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Binding characteristics of the adenosine A_2 receptor ligand [^{3}H]CGS 21680 to human platelet membranes

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Abstract—The binding characteristics of the selective adenosine A_2 agonist [^{3}H]CGS 21680 ([^{3}H]2-[*p*-(2-carboxyethyl)-phenethyl-amino]-5′-*N*-ethylcarboxamidoadenosine) were determined in human platelet membranes. Specific binding was saturable, reversible and dependent upon protein concentration. Saturation experiments revealed a single class of binding sites with K_d and B_{max} values of 1.4 μM and 5.9 pmol/mg of protein, respectively. Adenosine receptor agonists and antagonists competed for the binding of [^{3}H]CGS 21680 (50 or 200 nM) to human platelet membranes showing a rank order of potency consistent with that typically found for interactions at the adenosine A_2 receptor. Adenylate cyclase stimulation and platelet aggregation inhibition induced by adenosine agonists exhibited a rank order of potency close to that observed in binding experiments. However, the adenosine A_1 receptor agonists, *R*- and *S*-N^6-(2-phenylisopropyl)adenosine, (R-PIA) and (S-PIA), N^6-cyclohexyladenosine (CHA) and 2-chloro-N^6-cyclopentyladenosine (CCPA), which stimulate adenylate cyclase and inhibit platelet aggregation in the low μM range, displaced [^{3}H]CGS 21680 only in the high μM range. In conclusion, we have found that [^{3}H]CGS 21680, which is widely used as a specific A_2 agonist in binding studies on brain tissues, is not appropriate for the characterization of the human platelet adenosine A_2 receptor.

Key words: adenosine A_2 receptors; human platelets; [^{3}H]CGS 21680; 2-HE-NECA; cyclic AMP; platelet aggregation

Adenosine is known to exert most of its pharmacological effects by interacting with at least two different membrane receptor subtypes, A_1 and A_2. Binding of adenosine itself produces, respectively, inhibition or stimulation of adenylate cyclase with the consequent decrease or increase of the intracellular levels of the second messenger cAMP§ [1, 2]. Blood platelet surface contains adenosine A_2 receptors whose activation prevents platelet aggregation [3]. With the exception of a study carried out with the xanthine amine congener (XAC*) [4], in peripheral tissues most binding studies have been carried out using [^{3}H]-NECA as radioligand. Nevertheless the binding properties of this ligand do not fully agree with the pharmacology of the A_2 platelet receptor because some adenosine agonists, known to both stimulate adenylate cyclase and inhibit platelet aggregation, fail to displace [^{3}H]NECA binding even at high concentrations [5, 6]. A more detailed characterization of the human platelet A_2 receptor was described on solubilized extracts from human platelets by Lohse *et al.* [7] who found that [^{3}H]NECA may interact with other sites in addition to the A_2 adenosine receptor subtype. The only ligand currently available which interacts with high affinity and selectivity for A_2 receptors appears to be [^{3}H]CGS 21680 [8]. This ligand has been used to characterize A_2 binding sites on striatal membranes [8], but there is no information about its use for the study of A_2 human platelet receptors. The present paper describes the binding characteristics of [^{3}H]CGS 21680 recognition sites on washed human platelet membranes. In addition, the potency of the adenosine agonists has been determined in adenylate cyclase studies and compared with that obtained in our previous studies in anti-aggregatory tests [9].

* Abbreviations: cAMP, adenosine 3′,5′-cyclic monophosphate; CCPA, 2-chloro-N^6-cyclopentyladenosine; CGS 21680, 2-[*p*-(2-carboxyethyl)-phenethyl-amino]-5′-*N*-ethylcarboxamido-adenosine; CGS 15943, 5-amino-9-chloro-2-(2-furyl)1,2,4-triazolo[1,5-*c*]quinazoline; CHA, N^6-cyclohexyladenosine; CPCA, 5′-(*N*-cyclopropyl)carboxamidoadenosine; CV 1808, 2-phenylamino-adenosine; DPCPX, 1,3-dipropyl-8-cyclopentylxanthine; GTP, guanosine-5′-triphosphate; 2-HE-NECA, 2-hexynyl-5′-*N*-ethylcarboxamido-adenosine; NECA, 5′-*N*-ethylcarboxamidoadenosine; R-PIA and S-PIA, *R*- and *S*-N^6-(2-phenylisopropyl)-adenosine; Ro 20-1724, 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone; XAC, 8-[4-[[[[(2-aminoethyl)amino]-carbonyl]methyl]oxy]phenyl]-1,3-dipropyl-xanthine.

