= 2-hexynyl-5'-(N-ethyl-carboxamido)adenosine
CGS 21680= 2-(p-carboxyethyl-phenethylamino)-5'-(N-ethyl-carboxamido)adenosine

Scheme 1.

plot thereby providing new details on the nature of the forces governing binding at the molecular level.

## MATERIALS AND METHODS

*Materials.* NECA*, *R*-PIA, *S*-PIA, CHA, CPA, CGS 21680, caffeine, theophylline, 8-PT, IBMX and DPCPX were from Research Biochemicals Incorporated (Natick, MA, U.S.A.) HE-NECA was provided by Dr G. Cristalli (University of Camerino, Italy). [$^3$H]CGS 21680 (sp. act. 39.6 Ci/mmol), [$^3$H]-CHA (sp. act. 39.6 Ci/mmol), [$^3$H]CHA (sp. act. 34.4 Ci/mmol) and Aquassure were from NEN Research Products (Boston, MA, U.S.A.). Adenosine deaminase type VI, bovine serum albumin and Trizma base were from the Sigma Chemical Co. (St Louis, MO, U.S.A.).

Male Wistar rats were acquired from Nossan Laboratories (Varese, Italy). All other reagents were of analytical grade and obtained from commercial sources.

*Membrane preparation.* Male Wistar rats (200–250 g) were decapitated and the striatum dissected on ice. The tissue was disrupted in a Polytron homogenizer (Kinematica, Gmbh, Luzern, Switzerland) at a setting of 5 for 30 sec in 25 vol. of 50 mM Tris–HCl, pH 7.4, containing 10 mM $MgCl_2$. The homogenate was centrifuged at 48,000 *g* for 10 min and the pellet was resuspended in the same buffer containing 2 IU/mL of adenosine deaminase. After 30 min incubation at 37° the membranes were centrifuged and pellets were stored at −80°. Prior to freezing, an aliquot of homogenate was removed for protein assay according to a Bio-Rad method [23] with bovine albumin as reference standard.

*Binding assays.* Binding assays [24] were performed on rat striatum membranes in the presence of 10 mM $MgCl_2$ at 0, 10, 20, 25, 30 and 35°, in a thermostatic bath assuring a temperature of ±0.1°. All buffer solutions were adjusted to maintain a constant pH of 7.4 at the desired temperature.

Displacement experiments were performed in 1 mL of Tris–HCl buffer containing 10 mM $MgCl_2$, 5 nM of the selective adenosine $A_{2a}$ receptor ligand [$^3$H]CGS 21680 [24], and membranes from 5 mg (wet weight) of striatum. To determine $IC_{50}$ values (where $IC_{50}$ is the inhibitor concentration displacing 50% of labelled ligand) the test compounds were added in triplicate to binding assay samples at a minimum of six different concentrations. Saturation binding experiments were carried out using 8–12 concentrations of [$^3$H]CGS 21680 ranging from 1 to 150 nM. Separation of bound from free radioligand was performed by rapid filtration through Whatman GF/B filters which were washed three times with ice-cold buffer. Filter-bound radioactivity was measured by scintillation spectrometry after addition of 5 mL of Aquassure. Non-specific binding was defined as binding in the presence of 100 μM NECA; this was always ≤10% of the total binding. Incubation time ranged from 150 min at 0° to 75 min at 35° according to the results of previous time–course experiments. $K_i$ values were calculated from the Cheng–Prusoff equation [25]. All binding data were analysed using the non-linear regression curve-fitting

* Abbreviations: NECA, 5'-(*N*-ethyl)carboxamidoadenosine; *R*-PIA and *S*-PIA, *R*(-) and *S*(+)-$N^6$(2-phenylisopropyl)adenosine; CHA, $N^6$-cyclohexyl adenosine; CPA, $N^6$-cyclopentyladenosine; CGS 21680, 2-[*p*-(carboxy-ethyl)-phenethylamino]-5'-(*N*-ethyl) carboxamidoadenosine; 8-PT, 8-phenyltheophylline; IBMX, 3-isobutyl-1-methylxantine; DPCPX, 1,3-dipropyl-8-cyclopentyl adenosine; HE-NECA, 2-hexynyl-5'-(*N*-ethyl)-carboxamidoadenosine.

