



[³H]-SCH 58261 labelling of functional A_{2A} adenosine receptors in human neutrophil membranes

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1 The present study describes the direct labelling of A_{2A} adenosine receptors in human neutrophil membranes with the potent and selective antagonist radioligand, [³H]-5-amino-7-(2-phenylethyl)-2-(2-furyl)-pyrazolo[4,3-e]-1,2,4-triazolo[1,5-c]pyrimidine, ([³H]-SCH 58261). In addition, both receptor affinity and potency of a number of adenosine receptor agonists and antagonists were determined in binding, adenylyl cyclase and superoxide anion production assays.

2 Saturation experiments revealed a single class of binding sites with *K_d* and *B_{max}* values of 1.34 nM and 75 fmol mg⁻¹ protein, respectively. Adenosine receptor ligands competed for the binding of 1 nM [³H]-SCH 58261 to human neutrophil membranes, with a rank order of potency consistent with that typically found for interactions with the A_{2A} adenosine receptors. In the adenylyl cyclase and in the superoxide anion production assays the same compounds exhibited a rank order of potency identical to that observed in binding experiments.

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

4 It was concluded that in human neutrophil membranes, [³H]-SCH 58261 directly labels binding sites with pharmacological properties similar to those of A_{2A} adenosine receptors of other tissues. The receptors labelled by [³H]-SCH 58261 mediated the effects of adenosine and adenosine receptor agonists to stimulate cyclic AMP accumulation and inhibition of superoxide anion production in human neutrophils.

Keywords: Adenosine A_{2A} receptors; adenosine A_{2A} receptor ligands; [³H]-SCH 58261 binding; human neutrophil membranes; cyclic AMP assays; superoxide anion production; thermodynamic analysis

Introduction

Adenosine acts as a neuromodulator in the central and peripheral nervous systems and as a homeostatic regulator in a variety of other tissues, including smooth muscle cells, heart muscle, platelets, coronary arteries and cells involved in immune and inflammatory reactions (Schrier & Imre, 1986; Liang, 1992; Hussain & Mustafa, 1993). The majority of the effects of adenosine are mediated by four pharmacologically distinct adenosine receptor subtypes, designated A₁, A_{2A}, A_{2B} and A₃, all of which have been cloned and their binding profiles characterized in various cell lines (Fredholm *et al.*, 1994; Olah & Stiles, 1995).

Adenosine is believed to function as an endogenous anti-inflammatory agent (Cronstein & Hirschhorn, 1990) based on its ability to inhibit cellular adhesion (Cronstein *et al.*, 1992) and superoxide anion generation (Cronstein *et al.*, 1990). Tissue damage induced by inflammatory processes is due in part to the migration of neutrophils to the sites of inflammation (e.g. sites where there is infection, tissue injury, ischaemia or complement activation) followed by the release of membrane-damaging oxygen radicals. Adenosine inhibits both activities and therefore seems to be important in limiting abnormal and excessive inflammatory reactions. Ischaemia is a powerful stimulus for adenosine production (Rudolphi *et al.*, 1992) and the vascular endothelium may protect itself against damage from activated neutrophils by releasing adenosine.

Interestingly, a recent study (Bong *et al.*, 1996) showed that the central nervous system can also influence inflammatory responses by reducing local levels of adenosine, and by in turn regulating the accumulation of neutrophils. Based on results of functional studies, it is known that A₁ and at least one subtype of A₂ receptors (Cronstein *et al.*, 1985), recently proposed to be the A_{2A} receptor subtype (Fredholm *et al.*, 1996), are present on neutrophils. The activation of A₁ receptors leads to enhanced chemotactic response, whereas activation of the A₂ receptors reduces the oxidative burst. A_{2A} receptors are known to activate stimulant guanine-binding proteins (Gs proteins) and thus to stimulate adenylyl cyclase (Ongini & Fredholm, 1996) but the role of adenosine 3':5'-cyclic monophosphate (cyclic AMP) in mediating the effects of adenosine in reducing neutrophil oxidative burst is dubious (Cronstein, 1994). The lack of available radioligands that do not interact with the adenosine binding proteins (Lohse *et al.*, 1988; Hutchinson *et al.*, 1990) has until recently prevented the direct labelling and full pharmacological characterization of the A_{2A} adenosine receptors in peripheral tissues such as human neutrophils. 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 potent and selective A_{2A} receptor 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, [³H]-SCH

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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), human cloned receptors transfected in mammalian cells (Dionisotti *et al.*, 1997) and human lymphocytes (Varani *et al.*, 1997). The present paper describes the characterization of A_{2A} adenosine receptors in human neutrophils by using the selective A_{2A} receptor antagonist [³H]-SCH 58261. The effect of typical adenosine receptor agonists to increase the cyclic AMP intracellular levels and the potency of a series of antagonists to inhibit N-ethylcarboxamidoadenosine (NECA) induced stimulation of adenylyl cyclase activity was evaluated. Moreover, the effect of adenosine receptor agonists, to inhibit superoxide anion generation, and the antagonism of the effect of increasing concentrations of 2-hexynyl-5'-N-ethylcarboxamidoadenosine (HE-NECA) on superoxide anion production by fixed concentrations of adenosine receptor antagonists in N-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP)-stimulated neutrophils were studied. Finally, with the aim of obtaining new insights into the forces driving the coupling of human A_{2A} neutrophil receptor with a selective ligand, a thermodynamic analysis of [³H]-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.

