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Characterization of A_{2A} adenosine receptors in human lymphocyte membranes by [^{3}H]-SCH 58261 binding

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1 The present study describes for the first time the characterization of the adenosine A_{2A} receptor in human lymphocyte membranes with the new potent and selective antagonist radioligand, [^{3}H]-5-amino-7-(2-phenylethyl)-2-(2-furyl)-pyrazolo [4,3-*e*]-1,2,4 triazolo [1,5-*c*] pyrimidine, ([^{3}H]-SCH 58261). In addition, both receptor affinity and potency of reference adenosine receptor agonists and antagonists were determined in binding and adenylyl cyclase studies.

2 Saturation experiments revealed a single class of binding sites with K_d and B_{max} values of 0.85 nM and 35 fmol mg^{-1} protein, respectively. A series of adenosine receptor ligands were found to compete for the binding of 0.8 nM [^{3}H]-SCH 58261 to human lymphocyte membranes with a rank order of potency consistent with that typically found for interactions with the A_{2A}-adenosine receptor. In the adenylyl cyclase assay the same compounds exhibited a rank order of potency similar to that observed in binding experiments.

3 Thermodynamic data indicate that [^{3}H]-SCH 58261 binding to human lymphocytes is entropy and enthalpy-driven, a finding in agreement with the thermodynamic behaviour of antagonists for rat striatal A_{2A}-adenosine receptors.

4 It is concluded that in human lymphocyte membranes [^{3}H]-SCH 58261 directly labels binding sites showing the characteristic properties of the adenosine A_{2A}-receptor. The presence of A_{2A}-receptors in peripheral tissue such as human lymphocytes strongly suggests an important role for adenosine in modulating immune and inflammatory responses.

Keywords: Adenosine A_{2A} receptors; adenosine A_{2A} receptor ligands; [^{3}H]-SCH 58261 binding; human lymphocyte membranes; cyclic cAMP assays; thermodynamic analysis

Introduction

Adenosine, acting via specific cell surface receptors, modulates a number of physiological functions. Adenosine receptors have been classified into A_1, A_{2A}, A_{2B} and A_3 subtypes. All of these receptor subtypes have the structural features of receptors linked to G-proteins and positively (A_{2A} and A_{2B}) or negatively (A_1 and A_3) coupled to adenylate cyclase (Van Calker *et al*., 1979; Fredholm *et al*., 1994; Linden *et al*., 1994). In the periphery, A_{2A}-receptors are localized in different organs and tissues, including platelets (Dionisotti *et al*., 1996), neutrophil leukocytes (Fredholm *et al*., 1996) and vascular smooth cells (Belardinelli *et al*., 1996). In the central nervous system, A_{2A}-receptors have long been characterized with the non-selective receptor agonist [^{3}H]-NECA (5′-N-ethylcarboxamidoadenosine). However, this agonist binds to different states and/or subtypes of the adenosine receptors as well as to other non-specific proteins, such as adenotin (Schwabe *et al*., 1993). Over the last few years the availability of the A_{2A}-receptor agonist [^{3}H]-CGS 21680 (2-[p-(2-carboxyethyl)-phenethyl-amino]-5′-N-ethyl-carboxamidoadenosine) (Jarvis *et al*., 1989) has made possible the pharmacological characterization of A_2-receptors leading to the separation of two distinct A_{2A}- and A_{2B}-receptor subtypes (Ongini & Fredholm, 1996). This ligand has been widely used to characterize A_{2A}-binding sites on striatal membranes and recent data suggest that CGS 21680 also labels the A_{2A}-receptors present on human purified platelet membranes (Varani *et al*., 1996). However, CGS 21680 has been shown to label non-A_{2A} binding sites in the rat brain (Lindström *et al*., 1996). Moreover it is an established concept that in binding studies the use of antagonist radioligands is usually preferred because of a variety of advantages over receptor agonists. Recently, important progress has been made with the development of selective A_{2A}-receptor antagonists having an interesting pharmacological profile (Ongini & Fredholm, 1996). One of them, the non-xanthine compound 5-amino-7-(2-phenylethyl)-2-(2-furyl)-pyrazolo [4,3-*e*]-1,2,4-triazolo-[1,5 *c*]-pyrimidine (SCH 58261), a new potent and selective A_{2A} antagonist (Baraldi *et al*., 1994), has been widely characterized in a variety of binding and functional assays (Zocchi *et al*., 1996a). The tritium-labelled form, [^{3}H]-SCH 58261, has been found to label A_{2A}-receptors in the brain (Zocchi *et al*., 1996b), in peripheral tissue membranes such as porcine coronary arteries (Belardinelli *et al*., 1996), human platelets (Dionisotti *et al*., 1996) and human cloned receptors transfected in mammalian cells (Dionisotti *et al*., 1997). Adenosine has been found to stimulate adenosine 3′:5′-cyclic monophosphate (cyclic AMP) accumulation in human peripheral blood lymphocytes (Bonnafous *et al*., 1982), suggesting the presence of adenosine A_{2A}-receptors on these immune system cells. The ability of various adenosine analogues to increase cellular content of cyclic AMP was correlated with the inhibition of lymphocyte cytotoxicity and the modulation of lymphocyte function by suppressing or dampening of the immune response. However, adenosine is also being investigated in a number of alternative indications including the *in vitro* destruction of tumour cells. For example, cladribine which is an adenosine deaminase-resistant analogue of deoxyadenosine, has recently gained status in the treatment of various leukaemias and lymphomas (Carrera *et al*., 1994). The present paper describes the characterization of A_{2A}-adenosine receptors in human lymphocytes by using the selective A_{2A}-antagonist [^{3}H]-SCH 58261. Moreover, the abilities of typical agonists to increase the cyclic AMP intracellular levels and the potency of a series of antagonists in inhibiting NECA stimulated adenylyl cyclase activity have been evaluated. Finally, with the aim of obtaining new insights into the forces driving the coupling of A_{2A} human lymphocyte

