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Inhibition by xanthine derivatives of adenosine receptor-stimulated cyclic adenosine 3′, 5′-monophosphate accumulation in rat and guinea-pig thymocytes

Bertil B. Fredholm & Göran Sandberg

Department of Pharmacology and Department of Histology, Karolinska Institutet, Stockholm, Sweden

1 The effect of stable adenosine analogues, including adenosine 5′-N-ethylcarboxamide (NECA) and N^6-L-phenylisopropyl-adenosine (L-PIA), were studied on cyclic adenosine 3′, 5′-monophosphate (cyclic AMP) accumulation in rat and guinea-pig thymocytes.

2 NECA was approximately 10 times more potent than L-PIA, in thymocytes from both species. D-PIA was more potent in guinea-pig than in rat thymocytes. The effect of a number of adenosine analogues followed the order: NECA > 2-chloro-adenosine > L-PIA > N^6-cyclohexyl-adenosine (CHA), an order of potency characteristic for adenosine receptors of the A2-subtype. Thymocytes may be used as a model system to study the pharmacology of such receptors.

3 Several xanthines were studied as antagonists of the NECA (1 μM)-induced cyclic AMP accumulation. The order of potency was: 1,3-diethyl-8-phenylxanthine ≫ 8-phenyl-theophylline > IBMX = 8-*p*-sulphophenyltheophylline = verrophylline > theophylline > caffeine > enprofylline > theobromine > pentoxiphylline. The pA_2 value for 8-phenyltheophylline was 0.35 μM, and the antagonism was shown to be competitive. The order of potency of the xanthine is virtually identical to that found earlier in several other systems in which the receptors are of the A1-subtype. None of the xanthine derivatives tested thus seem to discriminate between A1 and A2-receptor-mediated adenosine actions.

Introduction

It is known that adenosine is able to stimulate cyclic adenosine 3′, 5′-monophosphate (cyclic AMP) accumulation in mouse thymocytes (Zenser, 1975), rat thymocytes (Nordeen & Young, 1977), guinea-pig thymocytes (Fredholm, Sandberg & Ernström, 1980), human peripheral blood lymphocytes (Schwartz, Stern & Polmar, 1978; Marone Plaut & Lichtenstein, 1978) and pig lymph-node lymphocytes (Bonnafous, Dornand & Mani, 1979a). The existence of an adenosine-stimulated adenylate cyclase has been demonstrated in mouse thymocytes (Zenser, 1975; Bonnafous, Dornand & Mani, 1979b; Bonnafous, Dornand, Favero & Mani, 1982) and human peripheral blood lymphocytes (Snider & Parker, 1977). As reviewed elsewhere (Strom, Lundin & Carpenter, 1977) elevation of lymphocyte cyclic AMP levels is associated with marked changes in lymphocyte function. In particular, adenosine is able to impair many aspects of thymocyte function including cytolysis (Wolberg, Zimmerman, Duncan, Singer & Elion, 1978; Zimmerman, Wolberg, Duncan, Rideout, Beachman, Krenitzky & Elion, 1978) and T-cell-mediated stimulation of B-cell immunoglobulin production (Moroz & Stevens, 1980). The proliferation of thymocytes *in vitro* is also influenced by endogenous adenosine (Sandberg & Fredholm, 1981; Sandberg, 1983).

It is known from experiments with other types of cells that adenosine is able to interact with at least two types of adenosine receptors, A1 (Ri) and A2 (Ra), to influence cyclic AMP production (for review see Londos, Cooper & Wolff, 1980; Fredholm, 1982). A1-receptors can be defined as those adenosine receptors at which phenylisopropyl-adenosine (L-PIA) and cyclohexyl-adenosine (CHA) are more potent than 2-chloroadenosine and adenosine 5′-ethylcarboxamide (NECA). These receptors can be studied by ligand binding techniques (Bruns *et al.*, 1980; Schwabe & Trost, 1980), and are frequently related to an inhibition of adenylate cyc-

lase. Adenosine A2-receptors, by contrast, are defined as those receptors at which NECA is more potent than 2-chloro-adenosine and much more potent than PIA and CHA. Adenosine analogue binding to these receptors is frequently, and possibly always, associated with an increase in adenylate cyclase activity. Irrespective of the type of adenosine receptor involved in a given adenosine response it can be inhibited by theophylline and other xanthine derivatives.

The present study was undertaken because we wanted to know what kind of adenosine receptor is involved in the adenosine-mediated increase in thymocyte cyclic AMP accumulation. We also studied the effect of ten different xanthine derivatives to see whether any of them were more potent in this system than in other systems (cf. Fredholm & Persson, 1982), thereby showing a degree of selectivity as inhibitors of the thymocyte adenosine receptors.

