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REVIEW

A_{2A} adenosine receptors in human peripheral blood cells

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Abbreviations: CCPA, 2-chloro-N^6-cyclopentyladenosine; CHA, N^6-cyclohexyladenosine; CHAPS, 3-[(3-cholamido-propyl)dimethylammonio]-1-propanesulphonate; CGS 21680, 2-[p-(2-carboxyethyl)-phenethylamino]-5′-N-ethylcarboxamidoadenosine; CGS 15943, 5-amino-9-chloro-2-(2-furyl)1,2,4-triazolo[1,5-*c*]quinazoline; DPCPX, 1,3-dipropyl-8-cyclopentylxanthine; FMLP, N-formyl-L-methionyl-L-leucyl-L-phenylalanine; HE-NECA, 2-hexynyl-5′-N-ethyl-carboxamidoadenosine; KF 17837, (E)-1,3-dipropyl-8-(3,4-dimethoxystyryl)-7-methylxanthine; NECA, 5′-N-ethylcarboxamidoadenosine; PEG, polyethylene glycol 8000; R-PIA and S-PIA (R(−) and S(+)-N^6-(2-phenylisopropyl)-adenosine; SCH 58261, 5-amino-7-(phenylethyl)-2-(2-furyl)-pyrazolo[4,3-*e*]-1,2,4-triazolo[1,5-*c*]pyrimidine; XAC, 8-[4-[[[[(2-aminoethyl)amino]-carbonyl]-methyl]oxy]-phenyl]-1,3dipropylxanthine

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Introduction

Adenosine mediates a wide range of physiological functions by activation of at least four cell surface receptors named A_1, A_{2A}, A_{2B} and A_3. Each receptor subtype has been cloned and shows unique ligand binding properties and a distinct pattern of tissue expression (Fredholm *et al*., 1994; Linden, 1994; Alexander & Peters, 1997; Ralevic & Burnstock, 1998). Adenosine is a highly active biologic compound with a variety of effects on numerous tissues, including heart muscle, coronary arteries, smooth muscle cells, platelets and cells involved in immune and inflammatory reactions. Through interaction with A_{2A} receptors, adenosine is involved in platelet antiaggregatory effects, neutrophil antiinflammatory responses as well as in modulation of immune cell function (Salmon & Cronstein, 1990; Sullivan *et al*., 1990; Hoskin *et al*., 1994; MacKenzie *et al*., 1994). The presence of A_{2A} receptors on all these different cell types i.e. monocytes, lymphocytes, neutrophils, basophils, mast cells should not be surprising because the cells involved in the immune and inflammatory responses arise from a common stem cell source in the bone marrow. Studies on the effects of adenosine and adenosine analogues on blood cells are important due to the proposed role of adenosine (1) in the pathogenesis of diseases such as severe combined immunodeficiency (ADA SCID) (Giblett *et al*., 1972; Huang *et al*., 1997), (2) in the regulation of normal immune processes (Bouma *et al*., 1997; Apasov *et al*., 1997), (3) as an endogenous anti-aggregatory and anti-inflammatory agent (Cronstein, 1995; Sullivan & Linden, 1998) and (4) in the use of adenosine analogues as pharmacologic agents (Jacobson *et al*., 1992; Olah & Stiles, 1995; Poulsen & Quinn, 1998). On this basis the present review summarizes the pharmacological, biochemical and functional data of A_{2A} receptors expressed in human platelets, lymphocytes and neutrophils of peripheral blood and describes the signal transmission mechanisms and the functional responses responsible for adenosine's biological effects.

Human platelets

Since 1963 adenosine has been postulated to be an endogenous inhibitor of platelet aggregation and secretion (Born & Cross, 1963). The putative efficacy of dipyridamole as an antiaggregatory agent could be explained at least in part by its effect to block the nucleoside transport system, and thereby increase the extracellular concentration of adenosine and potentiate its effects on membrane receptors. Platelets, though non-nucleated and, thus, strictly speaking, not entitled to be classified as cells, represent a uniform tissue possessing A_2 receptors (now known to be the A_{2A} receptor subtype) on the external membrane (Haslam & Cusack, 1981). It is believed that the antiaggregatory action of adenosine depends upon the inhibition, coupled with adenylate cyclase activation, of both calcium influx and mobilization of internal stores (Paul *et al*., 1990). Considering the relevance of platelet aggregation in a variety of cardiovascular and cerebrovascular disorders, several studies have been performed to characterize A_{2A} adenosine receptors in these blood components. The lack of a selective A_{2A} radioligand had hampered the direct characterization of the A_{2A} receptor subtype. The non-selective agonist NECA (A_1, A_{2A}, A_{2B}, A_3) was the first pharmacological tool to be used for labelling the A_{2A} receptor in the rat brain but it was also found to bind to different affinity states and subtypes of adenosine receptors (Yeung & Green 1984; Bruns *et al*., 1986). As in other peripheral tissues, in human platelet membranes the binding properties of [^{3}H]-NECA did not agree with the pharmacology of A_{2A} receptors (Hütteman *et al*., 1984; Ukena *et al*., 1984; Lohse *et al*., 1988; Nakata & Fujisawa, 1988; Keen *et al*., 1989). Specifically, while only 10% of [^{3}H]-NECA binding to human platelet membranes is displaced by N^6-substituted adenosine derivatives, which are known to both activate adenylate cyclase and inhibit platelet aggregation, about 95% of this binding could be displaced with unlabelled NECA suggesting that the majority of the [^{3}H]-NECA binding to human platelet membranes is to non-receptor sites. This ubiquitous adenosine A_2-like binding site has been separated chromatographically and distinguished pharmacologically from the A_{2A} adenosine receptor (Lohse *et al*., 1988). Subsequently, the NECA-binding site has been purified from human placental (Hutchison *et al*., 1990) and human platelet membranes (Fein *et al*., 1994) and was named 'adenotin', a low affinity binding protein of 98 kDa whose biological significance has not been clarified, and precluded the direct characterization of A_{2A} receptors in platelet membranes.