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receptors with a selective ligand, a thermodynamic analysis of [^{3}H]-SCH 58261 binding has been performed and the enthalpic (ΔH°) and entropic (ΔS°) contribution to the standard free energy (ΔG°) of the binding equilibrium were determined.

Methods

The cell preparations were isolated from heparin-treated peripheral blood (100–200 ml) from healthy human volunteers as described below:

Lymphocyte membrane preparation

Blood was centrifuged on Ficoll-Hypaque density gradients by the method of Boyum (1968). The mononuclear leukocytes fraction contained approximately 85% small lymphocytes, 13% monocytes, and less than 2% polymorphonuclear leukocytes by morphological criteria in Türk's solution. Membranes for binding studies were prepared essentially according to the method of Aarons *et al.* (1980). Mononuclear leukocyte fractions isolated were removed from the Ficoll-Hypaque gradients, washed in 0.02 M phosphate-buffered saline, pH 7.2 containing 5 mM $MgCl_2$ and 0.15 mM $CaCl_2$ (PBS) and passed twice over a nylon mesh column. This procedure, designed to remove monocytes and polymorphonuclear leukocytes, which adhere to the nylon mesh, resulted in a purified lymphocyte preparation containing at least 99% small lymphocytes identified by morphological criteria. After centrifugation at 400 *g* for 15 min at 4°C, the mononuclear cell pellets were resuspended in ice-cold glass distilled H_2O and centrifuged at 20,000 *g* for 10 min at 4°C. The supernatant was discarded and the pellet resuspended in 40 ml of ice-cold glass distilled H_2O and allowed to stand on ice for 60 min to ensure adequate lysis. The preparation was centrifuged at 20,000 *g* for 10 min and the resulting pellet was resuspended in 50 mM Tris-HCl (pH 7.4 at 25°C) at a concentration of 100–150 μg protein 100 μl^{-1} and this homogenate was used for the assay of [^{3}H]-SCH 58261 binding. The protein concentration was determined according to a Bio-Rad method (Bradford, 1976) with bovine albumin as reference standard.

Lymphocyte isolation

Lymphocytes were isolated from heparin-treated blood according to the method of Boyum (1968). These cells were removed from the gradients and washed in 20 ml of PBS. After centrifugation at 400 *g* for 15 min, the pellet was resuspended twice in the same volume of PBS and was centrifuged at 400 *g* for 10 min. The final pellet was resuspended in PBS at a concentration of 10^6 cells ml^{-1}. This cell suspension was used for measurement of cyclic AMP levels.

[^{3}H]-SCH 58261 binding assay in the lymphocyte membranes

Binding assays were carried out according to Dionisotti *et al.* (1996). In saturation studies, lymphocyte membranes were incubated with 8 to 10 different concentrations of [^{3}H]-SCH 58261 ranging from 0.08 to 8 nM. Inhibition experiments were carried out in duplicate in a final volume of 250 μl in test tubes containing 0.8 nM [^{3}H]-SCH 58261, 50 mM Tris HCl buffer, 10 mM $MgCl_2$, pH 7.4 and lymphocyte membranes (150 μg protein/assay) and at least 8–10 different concentrations of typical adenosine agonists and antagonists. Inhibitory binding constant, K_i, values were calculated from those of IC_{50} 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. Non-specific binding was defined as binding in the presence of 10 μM NECA and was about 30% of total binding. Incubation time was 60 min at 4°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 by use of a Brandel cell harvester. 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 & Rodbard, 1980), was used for computer analysis of saturation and inhibition experiments.