computer program LIGAND [26]. The affinities of NECA, CGS 21680 and 2-hexynyl-NECA to adenosine $A_1$ adenosine receptors were also determined on rat brain (minus striatum) homogenates, using 1 nM [$^3$H]CHA as a radioligand according to the Bruns *et al.* method [27]. Data for these three compounds, not previously studied, were necessary for a more complete comparison of $A_1$ and $A_{2a}$ binding.

*Calculations.* For the generic binding equilibrium $L + R = LR$($L$ = ligand, $R$ = receptor), the affinity constant is calculated as $K_A = [LR]/([L][R]) = [LR]/(L_{MAX} - LR][B_{MAX} - LR]) = 1/K_D$, where $[L_{MAX}]$ is the total concentration of the ligand added, $[B_{MAX}]$ the total concentration of the binding sites and $K_D$ the dissociation constant. Since $[LR]/[L_{MAX} - LR]$ = [Bound]/[Free] = $[B_{MAX}]K_A$-$K_A$[Bound], $K_A$ and $B_{MAX}$ values are obtained from the slope and the intercept of the Scatchard plot [Bound]/[Free] *versus* [Bound].

Standard free energy is calculated as $\Delta G° = -RT \ln K_A$ at 298.15 K, and standard enthalpy, $\Delta H°$, from the $\Delta G°$ measurements at different temperatures. To this end two general cases can be distinguished:

(1) the difference in standard specific heats at constant pressure of the equilibrium ($\Delta C_p°$) is nearly zero. In this case the equation ($\delta \ln K_A/\delta(1/T) = -\Delta H°/R$ gives a linear van't Hoff plot ln $K_A$ *versus* $(1/T)$. Standard enthalpy can be calculated from its slope, $-\Delta H°/R$ while standard entropy is calculated as $\Delta S° = (\Delta H° - \Delta G°)/T$ with $T = 298.15$ K and $R = 8.314$ JK$^{-1}$mol$^{-1}$.

(2) $\Delta C_p°$ is different from zero. The plot $\Delta G°$ *versus* $T$ is often parabolic and other mathematical methods [28] for calculating the thermodynamic parameters of the equilibrium are available.

In the present case, where the van't Hoff plots can be considered as being essentially linear (see below), the first method was applied.

## RESULTS

Table 1 reports the values of inhibitory binding constants ($K_i$) measured at the six chosen temperatures and for the selected 11 ligands, by displacement of [$^3$H]CGS 21680 from rat striatum adenosine $A_{2a}$ receptors. $K_D$ and $B_{MAX}$ values, derived from the saturation experiments of [$^3$H]CGS 21680, are also shown. Since $K_i$ values can be regarded as equivalent to the dissociation equilibrium constants ($K_D$) the $K_D$ value measured for [$^3$H]CGS 21680 can be considered to be homogenous with the other tabulated data. In addition, Table 1 reports $K_i$ values for the binding of NECA, CGS 21680 and 2-hexynyl-NECA to adenosine $A_1$ receptors, as measured on rat brain (minus striatum) membranes.

On average, adenosine agonists appear to have a higher affinity, at room temperature, than xanthine antagonists. While inhibitory constants change with temperature (see below), $B_{MAX}$ values obtained from [$^3$H]CGS 21680 saturation experiments appear to be largely independent of it.

Figure 1 illustrates three representative Scatchard plots at 0, 10 and 25° for the saturation equilibrium of [$^3$H]CGS 21680, for which concentrations ranging from 1 to 150 nM were used. The plots are linear at all temperatures investigated and computer analysis of the data [26] failed to show a significantly better fit to a two site than to a one site binding model, indicating that only one class of high affinity binding sites is present under our experimental conditions. Similar conclusions can be drawn from the analysis of the displacement curves concerning all other compounds in Table 1.