Methods

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

Preparation of cell suspensions

Human neutrophils were prepared according to the procedure of Markert *et al.* (1984) with modifications. Blood was supplemented with 20 ml of a solution consisting of 6% (by weight) Dextran T500. After gentle mixing, erythrocytes were allowed to settle down at 20°C for 60 min. The turbid upper layer containing leukocytes was carefully removed with polyethylene transfer pipette and placed into a 50 ml polypropylene centrifuge tube. Leukocytes were pelleted by centrifugation at 20°C for 12 min at 100 g. All other centrifugations were also performed in these tubes. Remaining erythrocytes were lysed by suspending the cell pellet in 10 ml of distilled water at 4°C under gentle agitation. After 30 s, isotonicity was restored by adding 3 ml of a solution containing 0.6 M NaCl. Cells were pelleted by centrifugation at 20°C for 5 min at 250 g, suspended in 10 ml of the Krebs Ringer phosphate buffer consisting of (mM): Na⁺ 150, K⁺ 5, Ca²⁺ 1.3, Mg²⁺ 1.2, Cl⁻ 155 and HEPES 10 at pH 7.45 and layered onto 10 ml of Fycoll-Hypaque. Neutrophils were sedimented by centrifugation at 20°C for 20 min at 250 g. This procedure allowed studies of cell suspensions containing 98 ± 2% neutrophils with few contaminating red blood cells or platelets. This cell suspension was used for measurement of cyclic AMP levels and superoxide anion production.

Preparation of neutrophil membranes

Human neutrophils were resuspended in 50 mM Tris HCl pH 7.4 containing 10 mM MgCl₂ and centrifuged at 11,000 g for 15 min at 4°C. The supernatant was discarded and the pellet resuspended in 10 ml of 50 mM Tris-HCl and centrifuged at

11,000 g for 15 min. 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 [³H]-SCH 58261 binding. The protein concentration was determined according to a Bio-Rad method (Bradford, 1976) with bovine albumin as reference standard.

[³H]-SCH 58261 binding assay in the neutrophil membranes

Binding assays were carried out according to Dionisotti *et al.* (1996). In saturation studies, neutrophil membranes were incubated with 8 to 10 different concentrations of [³H]-SCH 58261 ranging from 0.05 to 10 nM in a total volume of 250 µl containing 50 mM Tris HCl buffer, 10 mM MgCl₂, pH 7.4. In competition experiments, carried out to determine the IC₅₀ values, 1 nM [³H]-SCH 58261 was incubated with 100 µl of neutrophil 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 the IC₅₀ values according to the Cheng and 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 40% 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 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%). A weighted non linear least-squares curve fitting program LIGAND (Munson & Rodbard, 1980), was used for computer analysis of saturation and inhibition experiments.

Measurement of cyclic AMP levels in human neutrophils

Human neutrophils (10⁶ cells ml⁻¹) were suspended in 0.5 ml incubation mixture (Krebs Ringer phosphate buffer, containing 1.0 iu adenosine deaminase ml⁻¹ and 0.5 mM 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone (Ro 20-1724) as phosphodiesterase inhibitor) and preincubated 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 EC₅₀ and antagonist IC₅₀ 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 [³H]-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.

Superoxide anion production

O₂⁻ release was monitored continuously with a temperature-controlled spectrophotometer by the reduction of ferricytochrome c inhibited by superoxide dismutase (SOD), as described elsewhere (Spisani *et al.*, 1992). The mixture was incubated with either control or different concentrations of agonists for 5 min at 37°C. At 0 time, 0.1 µM FMLP was added and the amount of O₂⁻ produced was calculated by the differences in absorbance of the samples, with a 15.5 mM extinction coefficient at 550 nm for cytochrome c reduction. The net nanomol of O₂⁻ release were calculated from the formula: nanomol released by stimulated neutrophils minus nanomol released by resting neutrophils alone. Neutrophils were incubated with 5 µg ml⁻¹ cytochalasin B for 5 min before peptide activation.

Thermodynamic analysis

For a generic binding equilibrium $L + R = LR$ (L = ligand, R = receptor), the affinity constant is calculated as $K_A = [LR]/([L][R]) = [LR]/([L_{max} - LR][B_{max} - LR]) = 1/K_d$, where $[L_{max}]$ = total concentration of the ligand added, $[B_{max}]$ = total concentration of the binding sites and K_d = dissociation constant. As $[LR]/([L_{max} - LR]) = [Bound]/[Free] = [B_{max}] K_A [Bound]$, the K_A and B_{max} values can be obtained from the slope and intercept of the Scatchard plot (Scatchard, 1949) $[Bound]/[Free]$ versus $[Bound]$.

The standard free energy was calculated as $\Delta G^\circ = -RT \ln K_A$ at 298.15 K, the standard enthalpy, ΔH° , from the Van't Hoff plot $\ln K_A$ versus $(1/T)$ (the slope of which is $-\Delta H^\circ/R$) and the standard entropy as $\Delta S^\circ = (\Delta H^\circ - \Delta G^\circ)/T$ with $T = 298.15$ K and $R = 8.314$ J K⁻¹ mol⁻¹.

Saturation experiments of [³H]-SCH 58261 binding to the human neutrophil membranes were carried out at 0, 10, 20, 25°C with concentrations ranging from 0.05 to 10 nM.