Methods

Preparation of thymocyte suspensions

Male guinea-pigs (4–6 weeks old) or male Sprague-Dawley rats (180–280 g) were used as cell donors. The thymocytes were isolated as described by Fredholm *et al.* (1978). They were washed in a buffer consisting of equal parts of Hanks' balanced salt solution and Dulbecco's phosphate buffered saline, and suspended in RPMI 2640 medium (supplemented with 2 μmol L-glutamine, 0.5 μmol L-alanine, 100 iv penicillin and 100 μg streptomycin per l) to a final density of approximately 5×10^6 cells Ml^{-1}. Aliquots (1 ml) of this cell suspension was incubated in Falcon culture tubes (12 × 75 mm).

Cyclic AMP accumulation

After 2 h of preincubation adenosine deaminase (Boehringer, Mannheim) was added to all cell cultures in a final concentration of 0.5 units ml^{-1} to remove any endogenous adenosine. The phosphodiesterase inhibitor, rolipram (ZK 62, 711) was also added to a final concentration of 30 μM. After a further 2 h of incubation with these additions the adenosine analogues were added in the concentrations indicated below. The substances were added in such a way as not to resuspend the cultured cells. After 15 min the supernatants were discarded and 1 ml 0.4 M perchloric acid added. The perchloric acid extracts were frozen and kept at −20 °C until assay.

In the experiments in which the potency of the compounds as antagonists of adenosine action was tested the putative antagonists were added 5 min before NECA (1 μM). In other respects these experiments were similar.

Determination of cyclic AMP

Prior to assay for cyclic AMP the frozen samples were thawed and 0.1 ml 4 M KOH and 0.5 ml 1 M Tris base was added. The clear supernatant after centrifugation was used directly for the assay of cyclic AMP, essentially as described earlier (Fredholm *et al.*, 1978).

Chemicals

[^{3}H]-adenosine 3′, 5′-monophosphate was obtained from Radiochemical Centre Amersham. Adenosine and adenosine 3′, 5′-monophosphate were obtained from Boehringer, Mannheim, who also kindly supplied the stereoisomers of N^6-phenylisopropyladenosine. Erythro-9-(2-hydroxy-3-nonyl) adenine

Table 1 The effect of adenosine analogues on cyclic AMP accumulation in rat and guinea-pig thymocytes

	Conc. causing 100% increase in cyclic AMP (μM)	
Analogue	*Rat*	*Guinea-pig*
NECA	0.043 ± 0.030	0.044 ± 0.022
2-Cl-ado	0.107–0.066	0.32 ± 0.05
L-PIA	0.41 ± 0.05	0.66 ± 0.22
CHA	2.86 ± 0.44	1.85 ± 1.05
D-PIA	12.9 ± 8.0	1.14 ± 0.15
Ado	16.1 ± 2.3	70 ± 42
Ado + EHNA (3 μM)	0.28 ± 0.15	0.76 ± 0.15

Each analogue was tested in 3 to 8 concentrations, 3-12 determinations at each concentration. The increase in cyclic AMP over basal was calculated and by linear regression the concentration required to double cyclic AMP levels was determined. The results are given as mean ± s.e. (Zivin & Waud, 1982). The basal cyclic AMP levels were: rat 5.2 ± 0.4 pmol cyclic AMP ml^{-1}, guinea-pig 7.0 ± 1.0 pmol cyclic AMP ml^{-1}. The lymphocyte suspension contained approximately 5×10^6 cells ml^{-1}. The phosphodiesterase inhibitor rolipram was present throughout the incubation. NECA = adenosine 5′-ethylcarboxamide; 2-Cl-ado = 2-chloroadenosine; L-PIA phenylisopropyladenosine; CHA = cyclohexyladenosine; Ado = adenosine, EHNA = erythro-9-(2-hydroxy-3-nonyl)adenine.

(EHNA) was a gift from Wellcome Labs, Research Triangle Park, N.C.; 2-chloro-adenosine was from Sigma; adenosine 5'-ethylcarboxamide was from Byk-Gulden, Konstanz; theophylline (as the ethylenediamine salt), theobromine and caffeine were from ACO, Stockholm; enprofylline was a gift from AB Draco, Lund; 1,3-diethyl-8-phenylxanthine was from Research Biochemicals Inc., Wayland, MA and 8-phenyl-theophylline from Calbiochem. Both compounds were dissolved in 0.3% tetraphenyl boronate. 8-*p*-Sulphophenyl-theophylline was a gift from Dr F. Bruns, Ann Arbor, Mich. and verrophylline a gift from Dr K. Murphy.

Results

In agreement with previous results, adenosine was found to stimulate the accumulation of cyclic AMP in guinea-pig and rat thymocytes (Table 1). The effect of adenosine was weak unless an adenosine deaminase inhibitor, EHNA (3 μM), was included in the assay. The effect of adenosine was shared by several analogues of adenosine, the most potent of which was NECA (Table 1).