The temperature dependence of the affinity constants, $K_A = 1/K_i$, is exemplified by the van't Hoff plots, ln $K_A$ *versus* $1/T$, of Fig. 2 and Fig. 3, which report typical results for four agonists (CPA, *R*-PIA, CGS 21680, HE-NECA) and four xanthine antagonists (caffeine, theophylline, IBMX, 8-PT), respectively.

The Van't Hoff plots appear to be essentially linear in the range 0–30° for agonists and 0–35° for antagonists. Their linearity indicates that $\Delta C_p°$ is nearly zero for both agonists and antagonists. This seems to be a general property of most classical membrane receptors studied so far [29].

In the case of agonists the values of affinity constants show a sudden decrease at 35°, which can be ascribed more to a drop in receptor efficiency than to a systematic curvature of the van't Hoff plot due to $\Delta C_p° \neq 0$. This phenomenon has already been observed [9, 21, 30] and generally ascribed to the fact that, *in vitro*, disrupted membranes may undergo phase transitions with consequent modifications in the receptor conformation at lower temperatures than those physiological for mammals. It is noteworthy that the loss of receptor efficiency below 35° is more apparent for agonists than antagonists in the case of both adenosine $A_1$ and $A_{2a}$ receptors.

Slopes of van't Hoff plots are consistently positive for antagonists, whose affinities decrease with the increase in temperature, and negative for agonists, whose affinities are improved by an increase in temperature (Figs 2 and 3). Final thermodynamic parameters calculated for the binding equilibria of the different compounds investigated are reported in Table 2. $\Delta G°$ values range from $-50.2$ to $-27.2$ kJ/mol for agonists and from $-35.0$ to $-26.2$ kJ/mol for antagonists. Equilibrium standard enthalpy, $\Delta H°$, and entropy, $\Delta S°$, values show that *binding of agonists is totally entropy-driven* ($7 \leq \Delta H° \leq 50$ kJ/mol; $117 \leq \Delta S° \leq 278$ J K$^{-1}$ mol$^{-1}$), while *that of xanthine antagonists is essentially enthalpy-driven* ($-36 \leq \Delta H° \leq -7$ kJ/mol; $-33 \leq \Delta S° \leq 94$ J K$^{-1}$ mol$^{-1}$): i.e. *agonists and antagonists are thermodynamically discriminated.* The scatter plot -T$\Delta S°$ *versus* $\Delta H°$ is shown in Fig. 4, where the present data for adenosine $A_{2a}$ receptors are indicated by filled circles. For the sake of comparison, the corresponding data for adenosine $A_1$ receptors are also shown as open circles, these last values being taken from Borea *et al.* [21], with the exception of compounds **1'** (CGS 21680), **4'** (HE-NECA) and **5'** (NECA) whose thermodynamical parameters are given in the present study (Table 2). The standard deviations estimated for the present measurements are 3–7 kJ/mol for $\Delta H°$ and 9–23 J K$^{-1}$ mol$^{-1}$ for $\Delta S°$. The thermodynamic discrimination observed between agonists and antagonists (Fig. 4) is therefore considered as highly significant for both $A_1$ and $A_{2a}$ receptors. Since there has been much discussion on