Drugs

NECA (5'-N-ethylcarboxamidoadenosine); R-PIA and S-PIA (R(-)- and S(+)-N⁶-(2-phenylisopropyl)-adenosine); CGS 21680 (2-[p-(2-carboxyethyl)-phenethylamino]-5'-N-ethylcarboxamidoadenosine); CHA (N⁶-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) were from Research Biochemicals Incorporated (Natick, Mass., U.S.A.). CCPA (2-chloro-N⁶-cyclopentyladenosine); HE-NECA (2-hexynyl-5'-N-ethylcarboxamidoadenosine); KF 17837 ((E)-1,3-dipropyl-8-(3,4-dimethoxystyryl)-7-methylxanthine); 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). Ro 20-1724 (4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone) was the kind gift of Dr E. Kyburz, Hoffman-La Roche (Basel, Switzerland). Dextran and Ficoll-Paque were purchased from Pharmacia (Uppsala, Sweden). FMLP (N-formyl-L-methionyl-L-leucyl-L-phenylalanine), ferricytochrome c, cytochalasin B were from Sigma Chemicals Co. (St. Louis, MO). [³H]-SCH 58261 (specific activity 68.6 Ci mmol⁻¹) was

from the Schering-Plough Research Institute (Milan, Italy). [³H]-cyclic AMP (specific activity 21 Ci mmol⁻¹) was purchased from the Radiochemical Centre (Amersham, U.K.). Aquassure and Atomlight were from NEN Research Products (Boston, Mass., U.S.A.). All other reagents were of analytical grade and obtained from commercial sources.

Results

[³H]-SCH 58261 binding assays

Kinetic studies ($n=3$) showed that [³H]-SCH 58261 binding reached equilibrium after approximately 20 min and was stable for at least 3 h (Figure 1a). [³H]-SCH 58261 binding was rapidly reversed by the addition of 10 µM NECA (Figure 1b). Computer analysis revealed that both association and dissociation data fitted a one-component model significantly better than a two-component model ($P<0.05$) with the following rate constants: $K_{obs} = 0.09$ (0.05–0.11) min⁻¹ and

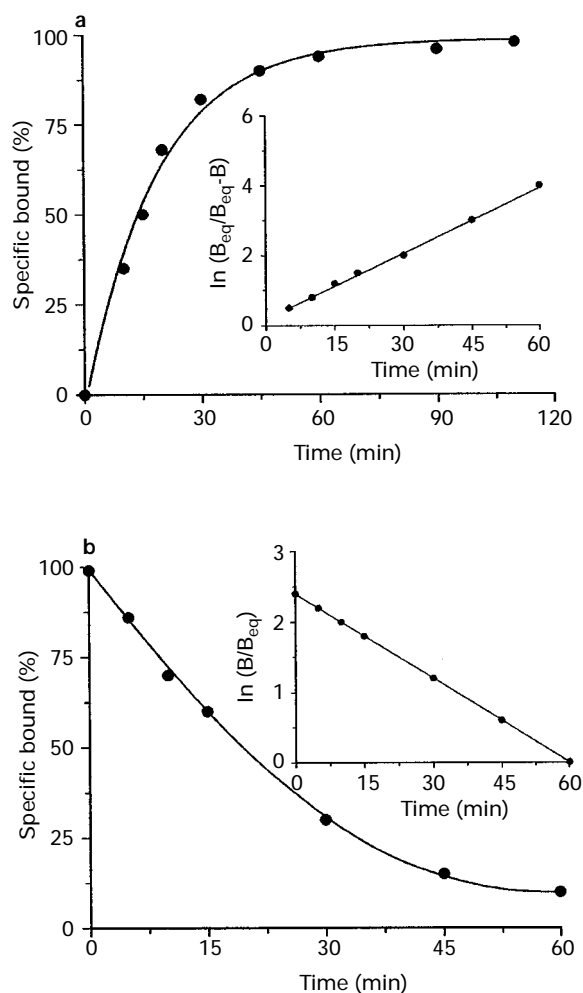


Figure 1 (a) Kinetics of 1 nM [³H]-SCH 58261 binding to human neutrophil membranes with association curves representative of a single experiment. Inset, first-order plots of [³H]-SCH 58261 binding. B_{eq} , amount of [³H]-SCH 58261 bound at equilibrium; B, amount of [³H]-SCH 58261 bound at each time. Association rate constant was: $K_{obs} = 0.088$ (0.051–0.110) min⁻¹. (b) Kinetics of 1 nM [³H]-SCH 58261 binding to human neutrophil membranes with dissociation curves representative of a single experiment. Inset, first-order plots of [³H]-SCH 58261 binding. Dissociation rate constant was: $K_{-1} = 0.046$ (0.026–0.075) min⁻¹. The incubation time was 60 min at 4°C.

$K_{-1} = 0.05$ (0.03 – 0.08) min^{-1} from a $t_{1/2} = 15$ min (14 – 18). These values gave a kinetic dissociation constant (K_d) of 1.1 (0.9 – 1.4) nM. Figure 2 shows a saturation curve of [³H]-SCH 58261 binding to A_{2A} adenosine receptors and the linearity of the Scatchard plot in the inset is indicative of the presence of a single class of binding sites with K_d value of 1.34 (1.23 – 1.48) nM and B_{max} value of 75 (70 – 85) 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 (about 60%). K_i values for various adenosine receptor agonists and antagonist, obtained in the competition of [³H]-SCH 58261 binding are summarized in Table 1. Figure 3a and b shows the competition curves of adenosine agonists and antagonists in human neutrophils, respectively. The rank order of potency of adenosine agonists to compete with [³H]-SCH 58261 binding to neutrophil membranes was: HE-NECA > NECA > CGS 21680 > R-PIA > CCPA > S-PIA > CHA. HE-NECA and NECA were the most potent agonists, their affinity being in the low

