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Pharmacological and biochemical characterization of purified A_{2a} adenosine receptors in human platelet membranes by [^{3}H]-CGS 21680 binding

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1 The binding properties of human platelet A_{2a} adenosine receptors, assayed with the A_{2a}-selective agonist, [^{3}H]-2-[*p*-(2-carboxyethyl)-phenethylamino]-5'-N-ethylcarboxamidoadenosine ([^{3}H]-CGS 21680), are masked by a non-receptorial component, the adenotin site. In order to separate A_{2a} receptors from adenotin sites, human platelet membranes were solubilized with 1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulphonate (CHAPS). The soluble platelet extract was precipitated with polyethylene glycol (PEG) and the fraction enriched in adenosine receptors was isolated from the precipitate by differential centrifugation.

2 The present paper describes the binding characteristics of the selective A_{2a} agonist, [^{3}H]-CGS 21680, to this purified platelet membrane preparation. In addition, receptor affinity and potency of several adenosine agonists and antagonists were determined in binding and adenylyl cyclase studies.

3 Saturation experiments revealed a single class of binding site with K_d and B_{max} values of 285 nM and 2.07 pmol mg^{-1} of protein respectively. Adenosine receptor ligands competed for the binding of 50 nM [^{3}H]-CGS 21680 to purified protein, showing a rank order of potency consistent with that typically found for interactions with the A_{2a} adenosine receptors. In the adenylyl cyclase assay the compounds examined exhibited a rank order of potency very close to that observed in binding experiments.

4 Thermodynamic data indicated that [^{3}H]-CGS 21680 binding to the purified receptor is totally entropy-driven in agreement with results obtained in rat striatal A_{2a} adenosine receptors.

5 It is concluded that in the purified platelet membranes there is a CGS 21680 binding site showing the characteristic properties of the A_{2a} receptor. This makes it possible to use this compound for reliable radioligand binding studies on the A_{2a} adenosine receptor of human platelets.

Keywords: [^{3}H]-CGS 21680 binding; human platelet membranes; purified A_{2a} adenosine receptors; cyclic AMP assays; thermodynamic analysis

Introduction

Adenosine modulates a variety of physiological functions acting via specific cell surface receptors which were initially divided into two subtypes, A_1 and A_2, on the basis of their ability to inhibit or stimulate adenylyl cyclase activity, respectively (Van Calker *et al.*, 1979). Molecular biology techniques indicate that at least four adenosine receptor subtypes exist: A_1, A_{2a}, A_{2b} and A_3 (Fredholm *et al.*, 1994; Linden *et al.*, 1994). The A_{2a} receptor subtype which is coupled to stimulation of adenylyl cyclase, is a high affinity receptor found in large amounts in the brain striatum (Bruns *et al.*, 1986). In the periphery, the A_{2a} receptor is localized in different organs and tissues, including platelets, neutrophils and vascular smooth muscle cells. For many years, brain A_{2a} receptors were characterized by use of the non selective agonist, [^{3}H]-NECA, which, however, was found to bind to different states and/or subtypes of the adenosine receptors as well as to other non-specific proteins (Hütteman *et al.*, 1984; Yeung & Green 1984; Bruns *et al.*, 1986; Lohse *et al.*, 1988). An additional group of non-receptor low affinity binding sites, named adenotin (Hutchison *et al.*, 1990; Schwabe *et al.*, 1993), impede the direct characterization of A_{2a} receptors in cell membranes of several mammalian tissues (Nakata & Fujisawa, 1988). The only high affinity and high selectivity ligand able to interact with A_{2a} receptors appears to be [^{3}H]-CGS 21680 (Jarvis *et al.*, 1989). CGS 21680 plays an important role in pharmacological subclassification of A_2 receptors and it has been suggested that it can discriminate between A_{2a} and A_{2b} receptors (Fredholm *et al.*, 1994). This ligand has been widely used to characterize A_{2a} binding sites on striatal membranes but few studies exist concerning its use in peripheral tissues (Varani *et al.*, 1994). Recent data suggest that CGS 21680 also acts on the A_{2a} receptors present on the human platelet membranes, but this interaction is masked by the presence of adenotin sites (Varani *et al.*, 1994). To overcome the many difficulties associated with this interaction between the A_{2a} adenosine receptors and adenotin sites, two approaches have been used to separate them: gel filtration (Lohse *et al.*, 1988; Fein *et al.*, 1994) and CHAPS solubilization followed by PEG precipitation and differential centrifugation (Zolnierowicz *et al.*, 1990). With this background, it is clear that a key advance in understanding the function of the A_{2a} receptors can be made with a specific substrate, such as platelets (Dionisotti *et al.*, 1992; Cooper *et al.*, 1995; Ongini *et al.*, 1995), where it is possible to examine both affinity and potency of adenosine ligands. In platelets, A_{2a} agonists inhibit aggregation (Dionisotti *et al.*, 1992) and stimulate the formation of the second messenger, cyclic AMP (Jacobson *et al.*, 1993). Recently other authors (Cristalli *et al.*, 1994a) have raised some doubt about the nature of A_{2a} platelet receptors.

The present paper is devoted to the pharmacological, biochemical and thermodynamic characterization of A_{2a} adenosine receptor in human platelets purified by CHAPS extraction of membranes and PEG precipitation. The purified receptor has been characterized by evaluating affinity constants of ten agonists and seven antagonists by the use, for the first time, of [^{3}H]-CGS 21680 as a selective A_{2a} radioligand, and the affinity values were compared with those obtained in platelet and striatal membranes. Moreover, the capabilities of the agonists

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to increase the cyclic AMP intracellular levels and the antagonist potency in inhibiting NECA-stimulated adenylyl cyclase activity have been evaluated. Finally, with the aim of obtaining new insights on the forces driving the coupling of A_{2a} human platelet receptor with a selective ligand, a thermodynamic analysis of the [^{3}H]-CGS 21680 binding has been performed and the enthalpic ($\Delta H°$) and entropic ($\Delta S°$) contribution to the standard free energy ($\Delta G°$) of the binding equilibrium were determined.