Table 1. Equilibrium binding parameters at six different temperatures

| | Ligand T(°K): | 273 | 283 | 293 | 298 | 303 | 308 |
|---|---|---|---|---|---|---|---|
| (a) Adenosine $A_{2a}$ receptor agonists | | | | | | | |
| **1.** | [$^3$H]CGS 21680 | $K_D$ = 47(±3) | 24(±1) | 18(±1) | 15(±1) | 14(±1) | 21(±1) |
| | | $B_{MAX}$ = 277(±19) | 210(±15) | 264(±18) | 223(±16) | 216(±15) | 202(±20) |
| **2.** | CPA | 3210(±220) | 1121(±50) | 920(±50) | 620(±30) | 310(±20) | 630(±30) |
| **3.** | CHA | 5822(±310) | 1454(±70) | 1230(±60) | 820(±50) | 490(±30) | 740(±40) |
| **4.** | HE-NECA | 6.4(±0.3) | 4.2(±0.2) | 3.6(±0.2) | 3.2(±0.2) | 2.4(±0.1) | 3.6(±0.2) |
| **5.** | NECA | 11.1(±0.5) | 9.3(±0.5) | 8.5(±0.4) | 8.3(±0.4) | 9.2(±0.5) | 12.3(±0.5) |
| **6.** | *R*-PIA | 1280(±60) | 680(±40) | 310(±20) | 240(±10) | 230(±10) | 350(±20) |
| **7.** | *S*-PIA | 47,100(±2400) | 13,900(±710) | 7900(±400) | 7120(±430) | 5010(±320) | 7620(±440) |
| (b) Adenosine $A_{2a}$ receptor antagonists | | | | | | | |
| **8.** | Caffeine | 6500(±300) | 8600(±400) | 19,800(±930) | 24,000(±1000) | 28,100(±1200) | 27,000(±1000) |
| **9.** | Theophylline | 2600(±100) | 3100(±150) | 5400(±300) | 6700(±400) | 6800(±400) | 5900(±300) |
| **10.** | DPCPX | 450(±20) | 520(±30) | 540(±30) | 550(±30) | 660(±40) | 890(±50) |
| **11.** | IBMX | 1800(±100) | 2100(±120) | 4100(±200) | 4210(±220) | 4940(±320) | 4810(±330) |
| **12.** | 8-PT | 1290(±70) | 1390(±80) | 1650(±80) | 2040(±90) | 1800(±80) | 1840(±90) |
| (c) Adenosine $A_1$ receptor agonists | | | | | | | |
| **1′.** | CGS 21680 | 2700(±140) | 920(±50) | 820(±40) | 820(±30) | 720(±30) | 1210(±73) |
| **4′.** | HE-NECA | 1400(±70) | 600(±30) | 520(±30) | 310(±20) | 220(±10) | 820(±40) |
| **5′.** | NECA | 16.5(±0.9) | 12.0(±0.7) | 9.2(±0.6) | 8.5(±0.4) | 7.6(±0.4) | 18.0(±0.9) |

(1) Dissociation constants $K_D$ (nM) and $B_{MAX}$ (fmol/mg protein) for compound **1** ([$^3$H]CGS 21680) derived from saturation experiments to rat striatum adenosine $A_{2a}$ receptors; (2) inhibitory constants $K_i$ (nM) for compounds **2–12** obtained by displacement of [$^3$H]CGS 21680 from the same receptors; and (3) inhibitory constants $K_i$ (nM) obtained by displacement of [$^3$H]CHA from rat brain minus striatum adenosine $A_1$ receptors.

Values are means of at least four experiments. Estimated standard deviations are in parentheses.

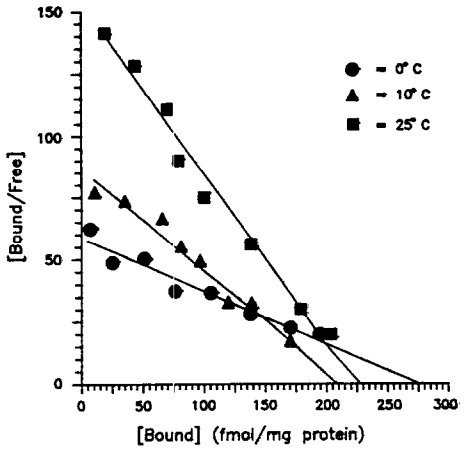



Fig. 1. Representative Scatchard plots for [$^3$H]CGS 21680 binding to rat striatum membranes at 0, 10 and 25°. The linearity of the plots indicates the presence of a single class of high affinity binding sites at all temperatures investigated. Correlation coefficients are systematically ≥0.98.