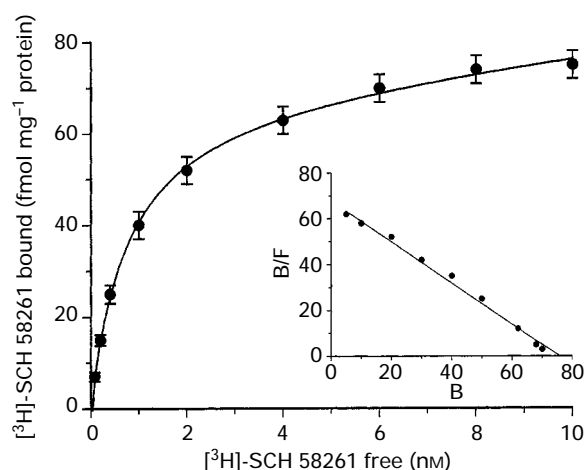


Figure 2 Saturation of [³H]-SCH 58261 binding to human neutrophil adenosine A_{2A} receptors. Experiments were performed as described in Methods. Values are the means and vertical lines s.e.mean of four separate experiments performed in triplicate. In the inset the Scatchard plot of the same data is shown. K_d value (nM) was 1.34 (1.23 – 1.48) and B_{max} value (fmol mg^{-1} protein) was 75 (70 – 85). Non-specific binding was determined in the presence of 10 μM NECA.

nanomolar range (8 – 10 nM), while the selective A₁ receptor agonists displayed affinity values in the micromolar range. Displacement of [³H]-SCH 58261 binding was stereoselective, with R-PIA ($K_i = 990$ nM) being approximately 5 times more active than its stereoisomer, S-PIA ($K_i = 4500$ nM). 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 most compounds (data not shown) were not significantly different from unity.

Cyclic AMP assay

Table 1 summarizes the EC_{50} values for forskolin-induced stimulation of cyclic AMP levels in human neutrophils 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 identical to that observed in binding affinities for the adenosine A_{2A} receptor. HE-NECA appeared to be the most potent compound ($\text{EC}_{50} = 30$ nM) followed by NECA and CGS 21680 (EC_{50} in the range 150 – 400 nM); R-PIA was more potent than its stereoisomer S-PIA ($\text{EC}_{50} = 3$ μM and 9 μM , respectively). Figure 4b shows the inhibition of cyclic AMP accumulation in human neutrophils by adenosine receptor antagonists and Table 1 summarizes the IC_{50} values of the same compounds. The most potent adenosine receptor antagonists were CGS 15943 and SCH 58261 ($\text{IC}_{50} = 15$ and 20 nM, respectively). The Spearman's rank correlation coefficient between affinity values of [³H]-SCH 58261 binding to A_{2A} adenosine receptor by selected receptor agonists and antagonists and the EC_{50} and IC_{50} values in the cyclic AMP assay was 1.00 ($P < 0.01$) (Table 2). A comparison of the K_i , EC_{50} and IC_{50} values indicated that a high correlation exists between data obtained from binding and cyclic AMP assays (Figure 6a).

Effects of adenosine agonists and antagonists on generation of superoxide anion by FMLP-stimulated neutrophils

We compared the ability of adenosine agonists to inhibit the generation of superoxide anion by neutrophils incubated in the presence of the metabolite cytochalasine B. All compounds inhibited superoxide anion generation stimulated by FMLP in

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

Compound	$[^3\text{H}]$ -SCH 58261 binding K_i (nM)	Cyclic AMP assay EC_{50} and IC_{50} (nM)		O_2^- production EC_{50} (nM)	
Agonists					
HE-NECA	7.66 (7.44–7.90)	30	(25–35)	3.0	(2.5–3.6)
NECA	9.84 (8.01–12.08)	150	(127–177)	30.0	(24.6–35.9)
CGS 21680	200 (184–216)	400	(371–430)	300	(278–323)
R-PIA	990 (841–1165)	3000	(2693–3339)	998	(890–1116)
CCPA	1980 (1683–2329)	7000	(6380–7680)	1995	(1779–2233)
S-PIA	4486 (4102–4905)	9000	(8502–9528)	3997	(3777–4230)
CHA	6199 (5366–7161)	11000	(10226–11838)	14978	(13888–16153)
Antagonists					
CGS 15943	0.15 (0.11–0.19)	15	(15–16)	–	–
SCH 58261	1.38 (1.25–1.51)	20	(18–22)	–	–
XAC	8.71 (7.76–9.77)	60	(56–64)	–	–
KF 17837	22 (19–24)	80	(71–91)	–	–
DPCPX	274 (255–295)	500	(459–544)	–	–

A comparison is made with stimulation and inhibition of NECA (1 μM) stimulated cyclic AMP levels by agonists (EC_{50}) and antagonists (IC_{50}) and with inhibition of O_2^- production by adenosine agonists (EC_{50}) in human isolated neutrophils. Each value is the geometric mean (with 95% confidence limits in parentheses) of at least four separate experiments performed in duplicate.

a dose-dependent manner (Figure 5a). The rank order of relative potency (concentration required for half-maximal response, EC₅₀ (95% confidence interval) nM) was: HE-NECA (3.0 (2.5–3.6)) > NECA (30.0 (24.6–35.9)) > CGS 21680 (300 (278–323)) > R-PIA (998 (890–1116)) > CCPA (1995 (1779–2233)) > S-PIA (3997 (3777–4230)) > CHA (14978 (13888–16153)). HE-NECA was a significantly more potent

inhibitor of superoxide anion generation than NECA or CGS 21680. To characterize further the mechanism involved we used a series of antagonists. The adenosine A₁ receptor-selective antagonist DPCPX, at a concentration of 100 nM did not antagonize the effect of HE-NECA. In contrast, SCH 58261 (100 nM) and CGS 15943 (100 nM) fully antagonized HE-NECA whereas XAC and KF 17837 had an intermediate