Methods

Human platelet membrane preparation

Membranes were obtained from platelet-rich plasma (PRP), supplied by healthy human volunteers; the platelets were prepared from blood previously anticoagulated and stabilized with ACD (1.4% citric acid, 2.5% sodium citrate, 2% D-glucose). Membranes for binding studies were prepared essentially according to the method of Schloos *et al.* (1987). PRP was centrifuged in polypropylene tubes at 17,500 *g* for 15 min at 25°C in a Sorvall type SS34 rotor. The platelets were then resuspended in 2/3 of the original volume in Tris-buffer A (50 mM Tris-HCl, 20 mM EDTA, 150 mM NaCl; pH 7.4 at 25°C) and recentrifuged at 17,500 *g* for 15 min at 25°C. Thereafter, the supernatant was discarded and the washed platelets were suspended in ice-cold hypotonic buffer B (5 mM Tris-HCl, 5 mM EDTA; pH 7.4 at 4°C) and homogenized with a Polytron for 30 s before centrifugation at 35,000 *g* for 15 min at 4°C. After a further resuspension and centrifugation (35,000 *g*, 15 min, 4°C) in ice-cold Tris buffer C (50 mM Tris HCl, 0.8 mM EDTA, pH 7.4 at 4°C) the resulting pellet was resuspended in 1/4 of the original PRP volume in the same buffer.

Partial purification of A_{2a} adenosine receptors

Human platelet membranes were prepared as described above and partially purified receptors were obtained essentially according to Zolnierowicz *et al.* (1990). The pellet (20 mg of protein per 10 ml) was homogenized with a Dounce homogenizer in 20 ml of 50 mM Tris HCl, pH 7.4, 10 mM $MgCl_2$, 2 mM EDTA, 0.1 mM phenylmethylsulphonyl fluoride (buffer E), and centrifuged at 48,000 *g* for 20 min. The pellet was suspended in buffer E (20 ml) and extracted with an equal volume of 2% CHAPS in buffer E (final concentration, 1% CHAPS) using a Dounce homogenizer (five strokes up and down). The mixture was stirred on ice for 30 min with a Teflon stirring bar. The suspension was spun at 48,000 *g* (20,000 r.p.m.) for 10 min in a Sorvall RC-5B centrifuge, the supernatant was collected and precipitated by the addition of 50% PEG in buffer E to a final concentration of 10%. After stirring for 30 min on ice, the precipitate was centrifuged at 48,000 *g* for 20 min as before. The PEG precipitate was homogenized with 10 ml of buffer E and spun at 1,000 *g* (3,000 r.p.m.) for 3 min in a Sorvall RC-5B centrifuge, and the supernatant was collected. The pellet was homogenized again in 10 ml of buffer E and spun for 3 min at 1,000 *g*. The supernatant, added to the previous one, was sedimented by spinning at 48,000 *g* for 20 min. The resulting supernatant, rich in adenosine A_2-like binding protein, was discarded. The pellet, a source of platelet A_{2a} adenosine receptor, was resuspended to obtain approximately 0.4 mg of protein ml^{-1} in the same buffer used in the binding experiments (buffer F). The protein concentration was determined according to a Bio-Rad method (Bradford, 1976) with bovine albumin as reference standard.

[^{3}H]-CGS 21680 binding assay in the platelet membranes

Binding assays were carried out essentially according to Varani *et al.* (1994). Membrane suspensions were washed three times in ice-cold Tris-buffer C with intervening centrifugations at 35,000 *g* for 15 min at 4°C. The pellet was finally resuspended in the assay buffer D, containing 50 mM Tris HCl, 0.8 mM EDTA and 10 mM $MgCl_2$ (pH 7.4 at 4°C), at a final concentration of 1 mg of protein ml^{-1} and assays were performed in a total volume of 250 μl, which consisted of 100 μl membrane suspension and 50 or 200 nM of [^{3}H]-CGS 21680 were used for inhibition experiments. Identical results were obtained by addition to the assay buffer of adenosine deaminase (1 iu ml^{-1}), as already observed by Hütteman *et al.* (1984). To determine the IC_{50} values of the adenosine ligands, solutions of each ligand were added in triplicate to the binding assay samples at a minimum of six different concentrations. The temperature-dependence of [^{3}H]-CGS 21680 binding to human platelets exhibited the highest specific binding at 0°C in agreement with the fact that the binding to high amounts of adenotin is detectable only at very low temperatures (Hütteman *et al.*, 1984). After a 120 min incubation at 0°C, the samples were centrifuged at 0°C for 3 min at 12,000 *g* in a Beckman microcentrifuge and the tip of the microcentrifuge tube containing the particulate pellet was cut off, transferred to vials containing 5 ml of Aquassure and counted by scintillation spectrometry. A centrifugation assay was used instead of a filtration assay because of the low affinity constant (in the μmolar range) of [^{3}H]-CGS 21680 for platelet membranes. Non-specific binding was defined as binding in the presence of 100 μM CGS 21680 and was about 35% of total binding.

[^{3}H]-CGS 21680 binding assay in the partially purified receptor

The binding of [^{3}H]-CGS 21680 to solubilized binding sites was measured in a total volume of 250 μl containing 50 mM Tris HCl pH 7.4, 10 mM $MgCl_2$, 2 mM EDTA and 1.0 unit of adenosine deaminase ml^{-1} (buffer F). In saturation studies, the solubilized A_{2a} receptors (40 μg protein 100 μl^{-1}) were incubated with 8 to 10 different concentrations of [^{3}H]-CGS 21680 ranging from 0.02 to 1 μM. In competition experiments, carried out to determine the IC_{50} values, 50 nM [^{3}H]-CGS 21680 was incubated with 100 μl of solubilized binding sites and 8 to 10 different concentrations of each of the adenosine agonists or antagonists examined. To identify A_{2a} adenosine receptors in platelet membranes, [^{3}H]-CGS 21680 binding was performed separately estimating the non-specific binding either with 100 μM CGS 21680 or 100 μM **R**(−)-N^6-(2-phenyl-isopropyl)-adenosine (**R**-PIA); its value was always $\leq$15% of the total binding. The same results were obtained in the presence of 100 μM NECA. [^{3}H]-CGS 21680 bound to the adenosine A_2-like binding protein was insensitive to displacement by 100 μM (**R**)-PIA as previously reported by Lohse *et al.* (1988). Accordingly, we used the ratio of [^{3}H]-CGS 21680 radioactivity displaceable by 100 μM (**R**)-PIA to [^{3}H]-CGS 21680 radioactivity displaceable by 100 μM CGS 21680 to indicate the relative abundance of A_{2a} adenosine receptors. Moreover, the inhibitory binding constant (K_i) values were calculated from the IC_{50} values according to the Cheng & Prusoff equation (Cheng & Prusoff, 1973), $K_i = IC_{50}/(1 + [C^*]/K_d)$, where [C*] is the concentration of the radioligand and K_d its dissociation constant. The binding of [^{3}H]-CGS 21680 to purified adenosine receptor was scarcely temperature-dependent as expected in the presence of a predominance of A_{2a} receptors with respect to adenotin (Lohse *et al.*, 1988). The incubation time was 90 min at 25°C according to the results of previous time-course experiments. Bound and free radioactivity were separated by filtering the assay mixture through Whatman GF/C glass-fibre filters that had been treated with 0.3% polyethyleneimine for at least 90 min (Bruns *et al.*, 1983). The incubation mixture was diluted with 3 ml of ice-cold incubation buffer, rapidly vacuum filtered and the filter was washed three times with 3 ml of incubation buffer. The filter-bound radioactivity was counted in a Beckman LS-1800 Spectrometer (efficiency 55%). A weighted non linear least-squares curve fitting programme LIGAND (Munson & Rod-