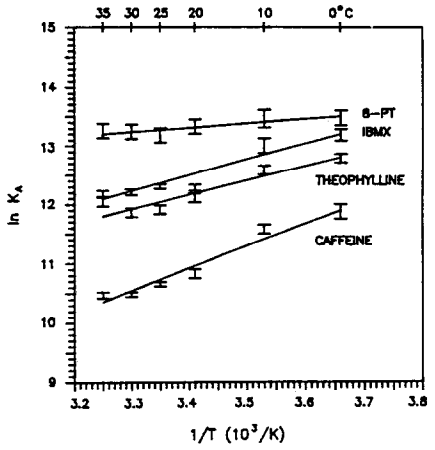



Fig. 3. Representative van't Hoff plots showing the effect of temperature on the equilibrium binding association constants, $K_A$, for a selection of four xanthine antagonists. All plots are essentially linear ($r \geq 0.92$) in the temperature range of 0–35°.

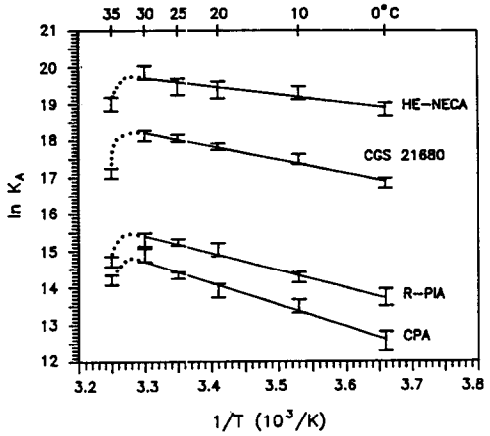



Fig. 2. Representative van't Hoff plots showing the effect of temperature on the equilibrium binding association constants, $K_A$, for a selection of four adenosine agonists. All plots are essentially linear ($r \geq 0.93$) in the temperature range of 0–30°.

error transmission in this type of measurement [31, 32], we expanded the portion of Fig. 4 concerned with the binding of agonists displaying confidence intervals at ±1 estimated standard deviation in Fig 5. The errors on $\Delta H°$, $\Delta(\Delta H°)$ and $\Delta S°$, $\Delta(\Delta S°)$, are linearly related because of the calculation method according to the equation $\Delta(\Delta S°) = (1/\beta')\,\Delta(\Delta H°)$ where $\beta'$ is the average experimental temperature [31, 32]. Accordingly, such errors can be represented as the short segments with slope $\beta'$ in Fig. 5 (more precisely $\Delta(\Delta H°)$ and $\Delta(\Delta S°)$ are the projections of the diagonal segments on the $\Delta H°$ and $\Delta S°$ axes). Moreover, the thickness of the segments is a measure of $\Delta(\Delta G°)$ and is very thin because such error is definitely smaller (see Table 2). This underlines the fact that the thermodynamical discrimination between agonists and antagonists holds up well even on a more accurate statistical basis.

## DISCUSSION

A preliminary analysis of Fig. 4 reveals that the thermodynamic parameters for $A_{2a}$ binding are similar to those for $A_1$ binding [21]. The thermodynamic discrimination is the same since for both $A_1$ and $A_{2a}$ receptors the binding of agonists is *entropy-driven* and that of antagonists is mostly *enthalpy-driven*. Moreover, data for both receptors appear to be arranged on a same regression line according to the equation

$$T\Delta S°(\text{kJ/mol at } 298.15\text{ K}) = 37(\pm 2) + 1.14(\pm 0.06)\Delta H°(\text{kJ/mol})$$
$$(n = 24,\ r = -0.971,\ s = 8.84,\ P \leq 0.01).$$

This equation can be rewritten as $\Delta H° = \beta \cdot \Delta S°$, which is the form for a case of enthalpy–entropy compensation [32, 33] with a compensation temperature of 262° K. This phenomenon seems to be a common feature in all cases of drug–receptor binding [29] and its biological implications have been discussed by different authors [14, 32].

The similarity between adenosine $A_1$ and $A_{2a}$ receptors is in indirect agreement with the finding that both are characterized by the presence of two hystidyl residues ($HIS^{250}$ and $HIS^{271}$ in canine adenosine $A_1$ and $A_2$ receptor sequences [34]) which are indispensable for agonist and antagonist receptor binding [35] and which, accordingly, are assumed to be located in the receptor binding cavity. This may suggest that they are not philogenetically independent in the sense that they may derive from a common and not very dissimilar ancestor.