Measurement of cyclic AMP levels in human lymphocytes

Human lymphocytes (10^6 cells ml^{-1}) were suspended in 0.5 ml incubation mixture (PBS buffer, 1.0 iu adenosine deaminase ml^{-1} and 0.5 mM 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone, Ro 20–1724, as a phosphodiesterase inhibitor) and preincubated for 10 min in a shaking bath at 37°C. Then, each adenosine receptor agonist plus forskolin (1 μM) was added to the mixture and the incubation continued for a further 5 min. The potency of receptor antagonists was determined by antagonism of 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 used to measure 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 bovine adrenals, was added to the samples previously incubated at 4°C for 150 min and, after the addition of charcoal, the samples 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° (ΔG° = −RT 1n K_a) which can be separated in its enthalpic and entropic contributions according to the Gibbs equation: ΔG° = ΔH° − ˜TΔS°. ΔG° is calculated as −RT 1n 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 at 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 (δ1n K_a/δ (1/T) = −ΔH°/R gives a linear van't Hoff plot 1n K_a versus (1/T) and standard enthalpy can be calculated from its slope, −ΔH°/R, while standard entropy is calculated as ΔS° = (ΔH° − ΔG°)/T with T = 298.15 K and R = 8.314 J mol^{-1} K^{-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]-SCH 58261 binding to the human lymphocyte membranes were carried out at 0, 10, 20, 25°C in a thermostatic bath assuring a temperature of ±0.1°C, with concentrations ranging from 0.8 to 8 nM. In the present case, where the van't Hoff plot can be considered as being essentially linear, the first method was applied.

Drugs

NECA (5′-N-ethylcarboxamidoadenosine), **R**-PIA and **S**-PIA (**R**(−)- and **S**(+)-N^6-(2-phenylisopropyl)-adenosine), 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) and cladribine were from Research Biochemicals Incorporated (Natick, MA, U.S.A.). CCPA (2-chloro-N^6-cyclopentyladenosine) and 2-HE-NECA (2-hexynyl-5′-N-ethyl-carboxamidoadenosine) were kindly provided by Dr G. Cristalli (University of Camerino, Italy). KF 17837 ((E)-1,3-dipropyl-8-(3,4- dimethoxystyryl) - 7 - methylxanthine) and SCH 58261 (5-amino-7-(phenylethyl)-2-(2-furyl)-pyrazolo[4,3-*e*]-1,2,4-triazolo[1,5-*c*]pyrimidine) were from Schering-Plough Research Institute (Milan, Italy). Adenosine deaminase (calf intestinal type VI), trizma base, aminophylline, cyclic AMP (adenosine 3′:5′-cyclic monophosphate) and forskolin were from Sigma Chemical Company (St. Louis, MO, 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]-SCH 58261 (specific activity 68.6 Ci $mmol^{-1}$) was from the Schering-Plough Research Institute (Milan, Italy). Aquassure and Atomlight were from NEN Research Products (Boston, MA, U.S.A.).

Results

[^{3}H]-SCH 58261 binding assays

Kinetic studies (n=3) showed that [^{3}H]-SCH 58261 binding reached equilibrium after approximately 20 min and was stable for at least 2 h (Figure 1a). [^{3}H]-SCH 58261 binding was rapidly reversed by the addition of 10 μM NECA (Figure 1b). Association and dissociation kinetic rate constants, determined as described by Burt (1986), from these experiments were: K_{obs}=0.077 (0.051−0.082) min^{-1}, K_{-1}=0.043 (0.031−0.065) min^{-1} from a $t_{1/2}$=16 min (14−18), and K_{+1}=0.067 min^{-1} nM^{-1}. A radioligand affinity constant (K_d) value of 0.64 (0.55−0.82) nM was derived from these experiments. Figure 2 shows a saturation curve of [^{3}H]-SCH 58261 binding to adenosine A_{2A}-receptors 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 a K_d value of 0.85 (0.73−0.98) nM and a B_{max} value of 35 (31−40) fmol mg^{-1} protein. The presence of 10 mM $MgCl_2$, 100 μM GTP or 2 iu adenosine deaminase in the assay mixture did not modify the percentage of specific binding. K_i values for reference adenosine receptor agonists and antagonists, obtained by competition with [^{3}H]-SCH 58261 binding are shown in Table 1. Figures 3a and 3b show inhibition curves for adenosine agonists and antagonists in human lymphocytes, respectively. The order of potency in [^{3}H]-SCH 58261 displacement assays for adenosine agonists was: 2-HE-NECA > NECA > CGS 21680 > **R**-PIA > CCPA > **S**-PIA = CHA > cladribine. 2-HE-NECA and NECA were the most potent compounds, with affinities in the low nanomolar range (14−22 nM), while the selective A_1-receptor agonists displayed affinity values in the micromolar range and the partially non-selective agonist, cladribine was the least potent compound. Displacement of [^{3}H]-SCH 58261 binding was stereoselective, with **R**-PIA (K_i=1.5 μM) being approximately 4 times more active than its