bard, 1980), was used for computer analysis of saturation and inhibition experiments.

Measurement of cyclic AMP levels in human platelets

Washed human platelets obtained from the peripheral blood of healthy volunteers were prepared as described by Korth *et al.* (1988). The final suspending medium was a Tyrode buffer, pH 7.4, of the following composition (mM): NaCl 137, KCl 2.68, $NaHCO_3$ 11.9, $MgCl_2$ 1.0, NaH_2PO_4 0.4, glucose 5.5. Platelets ($6-8 \times 10^4$ cells) were suspended in 0.5 ml incubation mixture (Tyrode buffer containing bovine serum albumin (BSA) 0.25%, 1.0 unit of adenosine deaminase ml^{-1} and 0.5 mM 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone (Ro 20-1724) as phosphodiesterase inhibitor) and pre-incubated for 10 min in a shaking bath at 37°C. Then adenosine agonists examined plus forskolin 1 μM were added to the mixture and the incubation continued for a further 5 min. The potencies of antagonists were determined by antagonism of the NECA (1 μM)-induced stimulation of cyclic AMP levels. Agonist EC_{50} and antagonist IC_{50} values were obtained from concentration-response curves after log-logit transformation of dependent variables by the weighted least square method (Finney, 1978).

The reaction was terminated by the addition of cold 6% trichloroacetic acid (TCA). The TCA suspension was centrifuged at 2,000 *g* for 10 min at 4°C and the supernatant was extracted four times with water-saturated diethyl ether. The final aqueous solution was tested for cyclic AMP levels by a competition protein binding assay carried out essentially according to Brown *et al.* (1971) and Nordstedt & Fredholm (1990). Samples of cyclic AMP standards (0–10 pmol) were added to each test tube containing the buffer used by Brown *et al.* (1971) (trizma base 0.1 M; aminophylline 8.0 mM; 2 mercaptoethanol 6.0 mM; pH 7.4) and [^{3}H]-cyclic AMP in a total volume of 0.5 ml. The binding protein, previously prepared from beef adrenals, was added to the samples previously incubated at 4°C for 150 min and, after the addition of charcoal, were centrifuged at 2,000 *g* for 10 min. The clear supernatant (0.2 ml) was mixed with 4 ml of Atomlight and counted in a LS-1800 Beckman scintillation counter.

Thermodynamic analysis

For the generic binding equilibrium L + R = LR (L = ligand, R = receptor) the affinity constant K_a is directly related to the standard free energy ΔG° ($\Delta G^\circ = -RT\ln K_a$) which can be separated in its enthalpic and entropic contributions according to the Gibbs equation: $\Delta G^\circ = \Delta H^\circ - T\Delta S^\circ$.

ΔG° is calculated as $-RT\ln K_a$ at 25°C, while the determination of the other thermodynamic parameters (ΔH° and ΔS°) is performed by K_a measurements at different temperatures ($K_a = 1/K_d$, where K_d is the dissociation constant of the equilibrium). Two general cases can be distinguished:

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

(2) ΔCp° is different from zero. The plot ΔG° versus T is often parabolic and other mathematical methods (Osborne *et al.*, 1976) for calculating the thermodynamic parameters of the equilibrium are available.

Saturation experiments of [^{3}H]-CGS 21680 binding to the purified protein were carried out at 0, 10, 20, 25°C using concentrations ranging from 0.02 to 1 μM and for ΔH° and ΔS° calculations the first method was applied.

Drugs

NECA (5'-N-ethylcarboxamidoadenosine); **R**-PIA and S-PIA (**R**(−) and S(+)-N^6-(2-phenylisopropyl)-adenosine); CV 1808 (2-phenylaminoadenosine); CPCA (5'-(N-cyclopropyl)carboxamidoadenosine); CGS 21680 (2-[*p*-(2-carboxyethyl)-phenethyl-amino]-5'-N-ethyl-carboxamidoadenosine); CHA (N^6-cyclohexyladenosine); CGS 15943 (5-amino-9-chloro-2-(2-furyl)1,2,4-triazolo[1,5-c]quinazoline); DPCPX (1,3-dipropyl-8-cyclopentylxanthine); XAC (8-[4-[[[[(2-aminoethyl)amino]-carbonyl]-methyl]oxy]phenyl]-1,3-dipropylxanthine); theophylline and caffeine were from Research Biochemicals Incorporated (Natick, Mass., U.S.A.). CCPA (2-chloro-N^6-cyclopentyladenosine); 2-HE-NECA (2-hexynyl-5'-N-ethyl-carboxamidoadenosine); SCH 59765 (2-(3-hydroxy-pentyn-1-yl)-5'-N-ethylcarboxamidoadenosine) were kindly provided by Dr Cristalli (University of Camerino, Italy). KF 17837((E)-1,3-dipropyl-8-(3,4-dimethoxystyryl)-7-methylxanthine); SCH 58261 (5-amino-7-(phenylethyl)-2-(2-furyl)-pyrazolo[4,3-c]-1,2,4-triazolo[1,5-c]pyrimidine) were kindly provided by Dr Ongini (Schering-Plough, Milan, Italy). CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulphonate); PEG (polyethylene glycol 8000); PMSF (phenylmethylsulphonyl fluoride); adenosine deaminase (calf intestinal type VI), trizma base, aminophylline, cyclic AMP (adenosine 3':5'-cyclic monophosphate), GTP, forskolin, and BSA (bovine serum albumin) were from SIGMA Chemical Company (St. Louis, Missouri, U.S.A.). RO 20-1724 (4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone) was a kind gift of Dr E. Kyburz, Hoffman- La Roche (Basel, Switzerland). All other reagents were of analytical grade and obtained from commercial sources. [^{3}H]-CGS 21680 (specific activity 39.6 Ci $mmol^{-1}$), Aquassure and Atomlight were from NEN Research Products (Boston, Mass., U.S.A.).