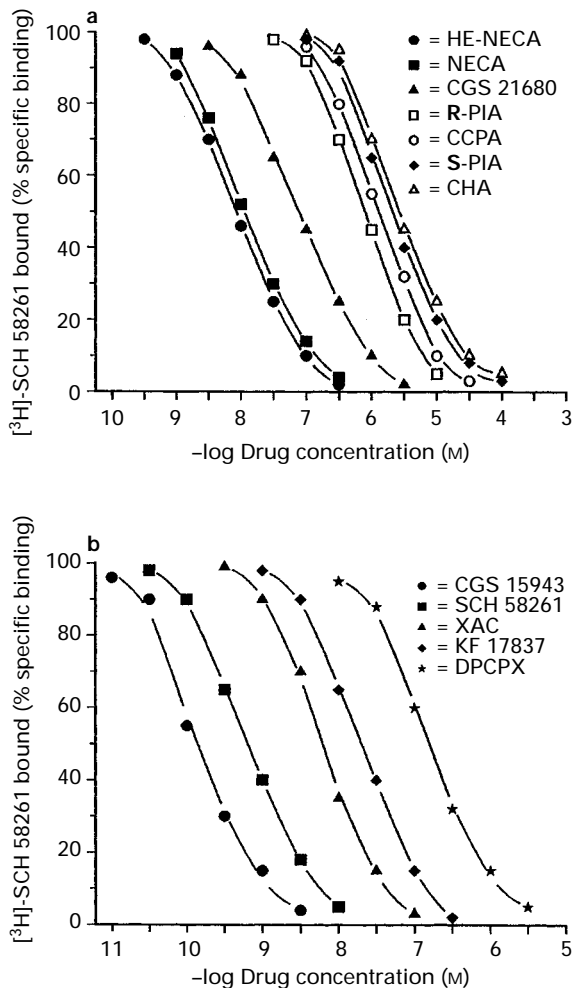


Figure 3 (a) Competition curves of specific [³H]-SCH 58261 (1 nM) binding to human neutrophil membranes by adenosine agonists. Curves are representative of a single experiment from a series of four independent experiments. Non-specific binding was determined in the presence of 10 μ M NECA. (b) Competition curves of specific [³H]-SCH 58261 (1 nM) binding to human neutrophil membranes by adenosine antagonists. Mean values of four experiments performed in duplicate are shown. Curves are representative of a single experiment from a series of four independent experiments. Non-specific binding was determined in the presence of 10 μ M NECA.

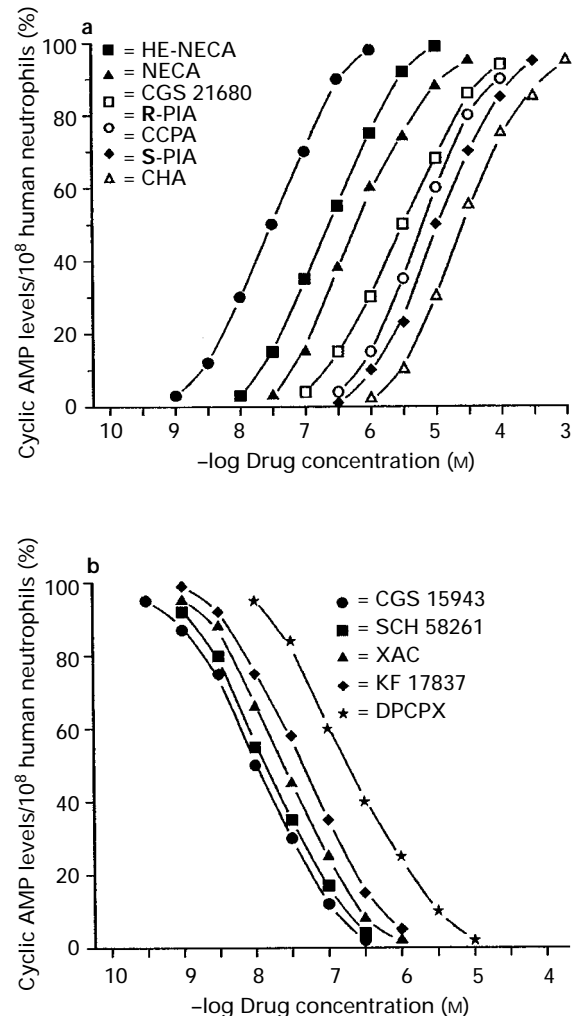


Figure 4 (a) Stimulation of cyclic AMP levels in human neutrophils by adenosine agonists. Curves are representative of a single experiment from a series of four independent experiments. (b) Inhibition of NECA (1 μ M) stimulated cyclic AMP levels (100%) in human neutrophils by adenosine antagonists. Curves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

Table 2 Correlation between affinity values of tested agonists and antagonists in the [³H]-SCH 58261 binding assay to human neutrophil membranes and corresponding *K_i* values, EC₅₀ and IC₅₀ values

Affinity data	n	Spearman rank order correlation coefficient	Probability (one-tailed test)	References
<i>K_i</i> rat striatal membranes	11	0.98	<0.01	Zocchi <i>et al.</i> (1996b)
<i>K_i</i> human platelet membranes	12	0.97	<0.01	Dionisotti <i>et al.</i> (1996)
<i>K_i</i> human lymphocyte membranes	12	0.98	<0.01	Varani <i>et al.</i> (1997)
<i>K_i</i> CHO cells	11	0.97	<0.01	Dionisotti <i>et al.</i> (1997)
EC ₅₀ and IC ₅₀ (cyclic AMP)	12	1.00	<0.01	Present study
EC ₅₀ (O ₂ ⁻ release)	7	1.00	<0.01	Present study

The *K_i* values were obtained in rat striatum, human platelets, human lymphocytes, CHO cells transfected with the human A_{2A} receptor, EC₅₀ and IC₅₀ values in the cyclic AMP assay, EC₅₀ in the superoxide anion production.

potency (Figure 5b). The rank order of potency of the antagonists to inhibit the effect of HE-NECA in neutrophils was: CGS 15943 (99.7 (89.0–111.6)) > SCH 58261 (78.2 (75.2–82.7)) > XAC (15.0 (13.9–16.2)) > KF 17837 (11.0 (10.4–11.6)) > DPCPX (3.2 (3.0–3.4)). The Spearman's rank correlation coefficient between affinity values of [³H]-SCH 58261 binding to A_{2A} adenosine receptor by selected receptor agonists and antagonists and the EC₅₀ and IC₅₀ values in superoxide anion production assay was 1.00 ($P < 0.01$) (Table 2). The comparison of K_i , EC₅₀ and IC₅₀ values indicated that a high correlation exists between data obtained from binding and O₂^{•−} release (Figure 6b).