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With the aim to overcome the disadvantages observed with NECA, different radioligands have been proposed for the characterization of the human platelet A_{2A} receptor. The A_1-selective antagonist [^{3}H]-XAC, having high levels of non-specific binding (Ukena *et al*., 1986), does not permit satisfactory binding studies of A_{2A} receptors. The A_{2A} selective agonist [^{3}H]-CGS 21680, which has been reported to interact with the high affinity A_{2A} adenosine receptor in both binding and adenylyl cyclase assays (Jarvis *et al*., 1989; Johansson *et al*., 1992; Mathot *et al*., 1995), has also been used by Varani *et al*. (1994) in human platelet membranes. Although saturation experiments showed that [^{3}H]-CGS 21680 interacts with one recognition site only, the affinity of this agonist for the receptor was in the micromolar range, therefore suggesting that this radioligand like NECA, labels also the low affinity non-receptor site (Table 1). Moreover, as previously reported for the platelet [^{3}H]-NECA binding (Hütteman *et al*., 1984), the displacement of [^{3}H]-CGS 21680 binding by a series of adenosine receptor agonists and antagonists revealed K_i values substantially higher than those obtained for either stimulation or inhibition of cyclic AMP levels (Table 2A,B). Thus, although CGS 21680 interacts with the platelet A_{2A} adenosine receptor, like NECA, it proved to be unsatisfactory for the characterization of A_{2A} human platelet receptors. This fact has led some authors to postulate that the adenosine receptor expressed in platelets may be different from the A_{2A} subtype identified in brain striatum (Cristalli *et al*., 1994). To overcome the many difficulties associated with the interaction between the A_{2A} adenosine receptors and adenotin sites it was attempted to characterize a purified platelet A_{2A} adenosine receptor preparation by using the selective radioligand [^{3}H]-CGS 21680 (Varani *et al*., 1996). The method employed (Zolnierowicz *et al*., 1990) as an alternative to the gel filtration chromatography (Lohse *et al*., 1988), avoids high non-specific binding by removing most of the adenotin binding protein using extraction of membranes with CHAPS followed by PEG precipitation. In the purified protein preparation [^{3}H]-CGS 21680 interacts with one recognition site only, with an affinity in the nanomolar range (K_d = 285 nM) and a binding capacity of 2 pmol mg^{-1} protein. These values are not far from those obtained for [^{3}H]-NECA in both solubilized A_{2A} receptors (Lohse *et al*., 1988) and partially purified preparation

Table 1 Comparison between K_d and B_{max} values obtained for [^{3}H]-NECA and [^{3}H]-CGS 21680 binding in human platelet membranes and in two different A_{2A} receptor preparations

	[^{3}H]-NECA		*[^{3}H]-CGS 21680*	
	K_d (nM)	B_{max} (pmol mg^{-1} prot^{-1})	K_d (nM)	B_{max} (pmol mg^{-1} prot^{-1})
Human platelet membranes	1000[a]	4[a]	1400[d]	5.9[d]
Solubilized A_{2A} receptor	46[b]	0.51[b]	–	–
Partially purified A_{2A} receptor	31[c]	0.23[c]	285[e]	2[e]

[a]Keen *et al*., 1989; [b]Lohse *et al*., 1988; [c]Zolnierowicz *et al*., 1990; [d]Varani *et al*., 1994; [e]Varani *et al*., 1996.

Table 2A Inhibition of [^{3}H]-CGS 21680 binding (K_i) by adenosine agonists to rat striatal membranes, human platelet membranes and partially purified A_{2A} adenosine receptors. Comparison is made with stimulation of cyclic AMP levels in human platelets

Compound	K$_i$ (μM) *rat striatal membranes*	K$_i$ (μM) *human platelet membranes*	K$_i$ (μM) *purified receptors*	*cyclic AMP assay* EC$_{50}$ (μM)
SCH 59765	0.012 (0.0108 – 0.0142)[a]	1.6 (1.09 – 2.35)	0.005 (0.004 – 0.006)	0.020 (0.020 – 0.030)
HE-NECA	0.002 (0.0019 – 0.0026)[b]	0.07 (0.06 – 0.08)[d]	0.006 (0.005 – 0.007)	0.041 (0.038 – 0.044)[d]
CPCA	0.006 (0.0053 – 0.0073)[b]	3.47 (3.33 – 3.62)[d]	0.052 (0.05 – 0.06)	0.22 (0.20 – 0.24)[d]
NECA	0.008 (0.0066 – 0.0091)[b]	2.13 (1.82 – 2.49)[d]	0.062 (0.06 – 0.07)	0.30 (0.26 – 0.34)[d]
CGS 21680	0.011 (0.0094 – 0.0129)[b]	1.92 (1.67 – 2.20)[d]	0.25 (0.24 – 0.26)	0.70 (0.58 – 0.83)[d]
CV1808	0.062 (0.0527 – 0.0724)[b]	6.76 (6.54 – 6.98)[d]	0.38 (0.36 – 0.40)	1.10 (1.01 – 1.19)[d]
R-PIA	0.164 (0.140 – 0.192)[b]	338 (319 – 357)[d]	1.40 (1.11 – 1.75)	2.98 (2.53 – 3.52)[d]
CCPA	0.650 (0.555 – 0.762)[b]	477 (391 – 580)[d]	2.51 (2.08 – 3.04)	8.98 (8.34 – 9.68)[d]
CHA	0.820 (0.780 – 0.860)[c]	1148 (977 – 1348)[d]	4.59 (4.27 – 4.93)	9.87 (8.24 – 11.84)[d]
S-PIA	0.882 (0.753 – 1.033)[b]	849 (822 – 877)[d]	8.45 (7.49 – 9.54)	14.96 (13.39 – 16.72)[d]