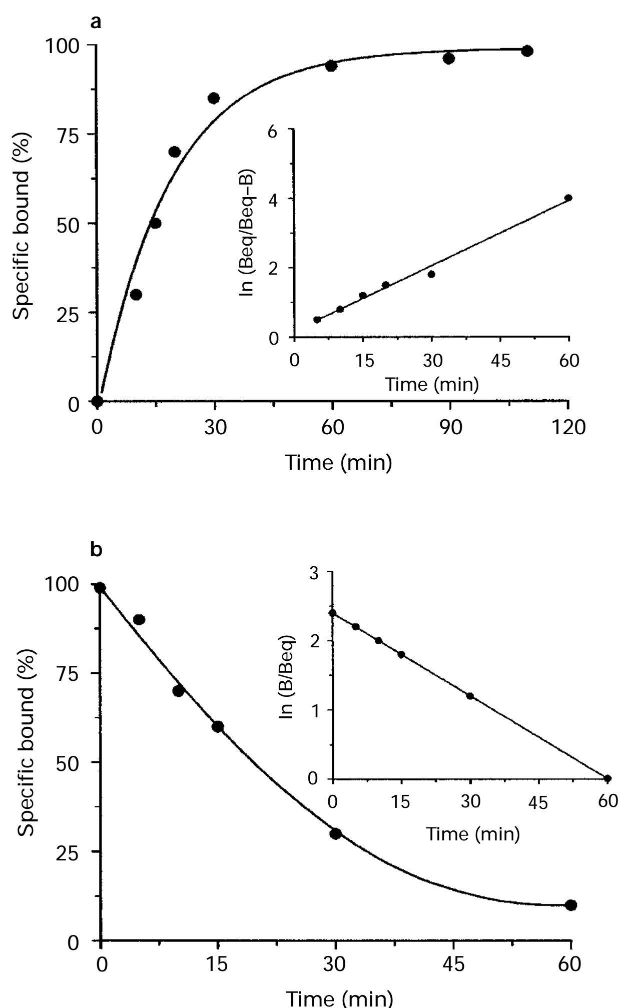

Figure 1 (a) Kinetics of 0.5 nM [^{3}H]-SCH 58261 binding to human lymphocyte membranes with association curves representative of a single experiment. Inset: first-order plots of [^{3}H]-SCH 58261 binding. Beq, amount of [^{3}H]-SCH 58261 bound to equilibrium; B, amount of [^{3}H]-SCH 58261 bound at each time. Association rate constant was: K_{+1}=0.067 min^{-1} nM^{-1}. (b) Kinetics of 0.5 nM [^{3}H]-SCH 58261 binding to human lymphocyte membranes with dissociation curves representative of a single experiment. Inset: first-order plots of [^{3}H]-SCH 58261 binding. Beq, amount of [^{3}H]-SCH 58261 bound to equilibrium; B, amount of [^{3}H]-SCH 58261 bound to each time. Dissociation rate constant was: K_{-1}=0.07 (0.06−0.09) min^{-1}.

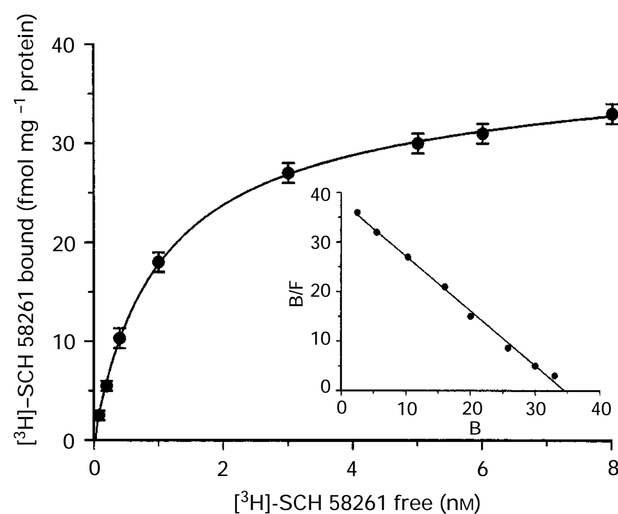

Figure 2 Saturation of [^{3}H]-SCH 58261 binding to human lymphocyte adenosine A_{2A}-receptors. Experiments were performed as described in Methods. Values are the means of four separate experiments performed in triplicate; vertical lines show s.e.mean. In the inset the Scatchard plot of the same data is shown. K_d value (nM) was 0.85 (0.73−0.98) and B_{max} value (fmol mg^{-1} protein) was 35 (31−40). Non-specific binding was determined in the presence of 10 μM NECA.