Thermodynamic binding assay

K_d and B_{max} values derived from the saturation experiments of [³H]-SCH 58261 binding to A_{2A} adenosine receptors performed at the four temperatures selected were found within the following range: $K_d = 1.34$ – 5.50 nM and $B_{max} = 75$ – 80 fmol mg^{−1} protein. While dissociation constants (K_d) changed with temperature, B_{max} values obtained from [³H]-SCH 58261 saturation experiments appeared to be largely independent of it. Scatchard plots were linear at all temperatures investigated

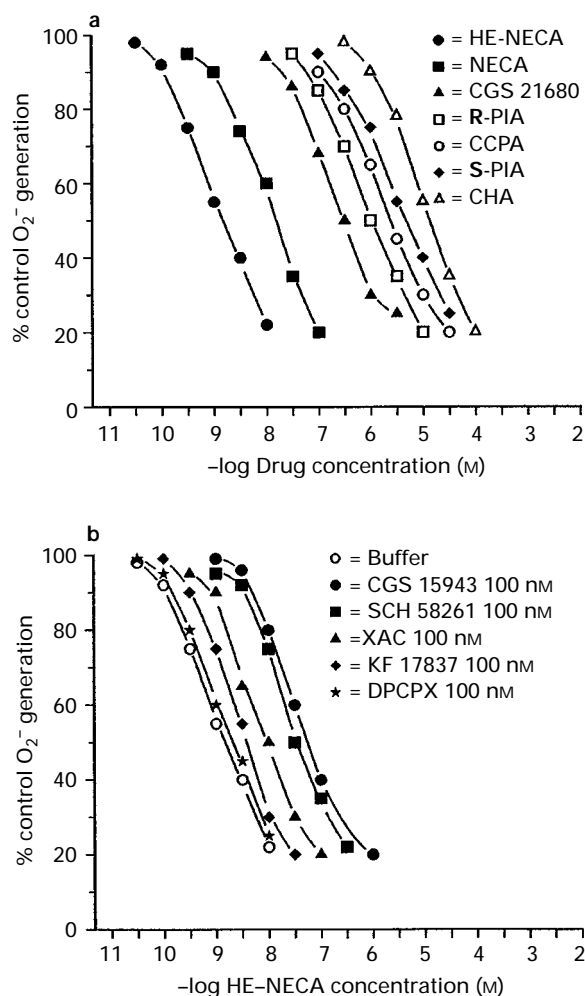


Figure 5 (a) Effect of adenosine receptor agonists on O₂^{•−} production induced by FMLP in human neutrophils. Curves are representative of a single experiment from a series of four independent experiments. (b) Antagonism of the effect of increasing concentrations of HE-NECA on O₂^{•−} production induced by FMLP in human neutrophils. Curves are representative of a single experiment from a series of four independent experiments.

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.

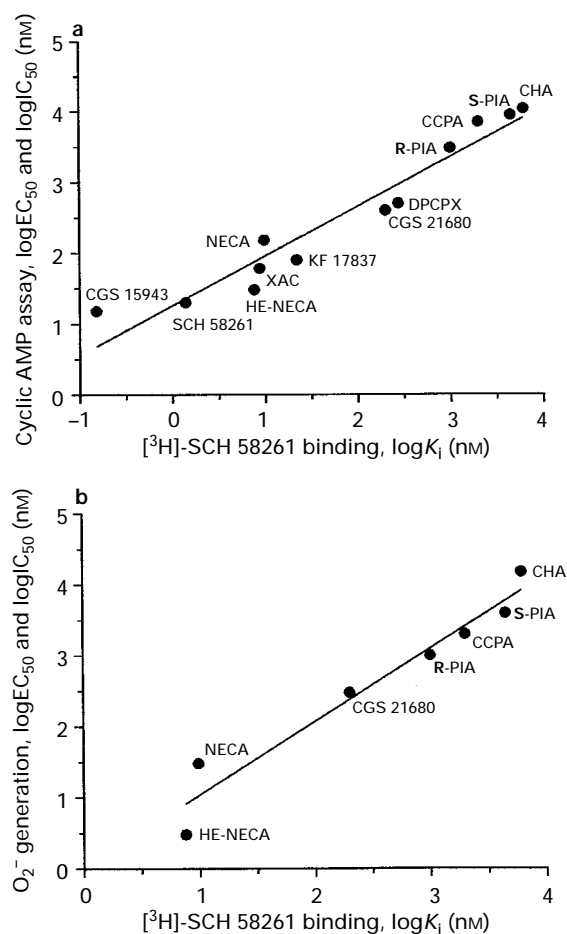


Figure 6 (a) Comparison between stimulation/inhibition of cyclic AMP levels and [³H]-SCH 58261 binding at A_{2A} adenosine receptors in human neutrophils ($r = 0.99$; $P < 0.01$). (b) Comparison between inhibition by agonists of O₂^{•−} production induced by FMLP and [³H]-SCH 58261 binding at A_{2A} adenosine receptors in human neutrophils ($r = 1.00$; $P < 0.01$).