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

Table 2B Inhibition of [^{3}H]-CGS 21680 binding (K_i) by adenosine antagonists to rat striatal membranes, human platelet membranes and purified A_{2A} adenosine receptors. Comparison is made with inhibition of NECA (1 μM) stimulated cyclic AMP levels in human platelets

Compound	K$_i$ (μM) *rat striatal membranes*	K$_i$ (μM) *human platelet membranes*	K$_i$ (μM) *purified receptors*	*cyclic AMP assay* IC$_{50}$ (μM)
CGS 15943	0.00095 (0.0008 – 0.0011)[a]	0.06 (0.05 – 0.07)[e]	0.006 (0.006 – 0.007)	0.012 (0.010 – 0.014)
SCH 58261	0.0023 (0.0020 – 0.0027)[b]	58.56 (53.49 – 64.12)	0.008 (0.007 – 0.009)	0.015 (0.012 – 0.018)
XAC	0.050[c]	0.68 (0.49 – 0.93)[e]	0.037 (0.03 – 0.04)	0.050 (0.04 – 0.06)
KF 17837	0.074 (0.061 – 0.089)[a]	14.66 (11.52 – 18.66)	0.042 (0.04 – 0.05)	0.070 (0.06 – 0.08)
DPCPX	0.55 (0.50 – 0.60)[d]	462 (426 – 501)[e]	0.33 (0.31 – 0.36)	0.60 (0.52 – 0.68)
THEOPHYLLINE	6.7 (6.06 – 7.50)[d]	1060 (981 – 1146)[e]	16.69 (15.12 – 18.41)	29.66 (25.00 – 35.20)
CAFFEINE	24.0 (20.73 – 27.37)[d]	4306 (3908 – 4746)[e]	66.78 (60.96 – 73.16)	174.06 (154.77 – 195.76)

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

(Zolnierowicz *et al*., 1990) and disagree with data obtained for [^{3}H]-CGS 21680 binding in human platelet membranes (Varani *et al*., 1994) suggesting the presence in this case of a large component of low affinity binding to non-receptor sites (Table 1). Moreover, the affinities of all tested compounds for the purified receptor are systematically higher, by one to three orders of magnitude, than their affinities for platelet membranes and are very similar to those obtained in rat striatum suggesting that, only under these experimental conditions, the agonist CGS 21680 appears to be an adequate radioligand to study purified A_{2A} adenosine receptors in human platelets (Table 2A,B). It is worth nothing that the discrepancies found between the affinity data obtained in cyclic AMP and receptor binding assays for many adenosine receptor ligands in human platelet membranes are markedly reduced in the partially purified platelet preparation in which K_i, EC_{50} or IC_{50} values are very similar for all compounds studied (Table 2A,B). These data indicate that in the purified platelet membrane preparation the presence of adenosine receptors is prevalent with respect to the non-receptor component (adenotin site).

Agonists at G protein-coupled receptors are not ideal radioligands (Stiles & Jacobson, 1987). Indeed, as in the case of the binding between agonist radioligands and other G protein-linked receptors, the binding of [^{3}H]-CGS 21680 to A_{2A} receptors is influenced by several factors including the state of the G proteins (Johansson *et al*., 1992). Thus, the availability of antagonist radioligands, having high A_{2A} receptor affinity and selectivity, represents a step forward in the characterization of this adenosine receptor subtype. Important progress has been made with the development of selective A_{2A} adenosine receptor antagonists (Ongini & Fredholm, 1996; Ongini *et al*., 1999). One of them, the non-xanthine compound SCH 58261 (Baraldi *et al*., 1994), has been widely characterized in a variety of binding and functional assays (Zocchi *et al*, 1996a) and has been shown not to interact with the adenotin binding site (Varani *et al*., 1996). The tritium-labelled form, [^{3}H]-SCH 58261, has been found to label A_{2A} receptors in the rat brain (Zocchi *et al*., 1996b; Fredholm *et al*., 1998), in peripheral tissue membranes such as porcine coronary arteries (Belardinelli *et al*., 1996), and human cloned receptors transfected in mammalian cells (Dionisotti *et al*., 1997). SCH 58261 has low affinity for A_1 receptors measured in rat brain cortex, does not interact with A_{2B} receptors and has low affinity (μM range) for either rat or human A_3 receptors (Zocchi *et al*., 1996a). On the basis of this pharmacological

Table 3 Comparison between K_d and B_{max} values obtained for [^{3}H]-SCH 58261 binding in rat striatal membranes and in human circulating blood cells; platelets, lymphocytes and neutrophils

	[^{3}H]-NECA		*[^{3}H]-SCH 58261*	
	K_d (nM)	B_{max} (fmol mg^{-1} prot.$^{-1}$)	K_d (nM)	B_{max} (fmol mg^{-1} prot.$^{-1}$)
Rat striatal membranes	3.54[a]	14.2[a]*	0.7[e]	971[e]
Human platelet membranes	1000[b]	4000[b]	0.85[f]	85[f]
Human lymphocyte membranes	750[c]	22700[c]	0.85[g]	35[g]
Human neutrophil membranes	230[d]	9.31×10^3 [d]**	1.34[h]	75[h]

[a]Yeung and Green, 1984; *fmol mg tissue^{-1}; [a]Bruns *et al*., 1986; [b]Keen *et al*., 1989; [c]Schultz *et al*., 1988; [d]Cronstein *et al*., 1985; ** sites/cell; [e]Zocchi *et al*., 1996b; [f]Dionisotti *et al*., 1996; [g]Varani *et al*., 1997; [g]Varani *et al*., 1998a.