As seen in Figures 1 and 2, the potency of NECA was considerably higher than that of L-PIA in thymocytes from both rats and guinea-pigs, and the maximal effect obtainable appeared to be larger with NECA than with PIA. This could indicate that N^6-substituted analogues such as L-PIA are partial,

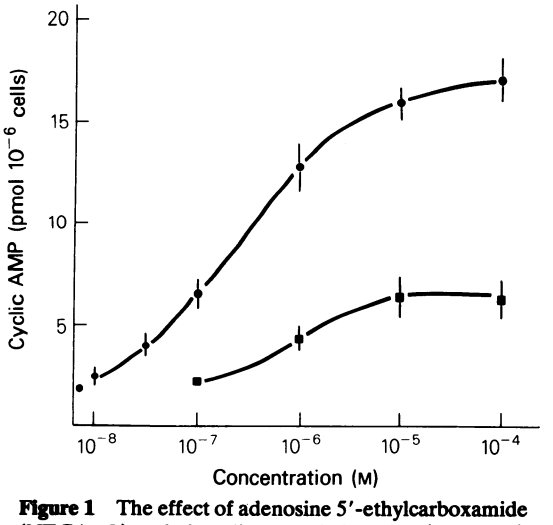

Figure 1 The effect of adenosine 5'-ethylcarboxamide (NECA, ●) and phenylisopropyladenosine (L-PIA, ■) on the cyclic AMP content of rat thymocytes. The results are given as mean with s.e. mean (vertical lines) of quadruplicate determinations from three separate experiments. The EC_{50} for NECA was estimated to be 3×10^{-7} M.

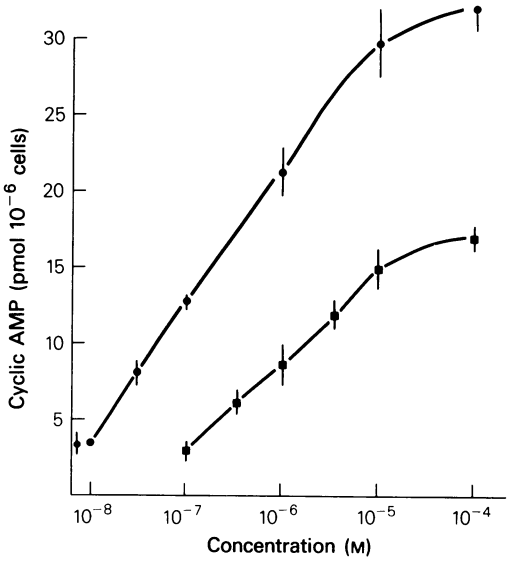

Figure 2 The effect of adenosine 5'-ethylcarboxamide (NECA, ●) and phenylisopropyladenosine (L-PIA, ■) on the cyclic AMP content of guinea-pig thymocytes. The results are given as means with s.e. mean (vertical lines) of a total of six determinations from three separate experiments. The EC_{50} for NECA was estimated to be 3.9×10^{-7} M.

rather than full, agonists on thymocyte cyclic AMP accumulation. The fact that the maximal response was different makes the comparison of potencies difficult: however, there is a 10–20 fold potency difference in the concentration giving half the respective maximal response between NECA and L-PIA. The absolute and relative potency of the two agonists appeared to be similar in the two species.

Since the maximal response appeared to differ between the analogues we have compared the potency of adenosine analogues by calculating the concentration required to double the cyclic AMP concentration. These comparisons are presented in Table 1. NECA was the most potent analogue followed by 2-chloro-adenosine, which was more potent than L-PIA and CHA. Adenosine (in the presence of EHNA) was approximately equipotent with L-PIA, which was more potent than D-PIA. Interestingly D-PIA was much more potent in guinea-pig than in rat thymocytes (Table 1).

The potency of a number of xanthine derivatives as antagonists of the adenosine-receptor-mediated accumulation of cyclic AMP was determined as shown in Tables 2 and 3. In agreement with results obtained in many other tissues, 8-phenyl-substituted xanthines were the most potent. Therefore 8-phenyl-theophylline was used to determine the type of an-

Table 2 The inhibitory effect of selected xanthine derivatives on the adenosine 5'-ethylcarboxamide (NECA, 1 μM)-induced stimulation of cyclic AMP accumulation in rat thymocytes

Xanthine	*IC_{50} (μM)*	K_i *(μM)*
Diethyl-8-phenyl-xanthine	0.47 ± 2.0	0.18 ± 0.46
8-Phenyl-theophylline	3.1 ± 1.6	0.72 ± 0.37
Verrophylline	5.9 ± 1.6	1.4 ± 0.37
Isobutyl-methylxanthine	6 ± 4.2	1.4 ± 1.0
8-*p*-Sulphophenyltheophylline	17 ± 1.5*	4.0 ± 0.35
Theophylline	45 ± 12	10 ± 2.8
Caffeine	98 ± 45*	22 ± 10
Enprofylline	650 ± 110*	150 ± 25
Theobromine	2500 ± 960*	580 ± 220
Pentoxyfylline	4220 ± 1600*	980 ± 340

*Signifies a Hill-slope significantly less than 1.