Materials and Methods

Chemicals. NECA, R-PIA and S-PIA, CV 1808, CPCA, CGS 21680, CHA, XAC, DPCPX, caffeine and theophylline were from Research Biochemicals Incorporated (Natick, MA, U.S.A.). CCPA and 2-HE-NECA were kindly provided by Prof. Cristalli (University of Camerino, Italy). CGS 15943 was provided by Ciba-Geigy (Summit, NJ, U.S.A.). [^{3}H]CGS 21680 (sp. act. 39.6 Ci/mmol), Aquassure and Atomlight were from NEN Research Products (Boston, MA, U.S.A.). [^{3}H]cAMP (sp. act. 24 Ci/mmol), was from Amersham Amity s.r.l. (Milano, Italy). Adenosine deaminase type VI, trizma base, aminophylline, cAMP, GTP, forskolin, 5′-guanylylimidodiphosphate and bovine serum albumin were from the Sigma Chemical Co. (St Louis, MO, U.S.A.). Ro 20-1724 was a kind gift of Dr E. Kyburz (Hoffman-La Roche, Basel, Switzerland). All other reagents were of analytical grade and were obtained from commercial sources.

[^{3}H]*CGS 21680 binding assay.* Blood anticoagulated and stabilized with ACD (1.4% citric acid, 2.5% sodium citrate, 2% D-glucose) was obtained from healthy human volunteers. Platelet membranes for binding studies were prepared according to Schloos *et al.* [10]. Binding assay was carried out essentially according to Jarvis *et al.* [8]. In a total volume of 250 μL (100 μg of protein/assay) saturation studies were performed using six different concentrations of [^{3}H]CGS 21680 ranging from 1 to 200 nM. In homologous displacement experiments 50 nM [^{3}H]CGS 21680 and 11 concentrations of unlabeled CGS 21680 (0.06–7.5 μM) were

used. Competition experiments were carried out using 50–200 nM of [^{3}H]CGS 21680. After incubation for 120 min at 0°, free and bound ligands were separated by centrifugation in a Beckman microcentrifuge for 5 min at 12,000 *g*. The pellets were washed twice without resuspension with incubation buffer and the tips of the plastic tubes were cut off, transferred into vials containing 5 mL of Aquassure liquid scintillation and counted in a Beckman LS-1800 Spectrometer (efficiency 55%). Non-specific binding measured in the presence of 100 μM NECA was about 35% of total binding in the whole concentration range. Protein concentration was determined according to a Bio-Rad method [11] with bovine albumin as reference standard.

Measurement of cyclic AMP levels in platelets. Washed human platelets were prepared as described by Korth *et al.* [12]. The final suspending medium was a Tyrode's 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 × 10^4cells) were suspended in 0.5 mL incubation mixture (Tyrode's buffer containing bovine serum albumin 0.25%, adenosine deaminase type VI, 1 IU/mL and 0.5 mM Ro 20-1724 as phosphodiesterase inhibitor) and preincubated for 10 min in a shaking bath at 37°. Then, the adenosine analogues and forskolin (1 μM) were added and the incubation continued for a further 5 min. The reaction was terminated by the addition of cold 6% trichloroacetic acid (TCA). TCA suspension was centrifuged at 2000 *g* for 10 min at 4° and the supernatant was extracted four times with water-saturated diethyl ether. The final aqueous solution was tested for cAMP levels by a competitive protein binding assay carried out essentially according to Brown *et al.* [13]. The sample (100 μL) or 50 μL cAMP standard (0–10 pmol) was added to each test tube containing trizma base 100 μM, aminophylline 8 mM, 2 mercaptoethanol 6 mM at pH 7.4 [^{3}H]cAMP (1 pmol, approximately 25,000 cpm) was added to each tube at a total assay volume of 500 μL. Binding protein, previously prepared from bovine adrenals, was added to the samples which were incubated at 4° for 150 min and, after the addition of charcoal, were centrifuged at 2000 *g* for 10 min. Clear supernatant (0.2 mL) was mixed with 4 mL of Atomlight and counted in a Beckman Spectrometer.

Statistical analysis. A weighted nonlinear least-square curve fitting program, LIGAND [14], was used for computer analysis of saturation and competition experiments. In the cAMP levels assay, EC_{50} values were obtained from concentration–response curves after log–logit transformation of dependent variables by weighted least-square method.