Table 4 Inhibition of [^{3}H]-SCH 58261 binding, (K_i), by adenosine agonists and antagonists to rat striatal, human platelet, lymphocyte and neutrophil membranes

	K_i (nM) *rat striatum*[a]	K_i (nM) *platelets*[b]	K_i (nM) *lymphocytes*[c]	K_i (nM) *neutrophils*[d]
Agonists				
HE-NECA	3.1 (2.4–4.0)	17 (10–27)	14 (11–18)	7.66 (7.44–7.90)
NECA	61 (48–77)	30 (21–43)	22 (20–24)	9.84 (8.01–12.08)
CGS 21680	111 (83–148)	753 (430–1317)	838 (731–960)	200 (184–216)
R-PIA	992 (834–1183)	1614 (1136–2292)	1490 (1302–1705)	990 (841–1165)
CCPA	3260 (2678–3968)	4311 (2200–8449)	3785 (3203–4472)	1980 (1683–2329)
CHA	2840 (2367–3408)	7829 (4638–13214)	6497 (5048–8363)	4486 (4102–4905)
S-PIA	8504 (8178–8843)	7833 (3754–16343)	6898 (6408–7425)	6199 (5366–7161)
Cladribine	–	–	50000 (45520–52870)	
Antagonists				
CGS 15943	0.38 (0.30–0.47)	0.22 (0.18–0.27)	0.20 (0.16–0.24)	0.15 (0.11–0.19)
SCH 58261	1.1 (0.84–1.3)	0.75 (0.40–1.4)	0.85 (0.83–0.87)	1.38 (1.25–1.51)
XAC	9.0 (6.6–12)	5.1 (3.7–7.0)	7.28 (5.51–9.63)	8.71 (7.76–9.77)
KF 17837S	9.4 (7.5–12)	48 (31–74)	19 (15–24)	22 (19–24)
DPCPX	234 (124–445)	501 (378–665)	492 (401–620)	274 (255–295)

Each K_i value is the geometric mean (with 95% confidence limits in parentheses) of at least four separate experiments performed in duplicate. [a]Zocchi *et al*., 1996b; [b]Dionisotti *et al*., 1997; [c]Varani *et al*., 1997; [d]Varani *et al*., 1998a.

profile [^{3}H]-SCH 58261 has been used to characterize A_{2A} receptors in human platelet membranes.

In saturation studies, [^{3}H]-SCH 58261 labels one class of binding sites only, with K_d of 0.85 nM (Dionisotti *et al*., 1996; Varani *et al*., 1998b) a value similar to that observed in rat striatal membranes (K_d=0.70 nM) (Zocchi *et al*., 1996b), but very different from that obtained with [^{3}H]-NECA in human platelet membranes (Table 3). In competition experiments, a series of typical adenosine ligands bound A_{2A} platelet receptors with an affinity and a rank order of potency similar to that found in rat striatal membranes (Table 4). Moreover K_i values of adenosine agonists and antagonists correlate well with results from functional studies such as stimulation of cyclic AMP levels or platelet aggregation inhibition (Dionisotti *et al*., 1992) (Table 5). Thus, [^{3}H]-SCH 58261 is the first radioligand that has allowed the binding characterization of the A_{2A} receptor subtype in human platelets. Recently, in A_{2A} receptor-knockout mice, it was reported that platelet aggregation was increased, indicating the importance of this receptor subtype in platelet function (Ledent *et al*., 1997). Activation of A_{2A} receptors in platelets causes an increase in cyclic AMP accumulation and a decrease in platelet aggregation. During ischemia and/or hypoxia, extracellular and plasma levels of adenosine increase to levels suficient to activate A_{2A} receptors and hence decrease platelet aggregability. The availability of [^{3}H]-SCH 58261 should stimulate further the elucidation of the changes, such as up or downregulation, in A_{2A} platelet receptors under a variety of physiologic and pathologic conditions.

Human lymphocytes

The interest in the immunomodulatory effects of adenosine arose after the discovery that hereditary deficiency of the enzyme adenosine deaminase (ADA) was associated with severe combined immune deficiency disease (SCID) (Giblett *et al*., 1972). SCID is a disease characterized by severe lymphocytopenia, affecting both B and T cells and marked susceptibility to infection. ADA SCID has been hypothesized to be due to the accumulation of intracellular products of adenosine metabolism leading to the depletion of lymphocytes (Hirschorn, 1995; Blackburn *et al*., 1996). However, the studies aimed at elucidating the mechanisms by which absence of ADA leads to immunodeficiency, first suggested the presence of adenosine receptors on lymphocytes to suppress or dampen the immune response (Wolberg *et al*., 1975). Subsequently, other studies reported that adenosine causes cyclic AMP accumulation in lymphocytes by interacting with a specific external membrane receptor and that the effect of a series of adenosine analogues to inhibit lymphocyte cytotoxicity was correlated with the potency of the same compounds to increase the cellular content of cyclic AMP (Marone *et al*., 1978; Schwartz *et al*., 1978; Bonnafous *et al*., 1981). Recently, it has been reported that exposure of T lymphocytes to extracellular adenosine causes inhibition of T cell effector functions. These immunosuppressive effects of adenosine in cytotoxic T lymphocytes (CTL) actions may be explained by activation of A_{2A} receptors followed by sustained increases in cyclic AMP that, in turn, antagonize T cell receptor (TCR)-triggered signaling including the FasL mRNA upregulation. The effect of the selective A_{2A} agonist CGS 21680 to inhibit the lysis of antigen-specific target cells by CTL strongly supports the identification of A_{2A} receptor as the major expressed receptor in T lymphocytes (Koshiba *et al*., 1997). Similarly, the effect of adenosine to inhibit T-cell proliferation and IL-2 receptor (CD 25) expression, is mimicked by low concentrations of 2-chloroadenosine and CGS 21680 suggesting the involvement of the A_{2A} subtype (Huang *et al*., 1997). The expression of CD25