Results

Purification procedure

Table 1 describes the results of the partial purification of human platelet adenosine A_{2a} receptors. In platelet membranes

Table 1 Partial purification of human platelet adenosine receptor

Step	*Total binding activity* (fmol)		*Ratio*[c]	*Specific activity* (fmol mg^{-1})		*Purification*	
	R-PIA[a]	CGS 21680[b]		R-PIA[a]	CGS 21680[b]	R-PIA[a]	CGS 21680[b]
Membranes	340	860	0.40	9.5	23.9	1	1
PEG precipitate	92	190	0.48	8.5	17.8	0.9	0.7
Purified receptor	60	77	0.78	126.0	161.0	13.3	6.7

Binding activity was estimated with 50 nM [^{3}H]-CGS 21680 as a radioligand at 25°C. The data are representative of four preparations. The differences among separate preparations were in the range of 15%. [a] Binding was estimated using 100 μM (**R**)-PIA for non-specific binding. [b] Binding was estimated using 100 μM CGS 21680 for non-specific binding. [c] Ratio of binding estimated in *a* to binding estimated in *b*.

and the PEG precipitate, the adenotin site predominates. The purified protein is relatively enriched for the **R**-PIA displaceable binding. There is about 22% contamination with adenotin sites. The final material obtained from human platelets had an 13.3 fold enrichment of adenosine A_{2a} receptor. The **R**-PIA displaceable binding of [^{3}H]-CGS 21680 to platelet purified receptor was saturable. Figure 1 shows a saturation curve of [^{3}H]-CGS 21680 binding to purified protein adenosine A_{2a} receptor and the linearity of the Scatchard plot in the inset is indicative, in our experimental conditions, of the presence of a single class of binding sites with K_d value of 285 ± 20 nM and B_{max} value of 2.07 ± 0.2 pmol mg^{-1} of protein. In homologous displacement experiments carried out with 50 nM [^{3}H]-CGS 21680 and 18 concentrations of unlabelled CGS 21680 (0.02–30 μM) very similar parameters were obtained (data not shown).

[^{3}H]-CGS 21680 competition binding assays

K_i values for selected agonists and antagonists, obtained in the competition of [^{3}H]-CGS 21680 binding and performed in both whole human platelet membranes and A_{2a} receptors, are shown in Tables 2 and 3, respectively. Figure 2 shows the corresponding inhibition curves in platelet membranes, while Figure 3 shows the competition curves obtained in the partially purified receptor. In human platelet membranes the order of potency in [^{3}H]-CGS 21680 displacement assays for adenosine agonists was: 2-HE-NECA > SCH 59765 > CGS 21680 = NECA > CPCA > CV 1808 > **R**-PIA > CCPA > **S**-PIA > CHA. The order of potency of the antagonist compounds was: CGS 15943 > XAC > KF 17837 > SCH 58261 > DPCPX > theophylline > caffeine. In the partially purified protein the order of potency of adenosine agonists was SCH 59765 > 2-HE-NECA > CPCA > NECA > CGS 21680 > CV1808 > **R**-PIA > CCPA > CHA > **S**-PIA. SCH 59765 and 2-HE-NECA were the most effective compounds with affinity in the low nanomolar range (5–6 nM), while the selective A_1 agonists displayed affinity values in the micromolar range. The displacement of [^{3}H]-CGS 21680 binding was stereoselective, with **R**-PIA ($K_i = 1.4$ μM) being approximately 6 times more active than its stereoisomer, **S**-PIA ($K_i = 8.4$ μM). The order of potency of adenosine antagonists was: CGS 15943 > SCH 58261 > XAC > KF 17837 > DPCPX > theophylline > caffeine. CGS 15943 and SCH 58261 were the most potent compounds (K_i values in the nanomolar range), while the xanthine antagonists, theophylline and caffeine, were the weakest compounds (K_i values in the micromolar range). The Hill coefficients of most compounds (data not shown) were not significantly different from unity with the exception of the antagonist, CGS 15943, which displayed a mean Hill slope of 0.72. The Spearman's rank correlation coefficient between affinity values of [^{3}H]-CGS 21680 binding to purified platelet A_{2a} adenosine receptor by selected agonists and antagonists with corresponding K_i values in [^{3}H]-CGS 21680 binding to striatal membranes was 0.995 ($P < 0.01$).

Cyclic AMP assay

Table 2 gives EC_{50} values for forskolin-induced stimulation of cyclic AMP levels in washed human platelets and Figure 4a shows the log dose-response curves for all adenosine agonists. All tested adenosine analogues were able to increase cyclic AMP levels displaying an order of potency similar to that observed in binding affinities for the purified adenosine A_{2a} receptor. SCH 59765 and 2-HE-NECA appear to be the most potent compounds ($EC_{50} = 20$ nM and 41 nM, respectively) followed by CPCA, NECA and CGS 21680 (EC_{50} in the range 220–700 nM), and **R**-PIA, was more effective than its stereoisomer **S**-PIA ($EC_{50} = 3$ μM and 15 μM, respectively). Figure 4b shows the inhibition of cyclic AMP levels in human platelets by adenosine antagonists and Table 3 gives the IC_{50} values of

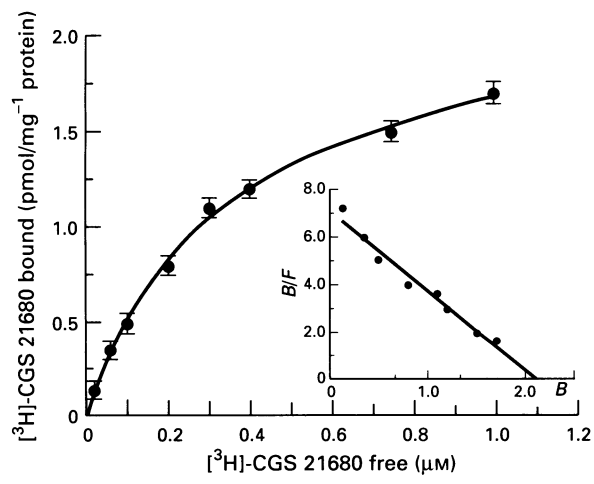

Figure 1 Saturation of [^{3}H]-CGS 21680 binding to partially purified human platelet adenosine A_{2a} receptors. Experiments were performed as described in Methods. Values are the means ± s.e. mean of four separate experiments performed in triplicate. In the inset, the Scatchard plot of the same data is shown. K_D value (nM) was 285 ± 20 and B_{max} value (pmol mg^{-1} protein) was 2.07 ± 0.2. Non-specific binding was determined in the presence of 100 μM CGS 21680.