Table 1 Inhibition of [^{3}H]-SCH 58261 binding (K_i) by adenosine agonists and antagonists to human lymphocyte membranes

	[^{3}H]-SCH 58261 binding		*Cyclic AMP assay*
Compound	K_i (nM)	*Hill coefficient*	EC_{50}/IC_{50} (nM)
2-HE-NECA	14 (11–18)	0.93 (0.86–1.00)	40 (38–42)
NECA	22 (20–24)	0.98 (0.93–1.01)	200 (184–216)
CGS 21680	838 (731–960)	0.97 (0.91–1.03)	897 (821–981)
R-PIA	1490 (1302–1705)	0.95 (0.89–1.02)	3936 (3206–4832)
CCPA	3785 (3203–4472)	0.93 (0.86–1.02)	5450 (4710–6320)
S-PIA	6497 (5048–8363)	0.91 (0.80–1.04)	8470 (7705–9311)
CHA	6898 (6408–7425)	1.01 (0.90–1.24)	12480 (11700–13310)
Cladribine	50000 (45520–52870)	0.82 (0.75–1.00)	>500000
Antagonists			
CGS 15943	0.20 (0.16–0.24)	0.97 (0.87–1.05)	12 (11–13)
SCH 58261	0.85 (0.83–0.87)	0.92 (0.82–1.04)	16 (14–18)
XAC	7.28 (5.51–9.63)	0.94 (0.88–1.01)	55 (51–59)
KF 17837	19 (15–24)	0.92 (0.85–1.02)	60 (53–67)
DPCPX	492 (401–620)	0.86 (0.72–1.06)	600 (560–670)

A comparison is made with stimulation by adenosine agonists (EC_{50}) or inhibition of NECA 1 μM-stimulated cyclic AMP levels by antagonists (IC_{50}) in isolated human lymphocytes. Each value represents the geometric mean, with 95% or 99% (Hill coefficients) confidence limits in parentheses, of at least four separate experiments performed in duplicate.

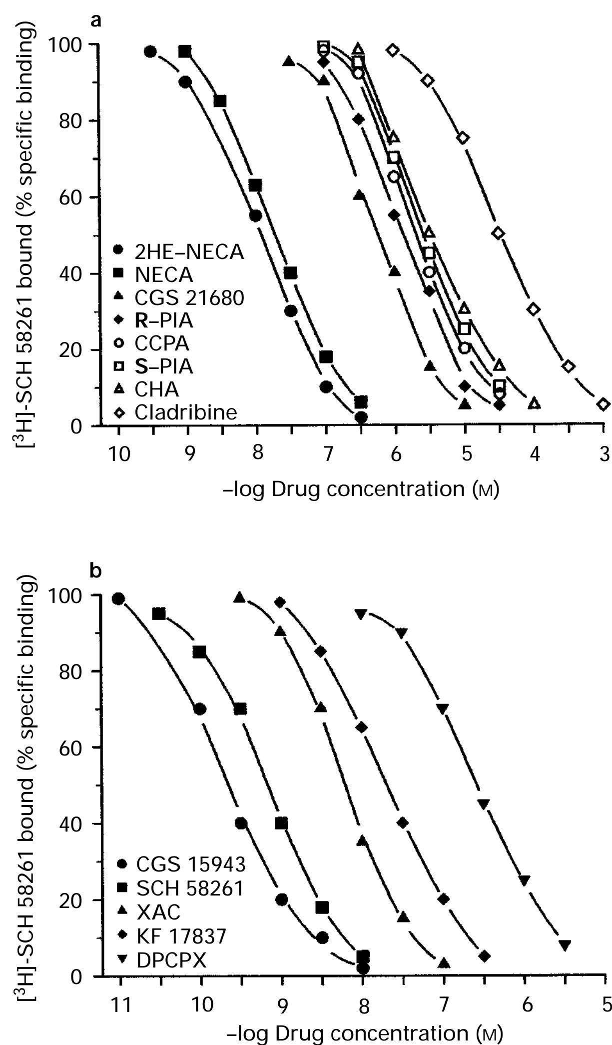

Figure 3 (a) Inhibition curves of specific [^{3}H]-SCH 58261 (0.8 nM) binding to human lymphocyte membranes by adenosine agonists. Curves are representative of a single experiment from a group of four independent experiments. Non-specific binding was determined in the presence of 10 μM NECA. (b) Inhibition curves of specific [^{3}H]-SCH 58261 (0.8 nM) binding to human lymphocyte membranes by adenosine antagonists. Curves are representative of a single experiment from a group of four independent experiments. Non-specific binding was determined in the presence of 10 μM NECA.