Table 5 Functional assays in human circulating blood cells; platelets, lymphocytes and neutrophils

	EC_{50}/IC_{50} [1] platelets[a]	IC_{50} [2] platelets[b]	EC_{50}/IC_{50} [3] lymphocytes[c]	EC_{50}/IC_{50} [4] neutrophils[d]	EC_{50} [5] neutrophils[d]
Agonists					
HE-NECA	41 (38–44)	100 (40–250)	40 (38–42)	30 (25–35)	3.0 (2.5–3.6)
NECA	300 (260–340)	490 (320–770)	200 (184–216)	150 (127–177)	30.0 (24.6–35.9)
CGS 21680	700 (580–830)	1090 (440–2740)	897 (821–981)	400 (371–430)	300 (278–323)
R-PIA	2980 (2530–3520)	7300 (2910–18300)	3936 (3206–4832)	3000 (2693–3339)	998 (890–1116)
CCPA	8980 (8340–9680)	19200 (7650–48200)	5450 (4710–6320)	7000 (6380–7680)	1995 (1779–2233)
S-PIA	9870 (8240–11840)	51900 (20700–130000)	8470 (7705–9311)	9000 (8502–9528)	3997 (3777–4230)
CHA	14960 (13390–16720)	–	12480 (11700–13310)	11000 (10226–11838)	14978 (13888–16163)
Cladribine	–		>500000	–	–
Antagonists					
CGS 15943	12 (10–14)		12 (11–13)	15 (15–16)	
SCH 58261	15 (12–18)		16 (14–18)	20 (18–22)	
XAC	50 (40–60)		55 (51–59)	60 (56–64)	
KF 17837	70 (60–80)		60 (53–67)	80 (71–91)	
DPCPX	60 (52–68)		600 (560–670)	500 (459–544)	

Stimulation and inhibition of NECA (1 μM) stimulated cyclic AMP levels by agonists (EC_{50}) and antagonists (IC_{50}) in isolated human platelets (1), lymphocytes (3) and neutrophils (4). Comparison with inhibition of platelet aggregation (IC_{50}) (2) and inhibition of neutrophils O_2^- generation (5). Each value (nM) is the geometric mean (with 95% confidence limits in parentheses) of at least four separate experiments performed in duplicate. [a]Varani *et al*., 1994; [b]Dionisotti *et al*., 1992; [c]Varani *et al*., 1997; [d]Varani *et al*., 1998a.

is an important indicator of lymphocyte activation, because failure of the production of either IL-2 or its receptor results in a failure of the T-cell immune response (Waldmann, 1986; Crabtree, 1989). The predominant expression of A_{2A} receptors, which has been established in functional assays using selective agonists and antagonists of A_{2A} receptors, was confirmed by Northern blot studies of A_1, A_{2A} and A_3 mRNA expression in lymphoid tissues: A_{2A} receptor mRNA, but not A_1 and A_3 receptor mRNA, was detected (Koshiba *et al*., 1997; Huang *et al*., 1997).

Thus, the accumulation of adenosine in ADA SCID or under hypoxic conditions causes an increased 'signalling' through adenosine receptors on T cells and immunosuppression providing a novel target for immunomodulation. Several authors propose that T cell depletion, immunodeficiency and autoimmunity could be due to extracellular adenosine-induced 'signalling', which in turn inhibits the TCR 'signaling', affecting the TCR-driven positive and negative selection of thymocytes (Sitkovsky, 1998; Smith *et al*., 1998; Koshiba *et al*., 1997). This is of interest in view of the potential pharmacologic use of adenosine analogues as possible antileukemic and antiinflammatory agents, but the development of new drugs that target specific adenosine receptors must be done with a full understanding of the role of these receptors in the immune system.