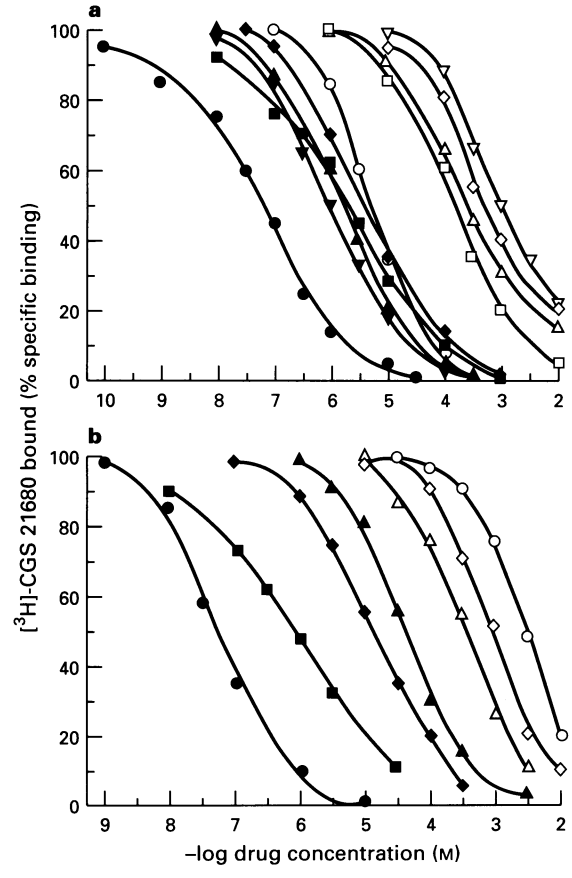

Figure 2 (a) Inhibition curves of specific [^{3}H]-CGS 21680 (50 nM) binding to human platelet membranes by adenosine agonists: (●) 2HE-NECA; (▼) SCH 59765; (■) NECA; (▲) CGS 21680; (◆) CPCA: (○) CV 1808; (□) **R**-PIA; (△) CCPA; (◇) **S**-PIA; (▽) CHA. (b) Inhibition curves of specific [^{3}H]-CGS 21680 (50 nM) binding to human platelet membranes by adenosine antagonists: (●) CGS 15943; (■) XAC; (▲) SCH 58261; (◆) KF 17837; (△) DPCPX; (◇) theophylline; (○) caffeine. Mean values of four experiments done in triplicate are shown. Non-specific binding was determined in the presence of 100 μM CGS 21680.

the same compounds. The most potent adenosine antagonists are CGS 15943 and SCH 58261 (IC_{50} = 12 and 15 nM, respectively), while theophylline and caffeine displayed IC_{50} values in the micromolar range. The Spearman's rank correlation coefficient between affinity values of [^{3}H]-CGS 21680 binding to purified platelet A_{2a} adenosine receptor by selected agonists and antagonists with EC_{50} and IC_{50} values in the cyclic AMP assay was 0.982 ($P<0.01$).

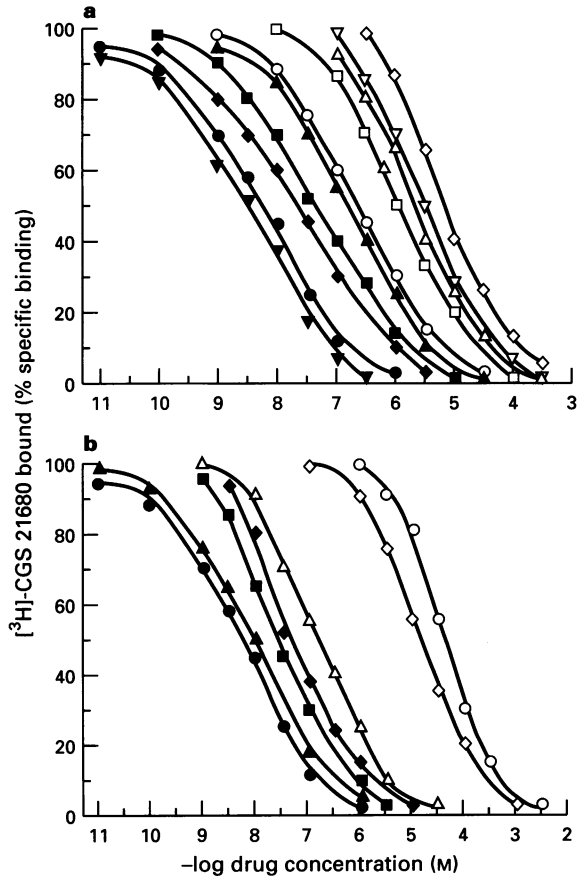

Figure 3 (a) Inhibition curves of specific [^{3}H]-CGS 21680 (50 nM) binding to human platelet partially purified A_{2a} receptors by adenosine agonists; ((▼) SCH 59765; (●) 2-HE-NECA; (■) NECA; (▲) CGS 21680; (◆) CPCA; (○) CV 1808; (□) **R**-PIA; (△) CCPA; (◇) S-PIA; (▽) CHA). (b) Inhibition curves of specific [^{3}H]-CGS 21680 (50 nM) binding to human platelet partially purified A_{2a} receptors by adenosine antagonists; (●) CGS 15943; (■) XAC; (▲) SCH 58261; (◆) KF 17837; (△) DPCPX; (◇) theophylline; (○) caffeine. Mean values of four experiments done in triplicate are shown. Non-specific binding was determined in the presence of 100 μM CGS 21680.