There are, however, differences between the

Table 2. Thermodynamic parameters for the equilibrium of binding of agonists and antagonists to adenosine $A_{2a}$ and $A_1$ receptors $\Delta G°$, $\Delta H°$ and $\Delta S°$ values are given at 298.15° K

| | Ligand | $\Delta G°$ (KJ/mol) | $\Delta H°$ (KJ/mol) | $\Delta S°$ (J/mol/degree) |
|---|---|---|---|---|
| (a) Adenosine $A_{2a}$ receptor agonists | | | | |
| **1.** | [$^3$H]CGS 21680 | −44.9(±0.1) | 27(±3) | 241(±9) |
| **2.** | CPA | −36.2(±0.2) | 44(±5) | 269(±18) |
| **3.** | CHA | −34.9(±0.3) | 48(±7) | 278(±23) |
| **4.** | HE-NECA | −50.2(±0.1) | 19(±4) | 232(±15) |
| **5.** | NECA | −45.8(±0.2) | 7(±5) | 117(±19) |
| **6.** | *R*-PIA | −35.3(±0.1) | 41(±6) | 258(±20) |
| **7.** | *S*-PIA | −27.2(±0.3) | 50(±5) | 259(±18) |
| (b) Adenosine $A_{2a}$ receptor antagonists | | | | |
| **8.** | Caffeine | −26.2(±0.2) | −36(±3) | −33(±11) |
| **9.** | Theophylline | −29.5(±0.1) | −25(±3) | 14(±11) |
| **10.** | DPCPX | −35.0(±0.2) | −7(±5) | 94(±18) |
| **11.** | IBMX | −30.4(±0.1) | −25(±3) | 18(±9) |
| **12.** | 8PT | −32.6(±0.1) | −9(±2) | 79(±10) |
| (c) Adenosine $A_1$ receptor agonists | | | | |
| **1′.** | CGS 21680 | −34.7(±0.3) | 26(±6) | 205(±21) |
| **4′.** | HE-NECA | −36.9(±0.3) | 38(±5) | 252(±19) |
| **5′.** | NECA | −46.7(±0.2) | 20(±5) | 220(±18) |

Estimated standard deviations are in parentheses.

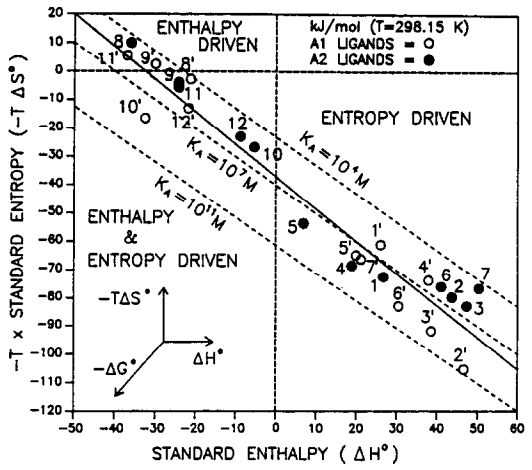



Fig. 4. Scatter plot of $-T\Delta S°$ *versus* $\Delta H°$ values for the adenosine $A_{2a}$ receptor ligands studied in the present paper (filled circles). Values for the binding to the adenosine $A_1$ receptors are reported for comparison (open circles). All points lie on the same regression (continuous) line having the equation $T\Delta S° = 37(\pm 2) + 1.14(\pm 0.06)\ \Delta H°$ ($N = 24$, $R = 0.971$), showing the definite effect of enthalpy–entropy compensation. The three dashed lines indicate the *loci* of the points representing possible combinations of $\Delta H°$ and $-T\Delta S°$ values giving rise to three different association constants ($K_A = 10^4$, $10^7$ and $10^{11}$ M).