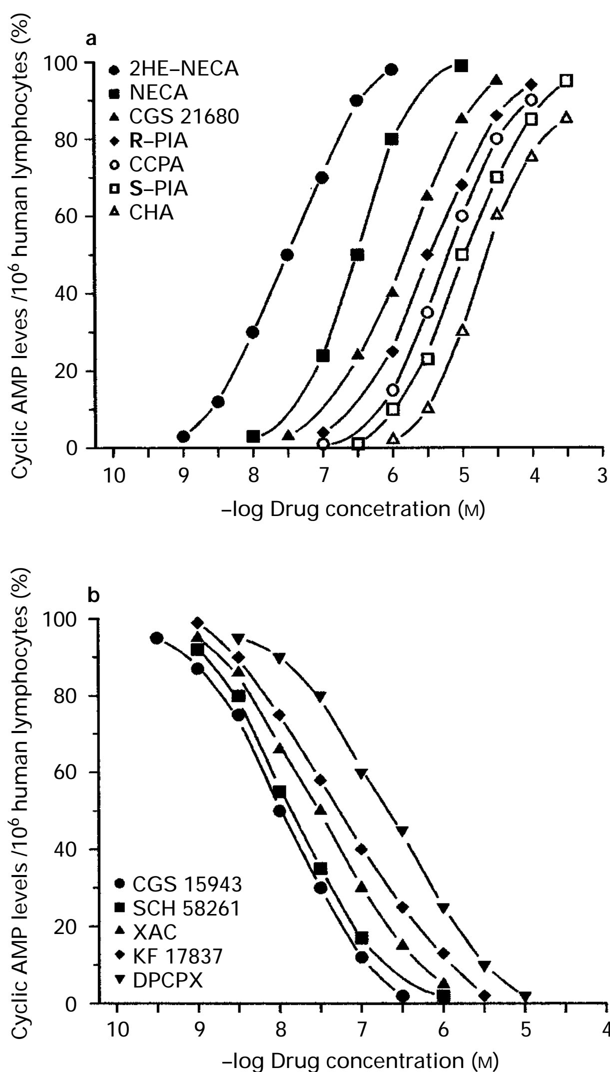

Figure 4 (a) Stimulation of cyclic AMP levels in human lymphocytes by adenosine agonists. Curves are representative of a single experiment from a group of four independent experiments. (b) Inhibition of NECA (1 μM) stimulated cyclic AMP levels (100%) in human lymphocytes by adenosine antagonists. Curves are representative of a single experiment from a group of four independent experiments.

stereoisomer, S-PIA (K_i = 6.6 μM). The order of potency of the receptor antagonists was: CGS 15943 > SCH 58261 > XAC > KF 17837 > DPCPX. CGS 15943 and SCH 58261 were the most potent compounds (K_i values in the low nanomolar range). Hill coefficients of all compounds (Table 1) were found to be not significantly different from unity.

Assay of cyclic AMP levels

Table 1 presents EC_{50} values for forskolin-induced stimulation of cyclic AMP levels in human lymphocytes and Figure 4a shows the log dose-response curve for typical adenosine receptor agonists. All adenosine analogues were able to increase cyclic AMP levels displaying an order of potency similar to that observed in binding affinities for the adenosine A$_{2A}$-receptor. 2-HE-NECA appeared to be the most potent compound (EC_{50} = 40 nM) followed by NECA and CGS 21680 (EC_{50} in the range 200–900 nM); **R**-PIA was more potent than its stereoisomer **S**-PIA (EC_{50} = 4 μM and 8.5 μM, respectively). Figure 4b shows the inhibition of NECA-stimulated cyclic AMP levels in human lymphocytes by adenosine receptor antagonists and Table 1 presents the IC_{50} values of the same compounds. The most potent adenosine receptor antagonists were CGS 15943 and SCH 58261 (IC_{50} = 12 and 16 nM, respectively). With the exception of cladribine, which at the highest concentration increased cyclic AMP by only 22%, all agonists produced a similar increase in cyclic AMP levels, suggesting that they are full agonists. The Spearman's rank correlation coefficient between affinity values of [^{3}H]-SCH 58261 binding to A$_{2A}$-adenosine receptors by selective receptor agonists and antagonists with EC_{50} and IC_{50} values in the cyclic AMP assay was 1.00 (P < 0.01).

Thermodynamic binding assay

K_d and B_{max} values derived from the saturation experiments of [^{3}H]-SCH 58261 binding to A$_{2A}$ adenosine receptors performed at the four chosen temperatures were found to be within the following range: K_d = 0.85–2.57 nM and B_{max} = 35–38 fmol mg^{-1} protein. While dissociation constants (K_d) changed with temperature, B_{max} values obtained from [^{3}H]-SCH 58261 saturation experiments appeared to be largely independent of temperature. 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 $\ln K_A$ versus 1/T of the [^{3}H]-SCH 58261 binding to the A$_{2A}$ adenosine receptor and the final equilibrium thermodynamic parameters (expressed as mean values ± s.e. of three independent determinations) were: $\Delta G°$ = −48.32 ± 0.10 kJ mol^{-1}; $\Delta H°$ = −36.74 ± 3.42 kJ mol^{-1}; $\Delta S°$ = 38.87 ± 4.51 J mol^{-1} K^{-1}. The linearity of the plot was statistically significant ($\Delta Cp° \approx 0$) and its slope ($-\Delta H°/R$) was positive, a property which has been found to be typical for antagonist binding to rat striatal A$_{2A}$ adenosine receptors (Borea *et al.*, 1995).