A binding study has been performed in lymphocyte membranes to characterize the affinity (K_d) and the density (B_{max}) of A_{2A} receptors (Schultz *et al*., 1988). The radioligand used was the non selective agonist NECA which had a K_d of 0.75 μM and a B_{max} of 22.7 pmol/mg of protein (Table 3). However, NECA has also been found to interact with non-receptor binding proteins in peripheral tissues (Hutchison *et al*., 1990) thus, due to the high amount of non-receptor NECA binding, the A_{2A} fraction of the binding could not be well characterized. The availability of [^{3}H]-SCH 58261 has facilitated the characterization of lymphocyte A_{2A} receptors. In human lymphocytes, [^{3}H]-SCH 58261 labelled a single class of recognition sites with affinity (K_d=0.85 nM) very similar to that observed in rat striatal and platelet membranes (K_d=0.7 and 0.85 nM, respectively; Table 2) (Varani *et al*., 1997, 1998b). In competition studies, typical adenosine receptor agonists and antagonists bound the lymphocyte A_{2A} receptor with a rank order of potency and affinities similar to those observed in [^{3}H]-SCH 58261 binding to human platelet membranes and in agreement also with those found in rat striatal membranes (Table 4). Functional data derived from the agonists-stimulation and antagonists-inhibition of cyclic AMP levels correlate well with binding parameters (Table 5). Finally, to evaluate the forces driving the coupling of A_{2A} human lymphocyte receptors with a selective antagonist, thermodynamic studies were performed and the enthalpic (ΔH°) and entropic (ΔS°) contributions 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 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 is entropy and enthalpy driven ($\Delta S^\circ = 38.87 \pm 4.51$ Jmol^{-1} K^{-1}, $\Delta H^\circ = -36.74 \pm 3.42$ kJ mol^{-1}), a behaviour found to be typical of the interaction of antagonists with A_{2A} receptors in rat striatum (Borea *et al*., 1995). Altogether all these data suggest that adenosine receptors present on lymphocytes have a pharmacological and biochemical profile typical of the A_{2A} receptor subtype. Information about the lymphocyte subset-specific expression of adenosine receptors would be useful, but it has been difficult to obtain sufficient quantities of cells to analyse expression of adenosine receptors in biochemical or radioligand binding assays, particularly in the minor sub-populations of lymphocytes. The role of A_{2A} adenosine receptors in the regulation of immune response has been investigated by determining the expression levels of this receptor in different subsets of functional lymphocytes (Koshiba *et al*., 1999). A monoclonal anti-A_{2A} receptor antibody was used to develop a flow cytometric assay. The levels of expression of A_{2A} receptors are much higher among T than B cells. T cells subsets are distinguished by the expression of TCR coreceptor molecules $CD8^+$ and $CD4^+$ involved in recognition of class I and class II major histocompatibility complex, respectively. More $CD4^+$ than $CD8^+$ T cells express A_{2A} adenosine receptors, but activation of T cells increases A_{2A} expression predominantly in $CD8^+$ T cells. $CD8^+$ T cells are mostly cytotoxic effector cells, whereas $CD4^+$ cells have been implicated in T helper cell activities. Studies of T helper cell subsets (TH1 and TH2) reveal that lymphokine-producing cells are much more likely to express A_{2A} receptors than are cells that do not produce lymphokines. A possible explanation is that inhibitory A_{2A} receptors are induced selectively in cells that produce cytokines, as a mean of limiting cytokine release (Koshiba *et al*., 1999).

Interestingly, on the basis that a deficiency of ADA produces combined immunodeficiency disease in humans, the lymphopenia observed in affected children has been attributed to the toxic effects of deoxyadenosine. Thus, cladribine which is an adenosine deaminase-resistant analogue of deoxyadenosine, has recently been proposed for the treatment of various leukemias and lymphomas (Carrera *et al*., 1994). Cladribine is an A_1/A_2 non-selective receptor agonist and it has been investigated in [^{3}H]-SCH 58261 binding assay to verify whether its mechanism of action underlying the antilimphoprolipherative activity is due to the interaction with A_{2A} receptors. However, cladribine showed a low affinity (K_i=50 μM) in the binding assay and was unable to fully stimulate cyclic AMP accumulation (EC_{50}>500 μM) suggesting that the interaction with A_{2A} receptors is not the only (or main) mechanism of action of this compound. Recently, the involvement of adenosine in the intrathymic apoptotic deletion of T-cells during development has been reported (Barbieri *et al*., 1998). In order to characterize the role of adenosine, the effect of both 2-chloro-adenosine and cladribine to trigger apoptosis of T-cells has been evaluated. The data suggest that, for 2-chloro-adenosine, apoptosis is partially induced by the activation of the A_{2A} receptor subtype, whereas no role has emerged for adenosine receptors in cladribine-dependent apoptosis. Moreover, in these cells, apoptosis could also be triggered through the activation of the A_3 receptor using selective agonists, but this mechanism is not involved in either 2-chloro-adenosine or cladribine-induced apoptosis. Thus, there are at least three different ways by which adenosine derivatives may induce apoptosis including the A_{2A}-like extracellular membrane receptor interaction, or the activation of the A_3 receptors, or the entry of nucleoside into cells and direct activation of intracellular events involved in the apoptotic process and this is the proposed mechanism of action of cladribine. In the future it would be of interest to study deoxyadenosine

Table 6 Correlation among K_i values of tested agonists and antagonists in the [^{3}H]-SCH 58261 binding to human neutrophil membranes and corresponding K_i values, EC_{50} and IC_{50} values

Affinity data	n	*Spearman rank order correlation coefficient*	*Probability (one-tailed test)*	*References*
K_i rat striatal membranes	11	0.98	<0.01	Zocchi *et al*., 1996b
K_i human platelet membranes	12	0.97	<0.01	Dionisotti *et al*., 1996
K_i human lymphocyte membranes	12	0.98	<0.01	Varani *et al*., 1997
EC_{50} and IC_{50} (cyclic AMP)	12	1.00	<0.01	Varani *et al*., 1998a
EC_{50} (O_2^- release)	7	1.00	<0.01	Varani *et al*., 1998a

Table 7 Correlation among EC_{50} and IC_{50} of adenosine receptor agonists and antagonists, respectively, obtained values in cyclic AMP assays using human blood cells

EC_{50} and IC_{50}	n	*Spearman rank order correlation coefficient*	*Probability (one-tailed test)*	*References*
Human platelets	12	1.00	<0.01	Varani *et al*., 1996
Human lymphocytes	12	1.00	<0.01	Varani *et al*., 1997

congeners resistant to deamination, in the hope that these agents might possess antileukemic and antilymphocyte activity.