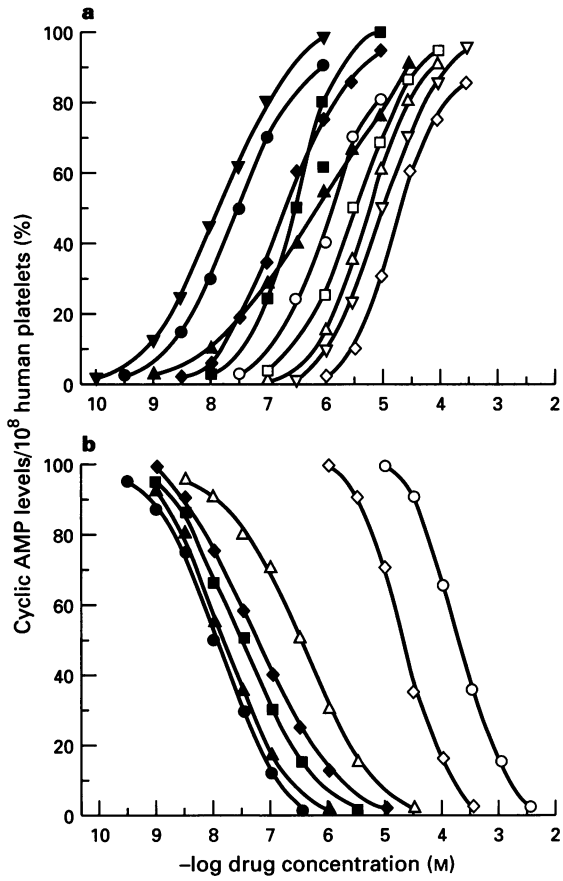

Figure 4 (a) Stimulation of cyclic AMP levels in human platelets by adenosine agonists: (▼) SCH 59765; (●) 2-HE-NECA; (◆) CPCA; (■) NECA; (▲) CGS 21680; (○) CV 1808; (□) **R**-PIA; (△) CCPA; (▽) CHA; (◇) S-PIA; the stimulation of cyclic AMP levels by NECA 10 μM was taken as 100%. (b) Inhibition of NECA (1 μM) stimulated cyclic AMP levels (100%) in human platelets by adenosine antagonists; (●) CGS 15943; (▲) SCH 58261; (■) XAC; (◆) KF 17837; (△) DPCPX; (◇) theophylline; (○) caffeine). Mean values of four experiments done in triplicate are shown.

Table 2 Inhibition of [^{3}H]-CGS 21680 binding by adenosine agonists to human platelet membranes, partially purified A_{2a} adenosine receptors and striatal membranes: comparison is made with stimulation of cyclic AMP levels in human platelets

Compound	*[^{3}H]-CGS 21680 binding* K_i (μM)*-membranes* (I)	*[^{3}H]-CGS 21680 binding* K_i (μM)*-receptors* (II)	*Cyclic AMP assay* EC_{50} (μM)	*[^{3}H]-CGS 21680 binding* K_i (μM) *striatal membranes*
SCH 59765	1.6 (1.09–2.35)	0.005 (0.004–0.006)	0.020 (0.020–0.030)	0.012 (0.0108–0.0142)[b]
2-HE-NECA	0.07 (0.06–0.08)[a]	0.006 (0.005–0.044)	0.041 (0.038–0.044)[a]	0.002 (0.0019–0.0026)[c]
CPCA	3.47 (3.33–3.62)[a]	0.052 (0.05–0.06)	0.22 (0.20–0.24)[a]	0.006 (0.0053–0.0073)[c]
NECA	2.13 (1.82–2.49)[a]	0.062 (0.06–0.07)	0.30 (0.26–0.34)[a]	0.008 (0.0066–0.0091)[c]
CGS 21680	1.92 (1.67–2.20)[a]	0.25 (0.24–0.26)	0.70 (0.58–0.83)[a]	0.011 (0.0094–0.0129)[c]
CV1808	6.76 (6.54–6.98)[a]	0.38 (0.36–0.40)	1.10 (1.01–1.19)[a]	0.062 (0.0527–0.0724)[c]
R-PIA	338 (319–357)[a]	1.40 (1.11–1.75)	2.98 (2.53–3.52)[a]	0.164 (0.140–0.192)[c]
CCPA	477 (391–580)[a]	2.51 (2.08–3.04)	8.98 (8.34–9.68)[a]	0.650 (0.555–0.762)[c]
CHA	1148 (977–1348)[a]	4.59 (4.27–4.93)	9.87 (8.24–11.84)[a]	0.820 (0.780–0.860)[d]
S-PIA	849 (822–877)[a]	8.45 (7.49–9.54)	14.96 (13.39–16.72)[a]	0.882 (0.753–1.033)[c]

Each value is the geometric mean, with 95% confidence limits in parentheses, of at least four separate experiments. [a] K_i and EC_{50} values are taken from Varani *et al.*, 1994; [b] K_i value is taken from Cristalli *et al.*, 1994; [c] K_i values are taken from Dionisotti *et al.*, 1992; [d] K_i value is taken from Borea *et al.*, 1995.

Table 3 Inhibition of [^{3}H]-CGS21680 binding by adenosine antagonists to human platelet membranes, partially purified A_{2a} adenosine receptor and striatal membranes: comparison is made with inhibition of NECA (1 μM) stimulated cyclic AMP levels in human platelets

Compound	*[^{3}H]-CGS21680 binding K_i (μM)-membranes* (I)	*[^{3}H]-CGS21680 binding K_i (μM)-receptors* (II)	*Cyclic AMP assay* IC_{50} (μM)	*[^{3}H]-CGS21680 binding K_i (μM) striatal membranes*
CGS15943	0.06 (0.05–0.07)[a]	0.006 (0.006–0.007)	0.012 (0.010–0.014)	0.00095 (0.0008–0.0011)[b]
SCH58261	58.56 (53.49–64.12)	0.008 (0.007–0.009)	0.015 (0.012–0.018)	0.0023 (0.0020–0.0027)[c]
XAC	0.68 (0.49–0.93)[a]	0.037 (0.03–0.04)	0.050 (0.04–0.06)	0.050[d]
KF17837	14.66 (11.52–18.66)	0.042 (0.04–0.05)	0.070 (0.06–0.08)	0.074 (0.061–0.089)[b]
DPCPX	462 (426–501)[a]	0.33 (0.31–0.36)	0.60 ((0.52–0.68)	0.55 (0.50–0.60)[e]
Theophylline	1060 (981–1146)[a]	16.69 (15.12–18.41)	29.66 (25.00–35.20)	6.7 (6.06–7.50)[e]
Caffeine	4306 (3908–4746)[a]	66.78 (60.96–73.16)	174.06 (154.77–195.76)	24.0 (20.73–27.37)[e]

Each value is the geometric mean, with 95% confidence limits in parentheses, of at least four separate experiments. [a] K_i values are taken from Varani *et al.*, 1994; [b] K_i values are taken from Dionisotti *et al.*, 1994; [c] K_i value is taken from Zocchi *et al.*, 1996; [d] K_i value is taken from Jarvis *et al.*, 1989; [e] K_i values are taken from Borea *et al.*, 1995.