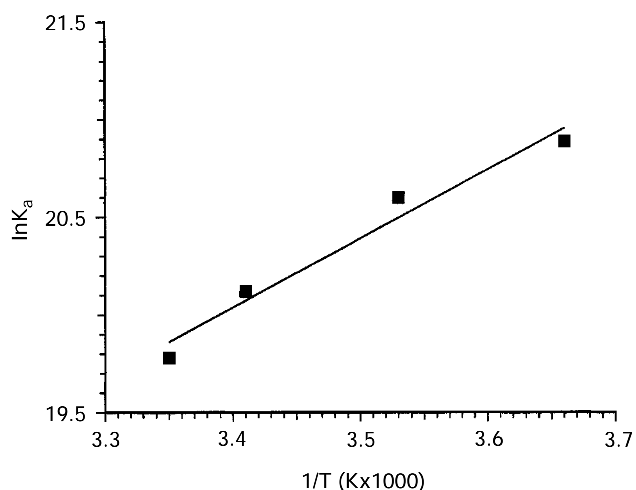

Figure 5 Van't Hoff plot showing the effect of temperature on the equilibrium binding association constant, $K_a = 1/K_d$, of [^{3}H]-SCH 58261. The plot was essentially linear in the temperature range investigated (0–25C°).

Discussion

In the past, the non-selective agonist radioligand [^{3}H]-NECA has been used successfully to label A$_{2A}$ adenosine receptors in rat striatal membranes (Bruns *et al.*, 1986). However, [^{3}H]-NECA has also been shown to interact with non-receptor binding proteins in both cerebral and peripheral tissues (Hutchison *et al.*, 1990). Over the past few years, the receptor agonist CGS 21680, a NECA derivative with both high affinity and selectivity for the A$_{2A}$ adenosine receptor, has become the radioligand of choice with which to investigate this receptor subtype on striatal membranes (Jarvis *et al.*, 1989) but few studies exist concerning its use in peripheral tissues, probably due to the presence of adenotin sites (Varani *et al.*, 1994). However, in binding studies the use of antagonist radioligands is preferred because they can overcome a variety of limitations associated with the high and low agonist affinity states. The development of A$_{2A}$ antagonist radioligands has been hampered by the lack of selective ligands (Ongini & Fredholm, 1996). Among adenosine receptor antagonists, xanthine amine congener ([^{3}H]-XAC), which is a non-selective adenosine receptor antagonist, was used to characterize the A$_{2A}$ adenosine receptor in human platelet membranes (Ji *et al.*, 1992). [^{3}H]-XAC binding was consistent with the labelling of A$_{2A}$ receptors, but specific binding was only 40% of the total binding. Another receptor antagonist radioligand, [^{3}H]-PD 115,199, was used, even though it has equal affinity for both A$_1$-and A$_{2A}$-receptors. Thus, in the presence of 20 nM DPCPX [^{3}H]-PD 115,199 labelled specifically A$_{2A}$-striatal receptors, but its radiostability was found to be poor (Bruns *et al.*, 1987). More recently, the selective A$_{2A}$-receptor antagonist [^{3}H]-KF 17837S has been shown to label directly A$_{2A}$-adenosine receptors in rat striatum (Nonaka *et al.*, 1994). The binding occurs at concentrations in the low nanomolar range, but non-specific binding (about 30–40%) is still high. The development of the new potent and selective A$_{2A}$-receptor antagonist SCH 58261, which does not to bind to adenotin sites (Varani *et al.*, 1996), and its radiolabelled form, [^{3}H]-SCH 58261, has facilitated the characterization of A$_{2A}$-receptors in a variety of tissues (Baraldi *et al.*, 1994; Zocchi *et al.*, 1996a,b; Dionisotti *et al.*, 1996).

In the present study we have characterized, for the first time, A$_{2A}$-adenosine receptors on human lymphocyte membranes by using [^{3}H]-SCH 58261. The biological importance of lymphocyte A$_{2A}$-adenosine receptors is emphasized by the fact that adenosine possesses immunosuppressive properties by activating adenylyl cyclase and in turn elevating cyclic AMP levels. In general, activation of adenosine receptors on lymphocytes diminishes immune and inflammatory responses. This is of interest in view of the fact that diminished adenosine receptor functions might contribute to diseases in which there is an abnormal self-directed immune response or an excess of inflammation.