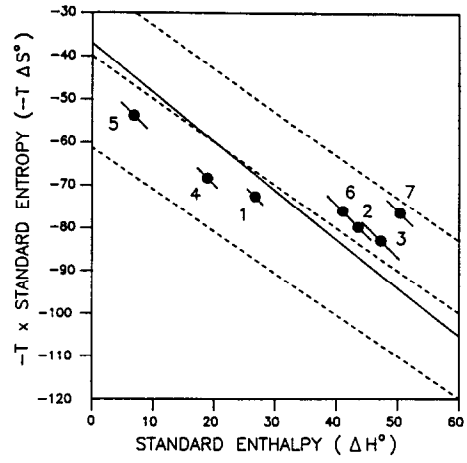
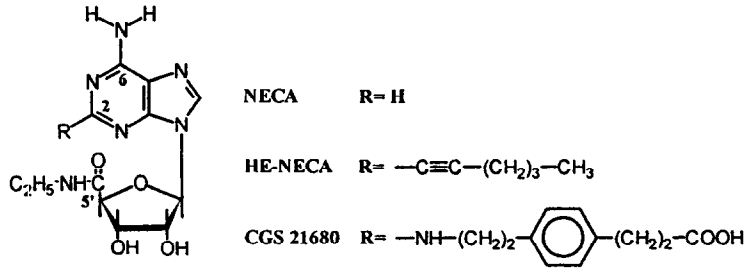



Fig. 5. Enlarged view of the part of Fig. 4 reporting $\Delta H°$ and $-T\Delta S°$ values for adenosine $A_{2a}$ receptor agonists together with their confidence intervals. These take the shape of thin diagonal segments because of the error transmission mechanism acting in this type of measurement (see above). Confidence intervals for $A_{2a}$ antagonists and $A_1$ agonists and antagonists are strictly comparable to those shown.

thermodynamic properties of the two receptors which are of some relevance for their pharmacologic implications. Such differences can be better appreciated in light of the actual meaning of the graphical representation given in Fig. 4. The plot is obviously the $-T\Delta S°$ *versus* $\Delta H°$ type, where any shift along the main diagonal towards the lower-right corner indicates a more entropy-driven and less enthalpy-driven process. Because of the linear dependence of $\Delta H°$, $\Delta S°$ and $\Delta G°$ ($\Delta G° = \Delta H° - T\Delta S°$), however, it contains further information on $\Delta G°$ or $K_A$ ($\Delta G° = -RT \ln K_A$) values of the




Scheme 2.

binding equilibrium. This is graphically represented in the bottom left corner of Fig. 4, where $-\Delta G° = -(\Delta H° - T\Delta S°)$ is the vector pointing downwards and nearly perpendicular to the main diagonal of the plot. The association constants increase in this direction while decreasing in the opposite one. The same value of $\Delta G°$ (and therefore of $K_A$) can be produced by all the linear combinations of different $\Delta H°$ and $-T\Delta S°$ pairs lying on a straight diagonal line on the plot. For example, the three dashed lines of Fig. 4 indicate the *loci* of the points corresponding to three different values of $K_A$ at the average experimental temperature of 298° K: $K_A = 10^4$, $K_A = 10^7$ and $K_A = 10^{11}$ M. In approximate terms, these represent the minimum, average and maximum values of $K_A$ for the present set of data.

The analysis of Fig. 4 shows that the binding of $A_2$ xanthine antagonists (compounds **8–12**) is, with the exception of caffeine (**8** and **8′**), more entropic and less enthalpic than that of $A_1$ antagonists, since the representative points of the former are shifted towards the bottom-right corner. This indicates that the binding site moiety interacting with the antagonists is less hydrophilic in adenosine $A_{2a}$ receptors because of a decreased ability to form one or more hydrogen bonds. The association constants, however, are only slightly modified, since the points essentially remain on the same diagonal line of constant $K_A$. The only exceptions are compounds **10** (DPCPX) and **12** (8-PT) which are far better binders for the $A_1$ than for the $A_{2a}$ receptor, consistent with their selectivity for the $A_1$ binding site.