Thermodynamic binding assay

K_d and B_{max} values derived from the saturation experiments of [^{3}H]-CGS 21680 binding to purified A_{2a} adenosine receptors performed at the four chosen temperatures were found within the following range: $K_d = 285-304$ nM and $B_{max} = 1.83-2.07$ pmol mg^{-1} of protein. These data indicate that K_d and B_{max} values are widely temperature-independent. Scatchard plots were linear at all temperatures investigated and computer analysis of the data (Munson & Rodbard, 1980) failed to show a significantly better fit to a two-site than to a one-site binding model, indicating that only one class of binding sites was present under our experimental conditions. Figure 5 shows the van't Hoff plot of $\ln K_A$ versus 1/T of the [^{3}H]-CGS 21680 binding to the partially purified receptor and the final equilibrium thermodynamic parameters were as follows: $\Delta G° = -37.14 \pm 0.10$ kJ mol^{-1}; $\Delta H° = 1.08 \pm 1.32$ kJ mol^{-1}; $\Delta S° = 128.35 \pm 4.55$ JK^{-1} mol^{-1}. The linearity of the plot is statistically significant ($\Delta Cp° \approx 0$) and its slope is practically zero.

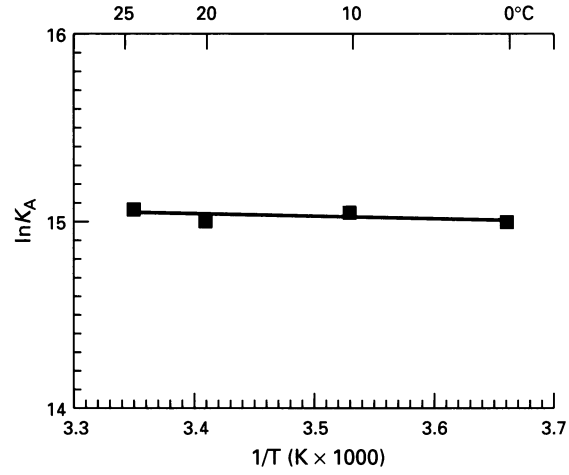

Figure 5 Van't Hoff plot showing the effect of temperature on the equilibrium binding association constant, $K_A = 1/K_D$, of [^{3}H]-CGS21680. The plot is essentially linear in the temperature range investigated (0–25°C).

Discussion

In most studies of peripheral A_{2a} adenosine receptors, the non-selective radioligand [^{3}H]-NECA has been used. In human platelet membranes this ligand has also been shown to bind non-receptor binding sites, which can explain discrepancies between the A_{2a} adenosine receptor activation kinetics of adenylyl cyclase and its pharmacological profile (Lohse *et al.*, 1988). In particular, it was reported that N^6-substituted adenosine derivatives, which in rat striatal membranes display affinity constant values in the high nanomolar or micromolar range of concentrations (Jarvis *et al.*, 1989), in platelet membranes display affinity constant values, obtained by inhibition of [^{3}H]-NECA binding, in the high micromolar or millimolar range of concentrations (Hüttemann *et al.*, 1984; Keen *et al.*, 1988). Based on these reports and on other findings, it was suggested that [^{3}H]-NECA also labels the adenotin binding site (Hutchison *et al.*, 1990). Trying to overcome these discrepancies we had studied (Varani *et al.*, 1994) the human platelet adenosine receptor using the A_{2a}-selective radioligand [^{3}H]-CGS 21680 which has been reported to interact with the high affinity A_{2a} adenosine receptor in both binding and adenylyl cyclase assays (Jarvis *et al.*, 1989; Webb *et al.*, 1992; Mathot *et al.*, 1995). Like NECA, CGS 21680 proved to be unsatisfactory for the characterization of A_{2a} human platelet receptors, since it also seemed to label the adenotin binding site (Varani *et al.*, 1994).

In the present study we have attempted, for the first time, to characterize a purified platelet A_{2a} adenosine receptor preparation by using the selective radioligand [^{3}H]-CGS 21680. The method employed (Zolnierowicz *et al.*, 1990) as an alternative to the gel filtration chromatography (Lohse *et al.*, 1988), avoids high non-specific binding by removing the majority of the adenotin binding protein using extraction of membranes with CHAPS followed by PEG precipitation. The major advantage of this procedure is that it allows the rapid batch preparation of large quantities of platelet membranes suitable for pharmacological studies of the adenosine A_{2a} receptor.

The major points to be discussed are the following:

(1) the affinities of all tested compounds for the partially purified receptor are systematically higher, from one to three orders of magnitude, than their affinities for platelet membranes and strictly similar to those obtained in rat striatum (Tables 2, 3);

(2) the binding thermodynamic profile of the interaction of CGS 21680 with the adenosine A_{2a} receptor from striatal membranes and with the partially purified platelet receptor are similar from a qualitative point of view;

(3) a high correlation exists between the affinities (K_i values) in binding to partially purified receptors and in the cyclic AMP assays (EC_{50} and IC_{50} values) (Tables 2, 3). All agonists were able to stimulate maximally the cyclic AMP levels and were therefore full agonists.

This is in agreement with similar results obtained by Cooper *et al.* (1995) who found that NECA and CGS 21680 are full agonists in this assay. Cristalli *et al.* (1994a), using a different biological assay (stimulation of adenylate cyclase activity)

found that CGS 21680 as well as HE-NECA, CV 1808 and CCPA, in platelet membranes behaved as partial agonists. The discrepancy existing between the present results, those of Cooper *et al.* (1995) and data obtained from Cristalli *et al.* (1994a) could be due to the fact that the stimulation of platelet membranes adenylate cyclase by agonists may not correspond to the accumulation of cyclic AMP in intact platelets.