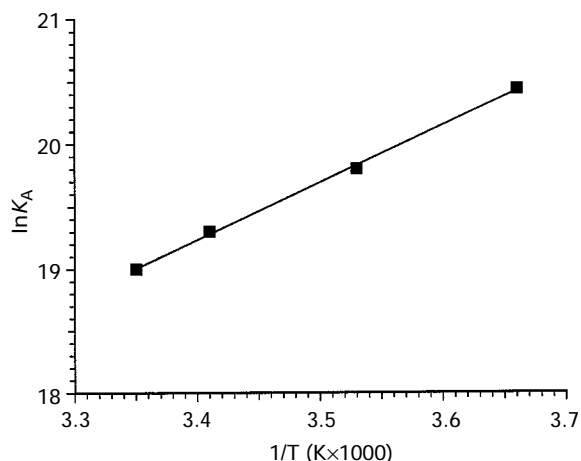


Figure 7 Van't Hoff plot showing the effect of temperature on the equilibrium binding association constant, $K_A = 1/K_d$, of [³H]-SCH 58261. The plot is essentially linear in the temperature range investigated (0–25°C).

Figure 7 presents the van't Hoff plot $\ln K_A$ versus $1/T$ of the [³H]-SCH 58261 binding to the A_{2A} adenosine receptor and the final equilibrium thermodynamic parameters (expressed as means values \pm s.e. of three independent determinations) were: $\Delta G^\circ = -47.07 \pm 0.12$ kJ mol⁻¹; $\Delta H^\circ = -35.67 \pm 3.38$ kJ mol⁻¹; $\Delta S^\circ = 38.46 \pm 3.52$ J mol⁻¹ K⁻¹. The linearity of the plot was statistically significant ($\Delta C_p^\circ \approx 0$) and its slope ($-\Delta H^\circ/R$) 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).

Discussion

The newly developed A_{2A} adenosine receptor antagonist radioligand, [³H]-SCH 58261, which has been recently shown to display negligible affinity for the adenotin sites (Varani *et al.*, 1996) and to label A_{2A} adenosine receptors in a variety of tissues including human platelets (Dionisotti *et al.*, 1996) and human lymphocytes (Varani *et al.*, 1997), represents a key advance towards the characterization of A_{2A} adenosine receptors.

In the present study we characterized the A_{2A} adenosine receptor on human neutrophil membranes by using [³H]-SCH 58261. Association and dissociation kinetic parameters of [³H]-SCH 58261 binding were similar to those observed in a previous study carried out in human platelets (Dionisotti *et al.*, 1996), and human lymphocytes (Varani *et al.*, 1997). In saturation experiments [³H]-SCH 58261 labelled a single class of recognition sites with affinity ($K_d = 1.3$ nM) in the same order of magnitude as that determined by kinetic experiments ($K_d = 1.1$ nM) and also similar to that observed in rat striatal and platelet membranes ($K_d = 0.7$ nM and 0.8 nM, respectively). In competition binding studies, various adenosine receptor agonists and antagonists bound the A_{2A} neutrophil receptor with a rank order of potency and affinity range similar to that observed for [³H]-SCH 58261 binding to human platelet membranes. Computer analysis of competition curves of both adenosine agonists and antagonists showed that [³H]-SCH 58261 interacted with only one recognition binding site (Hill coefficients not significantly different from unity). This is in agreement with previous data by Zocchi *et al.* (1996b).

Our data are also in agreement with those previously obtained in rat striatal, human platelet and lymphocyte membranes and CHO cells transfected with the human cloned A_{2A} receptor. It is worth noting that contrasting results have been obtained in the literature about the effect of A_{2A} adenosine receptor antagonists to reveal both high and low affinity state (Bruns *et al.*, 1987; Nonaka *et al.*, 1994). However, our data are in agreement with those obtained with [³H]-SCH 58261 (Zocchi *et al.*, 1996b; Belardinelli *et al.*, 1996) in striatal and coronary artery membranes and [³H]-PD 115,199 (Bruns *et al.*, 1987), which displaced A_{2A} striatal receptors with Hill coefficients close to unity 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 [³H]-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 [³H]-SCH 58261 binding in the human neutrophils indicates that the ΔC_p° 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 temperature range investigated (Borea *et al.*, 1995). The linearity of van't Hoff plots in a limited 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 [³H]-SCH 58261 binding to human neutrophils is entropy and enthalpy-driven ($\Delta S^\circ = 38.46 \pm 3.52$ J mol⁻¹ K⁻¹, $\Delta H^\circ = -35.67 \pm 3.38$ kJ mol⁻¹). This binding behaviour has been found, in rat striatum, to be typical of A_{2A} receptor antagonists (Borea *et al.*, 1995).