Human neutrophils

Neutrophils are the most abundant white cells in the peripheral blood and are usually the first cells to arrive at an injured or infected site. For more than a decade adenosine, by interacting with specific receptors on the surface of neutrophils, has been recognized as an endogenous antiinflammatory agent (Schrier & Imre, 1986; Cronstein *et al*., 1990, 1992a; Nolte *et al*., 1992; Sullivan *et al*., 1995). Like in other physiological systems, the effects of adenosine on inflammatory process are not all in one direction. Neutrophils have been found to express both A_1 and A_{2A} adenosine subtypes and the antiinflammatory actions of adenosine related to A_{2A} receptors are balanced by the proinflammatory effects mediated through the A_1 receptor subtype (Salmon & Cronstein, 1990; Fredholm *et al*., 1996). Thus, during the initiation of an inflammatory response low concentrations of adenosine promote neutrophil chemotaxis, phagocytosis (Cronstein *et al*., 1990; Salmon & Cronstein 1990) and endothelial cell adhesion (Becker *et al*., 1992; Schwartz *et al*., 1993); at the higher concentrations present in traumatizated tissues, acting *via* A_{2A} receptors, adenosine inhibits cellular adhesion and superoxide anion generation (Cronstein & Haines, 1992; Sullivan & Linden, 1998). Tissue damage induced by inflammation stems in part from the migration of neutrophils to the site of infection followed by the release of membrane-damaging oxygen radicals. Adenosine inhibits both activities and therefore could be important in limiting abnormal and excessive inflammatory reactions (Cronstein, 1994). More recently other antiinflammatory effects of adenosine acting at its receptors, have been documented. For example, activation of A_{2A} receptors seems to be associated with inhibition of tumour necrosis factor (TNF)-α, IL-6 and IL-8 release by activated mononuclear phagocytes. TNF-α and other cytokines are important in the pathogenesis of sepsis (Fong & Lowry, 1990) and ischemia-reperfusion injury (Seekamp *et al*., 1993) and the well-established beneficial effects of adenosine on ischemia-reperfusion injury have been attributed mainly to direct inhibition of neutrophil function (Marts *et al*., 1993). The antiinflammatory effects of adenosine and its receptor analogues were first suggested in 1983 when Cronstein *et al*. (1983) demonstrated that adenosine, released from neutrophils in suspension, selectively inhibited superoxide anion generation stimulated by FMLP, concanavalin A and the calcium ionophore A 23187. This effect was enhanced by dipyridamole which prevents adenosine uptake into the cells. The prevention of the oxidative burst by adenosine results from its interaction with a specific membrane receptor that has been identified as the A_2 subtype (now known to be the A_{2A} subtype) on the basis of some important characteristics such as the IC_{50} of adenosine for inhibition of O_2^- generation, the order of potency of adenosine analogues, the effect of methylxanthines to antagonize the actions of adenosine and the binding parameters of a radiolabelled adenosine analogue to intact cells (Cronstein *et al*., 1985). In general, occupancy of A_{2A} adenosine receptors stimulates accumulation of intracellular cyclic AMP, which acts as a second messenger to alter cellular function (Londos *et al*., 1980; Daly *et al*., 1981; Fredholm *et al*., 1994; Sullivan & Linden, 1998). However, there is reason to think that the actions of adenosine in mediating the inhibition of superoxide anion generation might not be due to cyclic AMP. This hypothesis is based on the observation that a non-methylxanthine phosphodiesterase inhibitor (Ro 20-1724) enhances intracellular cyclic AMP levels and inhibits superoxide anion generation (Cronstein *et al*., 1988). Both Ro 20-1724 and NECA inhibit superoxide anion generation in an additive way but Ro 20-1724 does not potentiate the functional effect of NECA; in addition it has been reported that KT 5720, an agent which inhibits cyclic AMP-dependent protein kinase A, reverses the effects of cell-soluble analogues of cyclic AMP (dibutyril cyclic AMP) on superoxide anion production but not of NECA (Cronstein *et al*., 1992b) supporting the hypothesis that cyclic AMP does not act as the intracellular messenger for adenosine inhibition of O_2^- generation. In contrast, there are studies in which KT 5720 and other protein kinase A blockers, such as Rp-cyclic adenosine 3′,5′-phosphorothioate (Rp-cAMP), are able to reduce the potency of adenosine (Sullivan *et al*., 1995, 1998; Fredholm *et al*., 1996). Thus, although the role of cyclic AMP in mediating the effects of adenosine analogues is in doubt, this last finding suggests that cyclic AMP may at least contribute and is in agreement with data reported by Varani *et al*. (1998a). The effects of adenosine on other steps in the activation pathway of the neutrophils have also been explored. Chemoattractant-stimulated generation of inositol 1,4,5-triphosphate leads to mobilization of intracellular Ca^{2+} in neutrophils. Adenosine does not affect the early and rapid increase in free cytosolic Ca^{2+} in stimulated neutrophils but inhibits the sustained

increase in intracellular Ca^{2+} that follows stimulation by using chemoattractants suggesting that adenosine does not interfere with the early steps in cell activation (Thiel & Bardenheuer, 1992). Pasini *et al*. (1985) have reported that adenosine may act as a Ca^{2+} channel blocker in neutrophils because it prevents superoxide anion generation in response to the calcium ionophore A23187. In contrast, other authors observed that adenosine inhibited chemoattractant-stimulated O_2^- generation even in the absence of extracellular Ca^{2+} and, as a consequence, the role of adenosine on Ca^{2+}-dependent step in neutrophils activation remains unclear (Cronstein *et al*., 1988, 1997).