(1) Saturation experiments in the purified protein preparation indicate that [^{3}H]-CGS 21680 interacts with only one recognition site with affinity in the nanomolar range, $K_d = 285$ nM, and with a binding capacity of 2.07 pmol mg^{-1} of protein. This K_d value is strictly similar to that obtained by Lorenzen *et al.* (1992) using [^{3}H]-NECA in solubilized A_{2a} adenosine receptors from human platelet membranes by gel filtration chromatography ($K_i = 227$ nM). Conversely, kinetic data obtained with [^{3}H]-CGS 21680 on human platelet membranes are different ($K_d = 1.4$ μM, $B_{max} = 5.9$ pmol mg^{-1} of protein) which suggests the presence, in this case, of a large component of low affinity binding to non-receptor sites (Varani *et al.*, 1994).

Competition experiments indicate that the behaviour of the selected adenosine ligands in human platelet membranes is very different from that observed in the purified preparation. The affinities of all agonists in the partially purified receptor are substantially higher than those obtained in platelet membranes (e.g. SCH 59765, K_i value of 5 nM vs 1.6 μM; 2-HE-NECA, K_i value of 6 nM vs 70 nM) and a similar situation is found for all antagonists (e.g. CGS 15943, K_i value of 6 nM vs 60 nM; SCH 58261, K_i value 8 nM vs 58.6 μM in purified membrane preparation compared with platelet membranes, respectively) (Tables 2, 3). The two high affinity ligands, SCH 59765 (agonist) and SCH 58261 (antagonist) display also the highest degree of selectivity for the A_{2a} receptor in comparison with adenotin (K_i ratios of 320 and 7000 respectively). This is of interest in view of the fact that the availability of selective A_{2a} agonists and antagonists could delineate future prospects for these drugs as, e.g., hypotensive, anti-aggregatory agents and in the treatment of neurological disorders such as Parkinson's disease (Fuxe *et al.*, 1993) or cerebral ischaemia (Gao & Phillis, 1994). N^6-substituted adenosine derivatives, which show a high affinity to A1 receptors, display K_i values from two to three orders of magnitude higher for the whole platelet membrane preparations with respect to the purified one, suggesting that the latter is essentially lacking the adenotin site. Moreover, [^{3}H]-CGS 21680 binding to rat striatum was displaced by adenosine receptor agonists and antagonists in a rank order of potency typical of A_{2a} adenosine receptors (Tables 2, 3) and it is worth noting that the values obtained for rat striatum (Jarvis *et al.*, 1989; Borea *et al.*, 1995) are in good agreement with K_i values found in the purified receptor, suggesting that the adenosine receptor of human platelets is similar to the high affinity A_{2a} subtype. The calculated Spearman's rank correlation coefficient ($r_s = 0.995$; $P < 0.01$) between the binding affinity constants (K_i) of selected agonists and antagonists to purified A_{2a} receptors and to striatal membranes is statistically highly significant.

(2) The linearity of the van't Hoff plot for [^{3}H]-CGS 21680 binding in the purified receptor indicates that the ΔCp° values of the drug-interaction is nearly zero which means that ΔH° and ΔS° values are not significantly affected by temperature variations at least over the range investigated. A similar result was found by Casadò *et al.* (1993) who reported that the ΔCp° of [^{3}H]-**R**-PIA binding to soluble adenosine A_1 pig brain receptor was nearly zero. On the other hand, such a linearity of van't Hoff plots in a restricted range of temperatures (usually 0-25/30°C) appears to be a common feature of practically all non-solubilized membrane receptors so far studied from a thermodynamic point of view (Gilli *et al.*, 1994). Interestingly this linearity is maintained also in solubilized receptors. Thermodynamic data obtained from the van't Hoff plot, indicate that [^{3}H]-CGS 21680 binding to purified receptor is totally entropy-driven ($\Delta S^\circ = 128.4$ JK^{-1}/mol, $\Delta H^\circ = 1.1$ kJ mol^{-1}). This result is in qualitative agreement with that previously reported (Borea *et al.*, 1995) for the binding of the same ligand to rat striatum adenosine A_{2a} receptors which also indicated an entropy-driven binding ($\Delta S^\circ = 241$ JK^{-1}mol^{-1}, $\Delta H^\circ = 27$ kJ mol^{-1}), the quantitative differences being, most probably, ascribable to diversities between animal species (human versus rat), tissue (platelets versus striatum) and physical state of the receptor (solubilized versus particulate).

(3) In human platelet membranes, several authors (Hüttemann *et al.*, 1984; Keen *et al.*, 1988; Varani *et al.*, 1994) report discrepancies between the affinity data obtained in cyclic AMP and receptor binding assays for many adenosine receptor ligands due to the fact that the inhibitory binding constant, K_i, values were higher (up to three orders of magnitude) than EC_{50} or IC_{50} values obtained for the stimulation or the inhibition of cyclic AMP levels (Tables 2, 3 for agonists and antagonists respectively). In our partially purified platelet membranes such a discrepancy is strongly reduced and K_i, EC_{50} and IC_{50} values are strictly similar from a quantitative point of view for all ligands studied. The calculated Spearman coefficient ($r_s = 0.982$) between the binding affinity constants (K_i) of selected agonists and antagonists to purified A_{2a} adenosine receptor and those obtained in the cyclic AMP assay indicates a very high rank correlation. 2-HE-NECA and SCH 59765 which are known to be the highest affinity adenosine analogues in rat striatum (Cristalli *et al.*, 1994b) and in purified platelets, are also the most potent in the cyclic AMP assay. The adenosine antagonists, CGS 15943 and SCH 58261, which have K_i and IC_{50} values in the nanomolar range ($K_i = 6$ nM and 8 nM; $IC_{50} = 12$ nM and 15 nM, respectively) are the compounds with the highest potency and can be considered very interesting as reference A_{2a} antagonists (Ongini *et al.*, 1995; Zocchi *et al.*, 1996). All these data agree in indicating that in the purified preparation the presence of adenosine receptors is prevalent with respect to the non-receptor component (adenotin site).

In conclusion, there is a strong correlation between the affinity data in the binding (K_i) of typical adenosine A_{2a} ligands to striatal membranes and to purified receptors and there is also a substantial similarity between thermodynamic parameters of interaction of [^{3}H]-CGS 21680 binding with purified platelet membranes and with rat striatum making it possible to propose that human platelet and rat striatum adenosine A_{2a} receptors are strictly similar. Moreover, K_i values in the binding assays correlated well with those of the cyclic AMP assay (EC_{50} and IC_{50}) further suggesting that the A_{2a} subtype predominates in our purified preparations. Finally, our results show, for the first time, that CGS 21680 is a reliable radioligand to study purified A_{2a} adenosine receptor in human platelets.

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