Results

[^{3}H]CGS 21680 binding assay. The study on temperature dependence of [^{3}H]CGS 21680 binding to human platelets exhibited the highest specific binding at 0° with an optimal pH between 7.0 and 7.4. After an incubation of 120 min, 50 nM [^{3}H]CGS 21680 bound to platelet membranes with specific binding of about 65%. Specific binding was found to increase linearly with respect to protein concentration over the range of 0–500 μg of protein/assay. GTP (100 μM), its hydrolysis-resistant analogue 5′-guanylimidodiphosphate and adenosine deaminase did not affect [^{3}H]CGS 21680 binding. Saturation studies carried out in the concentration range 1–200 nM failed to reach any plateau. Homologous displacement experiments, carried out as described in the Materials and Methods, demonstrated that [^{3}H]CGS 21680 bound a single class of receptors in human platelet membranes with a K_d of 1.4 (±0.2) μM and an apparent B_{max} value of 5.9 (±0.6) pmol/mg of protein (Fig. 1). Different adenosine agonists competed for the binding of [^{3}H]CGS 21680 (50 nM) to human platelet membranes showing the following order of potency: 2-HE-NECA > CGS 21680 = NECA > CPCA > CV 1808 > R-PIA > CCPA > S-PIA > CHA. 2-HE-NECA, a new potent and selective A_2 agonist [15], was the most effective compound with affinity in the nanomolar range (K_i = 70 nM). The order of potency of the antagonist compounds examined was CGS 15943A > XAC > DPCPX > theophylline > caffeine. Most of the agonist and antagonist compounds were found to interact with one binding component (Hill coefficient not significantly different from unity) with the exception of CPCA, S-PIA, and XAC. K_i values [16] and Hill coefficients are reported in Tables 1 and 2. K_i values were also calculated using 200 nM [^{3}H]-CGS 21680 and displayed a rank order of potency strictly similar to that obtained using 50 nM [^{3}H]CGS 21680.

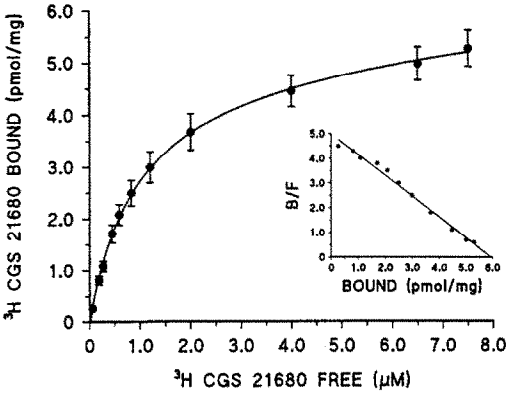

Fig. 1. Saturation isotherm and Scatchard plot (inset) of data transformed from competition experiments on human platelet membranes where 50 nM [^{3}H]CGS 21680 and 11 concentrations of unlabeled CGS 21680 ranging from 0.06 to 7.5 μM were used. Each value of the saturation isotherm is the mean ± SEM of four separate experiments. B is [^{3}H]CGS 21680 specifically bound (pmol/mg protein) and F is free [^{3}H]CGS 21680 (μM).

Measurement of cyclic AMP levels in platelets. EC_{50} values for forskolin-induced stimulation of cAMP levels in washed human platelets are reported in Table 1. All the tested adenosine analogues were able to increase cAMP levels displaying an order of potency similar to that observed in platelet binding studies. 2-HE-NECA appeared to be the most potent compound with an EC_{50} value of 0.041 μM. The A_2 selective adenosine agonists CPCA, NECA, CGS 21680 and CV 1808 showed EC_{50} values ranging from 0.22 to 1.10 μM.

Discussion

Over the last few years, CGS 21680, a NECA derivative with high affinity and selectivity for A_2 adenosine receptors [8], has become the radioligand of choice to investigate the A_2 receptor subtype in the central nervous system and selected cell cultures. In the present study, we have examined [^{3}H]CGS 21680 as a possible radioligand also for the A_2 adenosine receptors localized on human platelets. With the exception of the bound from free ligand separation and the presence of Mg^{2+} 10 mM, all the experimental conditions of [^{3}H]CGS 21680 binding were similar to those previously described by Hütteman *et al.* [5] for [^{3}H]NECA binding. Saturation experiments showed the interaction of [^{3}H]CGS 21680 with only one recognition site with affinity in the micromolar range, K_d = 1.4 μM, and binding capacity of 5.9 pmol/mg of protein. Similar binding parameters (K_d = 1.0 μM; B_{max} = 4.0 pmol/mg of protein) have been previously reported in [^{3}H]NECA binding studies to human platelets when carried out in the presence of Mg^{2+} 5 mM [6], whereas the heterogeneity of platelet [^{3}H]NECA

Table 1. Comparison of binding and functional activity data of various adenosine agonists in human platelets