Association and dissociation kinetic parameters of [^{3}H]-SCH 58261 binding were similar to those observed in a previous study carried out in human platelets (Dionisotti *et al.*, 1996). In saturation experiments, [^{3}H]-SCH 58261 labelled a single class of recognition sites with affinity (K_d = 0.85 nM) in the same order of magnitude as that determined by kinetic experiments (K_d = 0.64 nM) and also very close to that observed in rat striatal and platelet membranes (K_d values = 0.7 nM and 0.85 nM, respectively). In competition studies, reference adenosine receptor agonists and antagonists bound the lymphocyte A$_{2A}$-receptor with a rank order of po-

tency and affinities similar to those observed in [^{3}H]-SCH 58261 binding to human platelet membranes. Computer analysis of inhibition curves of both adenosine agonists and antagonists showed that [^{3}H]-SCH 58261 interacts with only one recognition binding site (Hill coefficients not significantly different from unity). Our data are in agreement with those previously found in rat striatal and platelet membranes. It is notable that contrasting results are present in the literature about the ability of adenosine A_{2A}-receptor antagonists to reveal both high and low affinity states (Bruns *et al*., 1987; Nonaka *et al*., 1994). In agreement with our results, adenosine receptor agonists displaced the antagonist radioligand [^{3}H]-PD 115,119 to the A_{2A} striatal receptor with Hill coefficients close to unity (Bruns *et al*., 1987) and the same results were obtained in striatal membranes with [^{3}H]-SCH 58261 (Zocchi *et al*., 1996b). In contrast, other authors found, in striatum, that adenosine receptor agonists inhibited the binding of the antagonists [^{3}H]-XAC (Ji *et al*., 1992) and [^{3}H]-KF 17837 (Nonaka *et al*., 1994) with shallow competition curves which are best described by a two site model. These latter results are not supported by the present data on agonist inhibition curves, where Hill coefficients were found to be not significantly different from unity, thus excluding the involvement of multiple coupling affinity states. This finding could be explained either by a tight coupling between receptor and G protein or, alternatively, by the existence of a uniform conformation of the receptor binding subunit regardless of G protein modulation (Nanoff *et al*., 1991; Nanoff & Stiles, 1993).

Thermodynamic analysis of [^{3}H]-SCH 58261 binding was performed and the enthalpic (ΔH°) and entropic (ΔS°) contribution to the standard free energy (ΔG°) of the binding equilibrium were determined. The linearity of the van't Hoff plot for [^{3}H]-SCH 58261 binding in the human lymphocytes indicates that the ΔCp° values of the drug-interaction is nearly zero which means that ΔH° and ΔS° values were not significantly affected by temperature variations, at least over the temperature range investigated (Borea *et al*., 1995). It is notable that 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 membrane receptor ligands so far studied, from a thermodynamic point of view (Gilli *et al*., 1994). Thermodynamic data obtained from the van't Hoff plot, indicate that [^{3}H]-SCH 58261 binding to human lymphocytes is entropy and enthalpy-driven ($\Delta S^\circ = 38.87 \pm 4.51$ Jmol^{-1} K^{-1}, $\Delta H^\circ = -36.74 \pm$ 3.42 kJmol^{-1}). This binding behaviour has been found, in rat striatum, to be typical of A_{2A} – receptor antagonists (Borea *et al*., 1995). Finally, another aim of the present study was to investigate the regulation of adenylyl cyclase activity to verify whether the binding parameters are correlated with the functional response. We evaluated EC_{50} or IC_{50} values obtained for the stimulation or inhibition, by receptor agonists and antagonists, respectively, of cyclic AMP levels. Interestingly, in the adenylyl cyclase assay, the compounds examined exhibited a rank order of potency very close to that observed in binding experiments. 2-HE-NECA and NECA, which are known to have the highest affinity of adenosine analogues for A_{2A}-receptors on human platelets and lymphocytes, were also the most potent in the cyclic AMP assay.

The adenosine receptor antagonists CGS 15943 and SCH 58261, which have K_i and IC_{50} values in the nanomolar range ($K_i = 0.2$ nM and 0.85 nM; $IC_{50} = 12$ nM and 16 nM, respectively), exhibit high affinity also in a variety of membranes (Ongini & Fredholm, 1996). The significant difference between the IC_{50} values for antagonism of NECA-induced activation of cyclic AMP accumulation and the corresponding K_i values obtained in the binding assays could be due to the high concentration (1 μM) of NECA used in the cyclic AMP assay.

In contrast, the A_1/A_2 non-selective agonist cladribine revealed a low affinity in the binding assay ($K_i = 50$ μM) and was unable to stimulate fully cyclic AMP levels ($EC_{50} > 500$ μM), suggesting that it may be a partial agonist. However, there is evidence to suggest that cladribine is effective against a variety of lymphoid neoplasms. The precise mechanism by which cladribine acts is as yet unknown and is further complicated by the fact that the drug is active against both dividing and non dividing cells. The present data strongly suggest that the interaction with A_{2A}-receptors is not the only (or main) mechanism of action of this compound. It would be of interest to study deoxyadenosine congeners resistant to deamination, in the hope that these agents might possess antileukemic and antilymphocyte activity.

In conclusion, all these data indicate, for the first time, that adenosine receptors present on lymphocytes have a pharmacological, biochemical and thermodynamic profile typical of the A_{2A} adenosine receptor subtype. The presence of A_{2A}-receptors in a peripheral cells, such as human lymphocytes, strongly suggests an important role for adenosine in modulating immune and inflammatory responses.

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