More recent studies indicate that adenosine receptor activation could also interfere with a subsequent step in signal transduction for chemoattractant receptors. It has been demonstrated that the direct activation of G proteins with NaF determines superoxide anion generation in neutrophils and the adenosine receptor occupancy does not reduce superoxide anion generation stimulated by NaF suggesting that adenosine inhibits the interaction between occupied chemotactic receptors and G proteins (Cronstein *et al*., 1990; Burkey & Webster, 1993). Possibly, the activation of adenosine receptors may determine the uncoupling of FMLP receptors from the signal transduction mechanism and the occupancy of adenosine receptors produces the association of chemoattractant receptors with the cytoskeleton, promoting their premature desensitization (Jesaitis *et al*., 1989; Cronstein & Haines, 1992; Revan *et al*., 1996).

Recent pharmacological studies comparing [^{3}H]-SCH 58261 binding with functional data in human neutrophils have shed some light on the signal transduction mechanism responsible for the adenosine-mediated inhibition of O_2^- production and the role of cyclic AMP (Varani *et al*., 1998a,b). In the past, radioligand binding studies in neutrophils have been performed with the non-selective agonist NECA (Cronstein *et al*., 1985) that showed a high background binding, interacting also with the adenotin site (Table 3). [^{3}H]-SCH 58261 has been found to label in human neutrophils a single class of recognition sites with K_d and B_{max} values (1.34 nM and 75 fmol mg^{-1} prot., respectively) with the same order of magnitude as that determined in human platelet and lymphocyte membranes (Table 3). In competition studies, typical adenosine agonists and antagonists bound the A_{2A} receptor with a rank order of potency and affinity range similar to that observed in [^{3}H]-SCH 58261 binding to human platelet and lymphocyte membranes (Table 4). Interestingly, in the stimulation of cyclic AMP accumulations, the compounds studied exhibited a rank order of potency similar to that observed in binding experiments (Table 5). Thus, like A_{2A} receptors in other mammalian tissues, those present on neutrophils are coupled with adenylate cyclase stimulation. Moreover, the studies of inhibition of superoxide anion production revealed that the receptor on neutrophils is of the A_{2A} subtype. Adenosine analogues were similar in both their effects to inhibit superoxide anion generation and to inhibit the binding of [^{3}H]-SCH 58261 (Tables 4 and 5). Similarly, the data on antagonists of HE-NECA actions in human neutrophils were in reasonable agreement with those obtained in binding assays. An excellent correlation was found also between cyclic AMP accumulation data and inhibition of O_2^- generation by adenosine receptor agonists investigated in this study, reported for comparison in Table 5, (Spearman rank correlation coefficient = 1.00, $P < 0.01$) suggesting that cyclic AMP could be involved in the action of A_{2A} receptors to inhibit superoxide anion generation. Finally, 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. Data obtained from this study indicate that [^{3}H]-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}), a behaviour similar to that found in rat striatum, which is typical of A_{2A} receptor antagonists (Borea *et al*., 1995) and in agreement with that found in human lymphocytes.

Conclusions

In conclusion, the therapeutic use of the antiaggregatory and antiinflammatory properties of adenosine as well as of its immunosuppressive effects requires a satisfactory characterization of the receptor subtype involved and the development of ligands selective for this receptor. Altogether, it can be stated that [^{3}H]-SCH 58261 has helped to characterize A_{2A} receptors which are present in tissues where they have been known to have a functional role such as platelets, lymphocytes and neutrophils. Thus, obstacles due to labelling of the adenotin site by [^{3}H]-NECA or [^{3}H]-CGS 21680 have been successfully overcome. A general conclusion from the most recent binding and functional data is that A_{2A} receptors have similar sensitivity in different tissues and animal species. This finding is in agreement with the molecular biology data showing that the A_{2A} receptor is a highly conserved structure in various animal species (Jacobson, 1995).

The available studies demonstrating the presence of A_{2A} adenosine receptors in human circulating blood cells, strongly suggest that adenosine could play an important role in modulating aggregatory, immune and inflammatory processes and that the activation of A_{2A} receptors may have therapeutic potential. For example, adenosine has been reported as a natural protectant of cells in ischemic tissue and although several regulatory mechanisms are involved, the activation of extracellular adenosine receptors is the most relevant. The activation of A_{2A} receptors prevents platelet aggregation, affects the immune response in cancer, auto-immune and neurodegenerative diseases and decreases the inflammatory reactions. This is particularly important based on recent evidence suggesting the presence of an inflammatory component in a variety of neurological disorders, including trauma, ischemia, sclerosis and various forms of encephalopathies (Abbracchio & Burnstock, 1998). The therapeutic uses of adenosine and its analogues are limited by severe side effects like hypotension and bradycardia that are due to the ubiquitous nature of the adenosine receptors as well as a short half life (Belardinelli *et al*., 1989). As an alternative strategy, agents that enhance endogenous adenosine concentrations at sites of inflammation might be useful. Recent studies indicate that enhanced release of adenosine mediates the antiinflammatory effects of both methotrexate and sulphasalazine, the two most widely used agents for the treatment of rheumatoid arthritis (Cronstein & Weissmann 1993; Gadangi *et al*., 1996). Both methotrexate and sulphasalazine inhibit 5 aminoimidazole-4-carboxamide ribonucleotide (AICAR) transformylase with consequent intracellular acumulation of its substrate, AICAR. AICAR accumulation augments the release of adenosine from the endothelium damaged by the presence of adherent and activate PMN and the end-point of this biochemical interference is a reduced PMN adhesion. Therefore, in the future, site and receptor selective targeting strategies are needed to develop novel pharmacologic agents having optimal efficacy with minimal unwanted effects for the treatment of a variety of diseases linked with adenosine-signalling pathways.

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