A first observation on agonists concerns the different behaviour of compound **5** (NECA) with respect to compound **4** (HE-NECA) which is a 2-hexynyl NECA derivative (Scheme 2). Data in Table 2 and Fig. 4 show that the introduction of the hexynyl group makes the binding more entropic and more efficient. This is in agreement with previous suggestions that a specific hydrophobic accessory binding site for the 2-substituent (AS2) is present in $A_{2a}$ but not in $A_1$ receptors, as schematically illustrated by the binding site models of Fig. 6a and b.

The most remarkable differences between $A_1$ and $A_{2a}$ receptors are elucidated by the comparison of thermodynamical data of $N^6$-substituted agonists (compounds **1–7** of Table 2, displayed in the lower-right corner of Fig. 4). It has already been reported [22] that $N^6$-substituted $A_1$ agonists show a typical

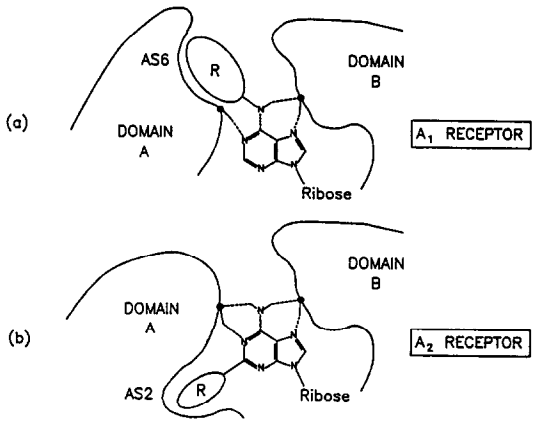



Fig. 6. A hypothetical model of adenosine $A_1$ (a) and $A_{2a}$ (b) receptors in the part deputed to agonistic binding. A more comprehensive model has been discussed elsewhere [21]. In both cases binding of the agonist is assumed to produce its biological effect by bringing the A and B domains of the receptor together. The attraction forces are determined by the hydrogen bond net shown in (b) for the endogenous adenosine ligand, which is substituted or reinforced by the AS6 or AS2 hydrophobic accessory sites for $A_1$ or $A_{2a}$ selective ligands, respectively. The two sites are named according to the substitution in the adenosine molecule ($N^6$ for $A_1$ and $C^2$ for $A_{2a}$ selective ligands). (●) = X – H (X = nitrogen or oxygen atom).

pattern of binding properties for which *affinity constants* and *entropy-driving character* increase together in the order **7′** (*S*-PIA) <**6′** (*R*-PIA) <**3′** (CHA) <**2′** (CPA), as clearly illustrated by the positions of their representative points in Fig. 4 (open circles). This has been ascribed to the presence of the $A_1$ accessory binding site, AS6 (sketched in Fig. 6a) which is of fundamental importance for determining both binding and intrinsic activity properties [22]. This accessory site is not present in the $A_{2a}$ receptor, Fig. 6b, as can be shown by analysing Figs 4 and 5. The points representing the $N^6$-substituted ligands to the $A_{2a}$ receptor (full circles marked 2, 3, 6 and 7) now cluster together and are no longer differentiated by the nature of the $N^6$-substituent. Moreover, they are displaced backwards with respect to the dashed diagonal line corresponding

to the average association constant ($K_A = 10^7$ M); that is, they become much weaker binders for the $A_{2a}$ than for the $A_1$ receptors.

In general binding equilibrium thermodynamic data (Table 2 and Fig. 4) agree in indicating that the adenosine $A_1$ and $A_{2a}$ receptors are very similar as far as their binding sites are concerned, indicating that they may be related from a philogenetic point of view. The two receptorial populations differ in the positions of two different hydrophobic accessory binding sites (AS6 and AS2 in the sketches of Fig. 6a and b, respectively) which are believed to be responsible for $A_1/A_{2a}$ selectivity properties [34]. It seems of interest that these accessory sites do not have any physiological role *per se* in evolutionary control, because the endogenous ligand, adenosine, cannot gain any advantage in binding to them. The fact that the accessory binding sites are not causally connected with the functional features of the adenosine binding site itself could explain, at least in part, why adenosine $A_1$ receptor ligands often display dissimilar binding parameters in different mammalian species [36, 37].

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