Another aim of the present study was to investigate the regulation of adenylate cyclase activity to determine whether the binding parameters correlated with the functional response. We determined the EC₅₀ and IC₅₀ values obtained for either stimulation or inhibition of cyclic AMP levels, by receptor agonists and antagonists, respectively. Interestingly, in the adenylate cyclase assay, the compounds studied exhibited a rank order of potency similar to that observed in binding experiments. For example, HE-NECA and NECA, adenosine receptor agonists known to have the highest affinity for human platelets, lymphocytes and neutrophils, were also the most potent compounds in the cyclic AMP assay. Additionally, CGS 21680, an agonist considered potent and highly selective for A_{2A} receptors, was found to have an affinity and potency similar to that of DPCPX, a potent and selective A₁ adenosine receptor antagonist. This finding cannot be easily explained. Nevertheless, it shows that DPCPX at concentrations >100 nM is not selective for A₁ receptors, and that CGS 21680 has low affinity for A_{2A} receptors in neutrophils. In addition, in a recent study, Lindström *et al.* (1996) provided evidence that CGS 21680 binds not only to classical A_{2A} receptors, but also to sites that differ from defined adenosine receptors. CGS 21680 was 20 fold less potent than NECA, and this is in good agreement with that found in human platelets (Dionisotti *et al.*, 1996) and lymphocytes (Varani *et al.*, 1997). Altogether these findings indicate that HE-NECA, because of its high affinity, is perhaps more suitable than CGS 21680 to be used as standard agonist for identifying A_{2A} receptors. Likewise, adenosine receptor antagonists CGS 15943 and SCH 58261 are potent in binding ($K_i = 0.1$ nM and 1.4 nM, respectively) and functional assays (IC₅₀ = 15 nM and 20 nM), as observed in a variety of cell membranes (Ongini & Fredholm, 1996). Thus, like A₂ receptors in other mammalian tissues, those present on neutrophils are coupled with adenylate cyclase

Table 3 Correlation between EC₅₀ and IC₅₀ values of adenosine receptor agonists and antagonists, respectively, obtained in cyclic AMP assays with human neutrophils, platelets, lymphocytes and CHO cells transfected with the human A_{2A} receptor

	n	Spearman rank order correlation coefficient	Probability (one-tailed test)	References
Human platelets	12	1.00	<0.01	Varani <i>et al.</i> (1996)
Human lymphocytes	12	1.00	<0.01	Varani <i>et al.</i> (1997)
CHO cells	9	0.82	<0.01	Dionisotti <i>et al.</i> (1997)

stimulation. Our studies of inhibition of superoxide anion generation by adenosine analogues indicate that the receptor on neutrophils is of the A_{2A} subtype. Adenosine analogues were similar in both their ability to inhibit superoxide anion generation and their ability to inhibit the binding of [³H]-SCH 58261. Thus, the rank order of potency of the agonists to inhibit superoxide anion generation was HE-NECA > NECA > CGS 21680 > R-PIA > CCPA > S-PIA > CHA, a finding that is in perfect agreement with the affinities obtained in the binding assays. Similarly, the data on antagonists of HE-NECA actions in human neutrophils are in reasonable agreement with those obtained in the binding assay. Moreover a good correlation was found between cyclic AMP accumulation data and inhibition of O₂⁻ generation by adenosine receptor agonists used in the present study (Spearman rank correlation coefficient = 1.00, $P < 0.01$). This finding suggests that cyclic AMP could be involved in the action of A_{2A} receptors to inhibit superoxide anion formation. The finding that the potencies of adenosine receptor agonists and antagonists obtained in the binding assays were greater than the potencies of the same receptor agonists and antagonists obtained in the cyclic AMP and O₂⁻ production assays is probably due to the different temperatures used in the experiments. However, it is worth noting that the rank order of potencies of the receptor agonists and antagonists obtained in the binding assays were identical to that obtained in the functional assays (cyclic AMP and superoxide anion production).

Finally, we calculated the Spearman's rank correlation coefficient between receptor affinity values of [³H]-SCH 58261 binding to human neutrophils A_{2A} receptors and K_i values of selected agonists and antagonists obtained in different membrane preparations, such as rat striatal, human platelet and lymphocyte membranes, CHO cells transfected with the human A_{2A} receptors. It is worth noting that the correlation coefficients were highly statistically significant, suggesting that A_{2A} receptors are similar in all preparations described above, and also reasonably good agreement was found between binding results and functional data in human lymphocytes for adenosine receptor ligands (Table 2). Likewise, we also calculated the Spearman's rank correlation coefficient between EC₅₀ and IC₅₀ values in the cyclic AMP assay of the adenosine ligands examined leading us to conclude that, as far as the

effector system is concerned, no relevant differences in the signal transmission pathways were found (Table 3). Regression analysis between A_{2A} receptor binding affinities and pharmacological activity data on neutrophils showed a significant positive correlation (Figure 6b). Altogether, these data indicate that the adenosine receptors present in human neutrophil membranes are of the A_{2A} subtype. This conclusion is consistent with the RT-PCR study carried out by Fredholm *et al.* (1996) on human neutrophils.

Neutrophils are the most abundant circulating white blood cells and are usually the first cells to respond to infectious or inflammatory stimuli. Like monocytes, neutrophils can phagocytose appropriately opsonized bacteria (coated with either specific antibodies or complement components) or other particulate matter. Upon stimulation, these cells may also secrete potentially toxic oxygen metabolites (Zhang & Fredholm, 1994) and lysosomal enzymes into the supernatant medium. When stimulated, these cells reach extravascular foci of infection or inflammation by adhering to vascular endothelium and migrating towards the source of the chemoattractants. Adenosine itself or compounds which stimulate A₂ receptors selectively inhibit neutrophil adhesion to endothelium, O₂⁻ generation and inhibit phagocytosis of immunoglobulin-coated red blood cells (Cronstein *et al.*, 1990). Thus, the A_{2A} receptor can be involved in modulating the biological activities of neutrophils.

In conclusion, the results of our experiments indicate that human neutrophils have an A_{2A} adenosine receptor with a pharmacological, biochemical and thermodynamic profile typical of the A_{2A} receptor subtype of other cell types. Because the products released by activated neutrophils are implicated in a variety of inflammatory disorders, selective A_{2A} receptor ligands may prove to be useful pharmacological tools to investigate inflammatory mechanisms. The presence of A_{2A} receptors associated with the functional response of human neutrophils is also strongly supportive of an important role for adenosine in modulating inflammatory responses in a variety of pathophysiological events.

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