Compound	[^{3}H]CGS 21680 binding K_i (μM)	Hill coefficient	cAMP assay EC_{50} (μM)	Platelet aggregation† IC_{50} (μM)
2-HE-NECA	0.07 (0.06–0.08)	0.93 (0.85–1.00)	0.041 (0.038–0.044)	0.10 (0.04–0.25)
CGS 21680	1.92 (1.67–2.20)	0.97 (0.91–1.03)	0.70 (0.58–0.83)	1.09 (0.44–2.74)
NECA	2.13 (1.82–2.49)	0.94 (0.86–1.01)	0.30 (0.26–0.34)	0.49 (0.32–0.77)
CPCA	3.47 (3.33–3.62)	0.73 (0.49–0.98)*	0.22 (0.20–0.24)	0.42 (0.17–1.07)
CV1808	6.76 (6.54–6.98)	0.86 (0.69–1.02)	1.10 (1.01–1.19)	3.05 (1.20–7.74)
R-PIA	338 (319–357)	0.93 (0.86–1.00)	2.98 (2.53–3.52)	7.30 (2.91–18.30)
CCPA	477 (391–580)	0.88 (0.71–1.04)	8.98 (8.34–9.68)	19.20 (7.65–48.20)
S-PIA	849 (822–877)	0.62 (0.39–0.86)*	14.96 (13.39–16.72)	51.90 (20.7–130.0)
CHA	1148 (977–1348)	0.74 (0.45–1.02)	ND‡	ND

Each value is the geometric mean, with 95% confidence limits in parentheses, of at least four separate experiments.
* Hill coefficient significantly different from unity ($P < 0.01$).
† Data from Dionisotti *et al.* [9].
‡ ND, not determined.

Table 2. Affinity data, expressed as K_i values, of some typical adenosine antagonists in human platelets

Compound	[^{3}H]CGS 21680 binding K_i (μM)	Hill coefficient
CGS 15943	0.06 (0.05–0.07)	1.04 (0.92–1.18)
XAC	0.68 (0.49–0.93)	0.52 (0.30–0.92)*
DPCPX	462 (426–501)	0.96 (0.92–1.02)
Theophylline	1060 (981–1146)	0.88 (0.77–1.08)
Caffeine	4306 (3908–4746)	1.01 (0.95–1.06)

Each value is the geometric mean, with 95% confidence limits in parentheses, of at least four separate experiments.
* Hill coefficient significantly different from unity ($P < 0.01$).

binding sites observed in the absence of Mg^{2+} by Hütteman *et al.* [5] and Edwards *et al.* [17] was also confirmed in the present study using [^{3}H]CGS 21680 (data not shown). It is therefore clear that [^{3}H]CGS 21680 labels not only the high affinity A_2 receptors which are found in platelets when solubilized extracts and [^{3}H]NECA as radioligand are used ($K_d = 46$ nM; $B_{max} = 0.51$ pmol/mg of protein [7]). Moreover, GTP was unable to affect the specific binding of [^{3}H]CGS 21680, either in the presence or absence of Mg^{2+}, a finding similar to that previously described [5, 6] for platelet [^{3}H]NECA binding and indicating that most of the binding sites labelled by [^{3}H]CGS 21680 are not related to interactions with the typical receptors coupled to G proteins. However, in competition experiments, the adenosine agonists and antagonists examined displaced the specific [^{3}H]CGS 21680 binding to human platelet membranes with a rank order of potency consistent with the typical interaction occurring at adenosine A_2 receptors in brain tissues [8, 18]. Functional studies on adenylate cyclase and platelet aggregation [9] showed for adenosine agonists a rank order of potency almost similar to that observed in binding experiments (Table 1). Nevertheless, as previously reported [5] for the platelet [^{3}H]NECA binding, the displacement of [^{3}H]CGS 21680 binding revealed K_i values substantially higher than those obtained for the stimulation or the inhibition of the functional responses. Thus, it is evident that CGS 21680 interacts with the platelet adenosine A_2 receptor but this specific binding is undetectable, presumably because the receptor density is so low as to be masked by other non-specific components.

The present data could be interpreted by considering that in platelets [^{3}H]CGS 21680 labels the non-receptor binding site (adenotin site) which has been described for [^{3}H]NECA binding in human platelets [7], bovine striatum [18], PC12 cell membranes [19] and other peripheral tissues [20]. For [^{3}H]CGS 21680, this may occur in platelets but not in cerebral tissues where the compound is highly specific and exhibits very low affinity ($K_i = 34.9$ μM) to the adenotin binding site in comparison with NECA ($K_i = 0.33$ μM) [18]. It is not known whether [^{3}H]CGS 21680 shows similar binding profile in other peripheral tissues. Another hypothesis is that the type of A_2 receptor present on platelet membranes is different from that found in other tissues. This interpretation could be in agreement with other findings showing a discrepancy between anti-aggregatory activity and binding data for certain classes of A_2 agonists [21].

In conclusion, although there is evidence that [^{3}H]CGS 21680 is useful and specific A_2 agonist in binding studies on brain tissues or specific cell lines, the results from the present work indicate that this radioligand is not satisfactory for the characterization of A_2 platelet receptors.

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