The inhibitory potency was determined by Hill-plots of 2 to 5 determinations at three or more concentrations of the xanthines; s.e. calculated as described by Zivin & Waud (1982) is also given. The results are presented as the IC_{50} values and the calculated K_i values. The K_i values were calculated from the expression: $K_i = IC_{50}(1+S/K_D)$, where S is the concentration of NECA (1 μM) and the K_D for NECA was estimated from the data presented in Figure 1 and was taken to be equivalent to the EC_{50} value (0.3 μM).

tagonism. As seen in Figure 3 the effect of increasing concentrations of the xanthine was to cause a parallel shift of the NECA dose-response curve. These data could be used to determine a pA_2 value (Figure 3 inset). This value was in reasonable agreement with the pK_i value determined in guinea-pigs as shown in Table 3, and in complete agreement with the pA_2 value determined in rat thymocytes (0.35 μM) (not shown). There were slight differences in the absolute potency of the xanthine derivatives in the two species. For example, 8-*p*-sulphophenyltheophylline appeared to be more potent and caffeine and verrophylline less potent in the guinea-pig than in the rat. None of these differences proved to be statistically significant, however.

Discussion

The present results suggest that the adenosine receptors that mediate an increase in thymocyte cyclic AMP accumulation are of the A2-subtype both in the rat and the guinea-pig. This is not surprising since this type of receptor generally mediates increases in cyclic AMP accumulation (Londos *et al.*, 1980; Fredholm, 1982). The absolute and relative potencies of the adenosine analogues were generally similar in thymocytes from both species, suggesting an essential similarity in receptors. Moreover, D-PIA was much more potent in guinea-pig than in rat thymocytes. A similar species difference was previously described for hippocampal slices (Fredholm, Jonzon, Lindgren & Lindström, 1982). In this respect there might therefore be a species difference in adenosine receptors.

The effect of several xanthine derivatives as antagonists at these sites was studied. The order of potency was quite similar to that reported earlier for antagonism of adenosine actions in fat cells, and archetypal A1 response (Fredholm & Persson, 1982). The order of potency was also similar to that observed for the displacement of bound tritiated

Table 3 The effect of selected xanthine derivatives on the adenosine 5'-ethycarboxamide (NECA, 1 μM)-induced accumulation of cyclic AMP in guinea-pig thymocytes

	IC_{50} (μM)	K_i *(μM)*
Diethyl-8-phenylxanthine	0.50 ± 1.05	0.14 ± 0.30
8-Phenyl-theophylline	0.89 ± 1.24	0.25 ± 0.35
8-*p*-Sulphophenyltheophylline	2.6 ± 1.4	0.70 ± 0.40
Isobutyl-methylxanthine	10 ± 2*	2.8 ± 0.6
Verrophylline	31 ± 15	8.9 ± 4.3
Theophylline	150 ± 120	43 ± 30
Caffeine	548 ± 120	156 ± 30
Enprofylline	768 ± 38*	220 ± 11
Theobromine	2530 ± 780*	720 ± 220
Pentoxifylline	4670 ± 1540*	1330 ± 450

See Table 2 for further details. The ratio S/K_d was 2.5 in these experiments.

*Signifies a Hill-slope significantly less than 1.

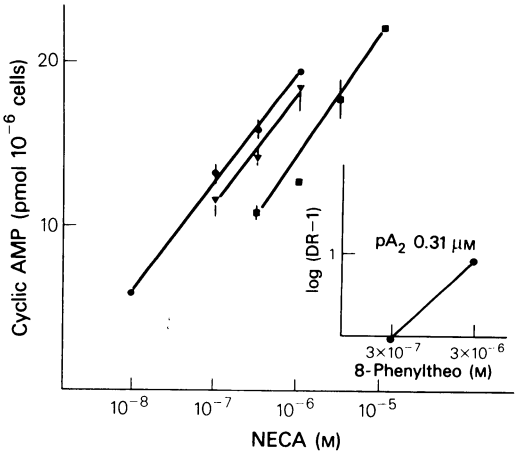

Figure 3 The dose-response curve to adenosine 5'-ethylcarboxamide (NECA) in guinea-pig thymocyte in the absence (●) and presence of 8-phenyl-theophylline (3×10^{-7} M' ▲; and 3×10^{-6} M, ■). Inset: Schild plot of data.

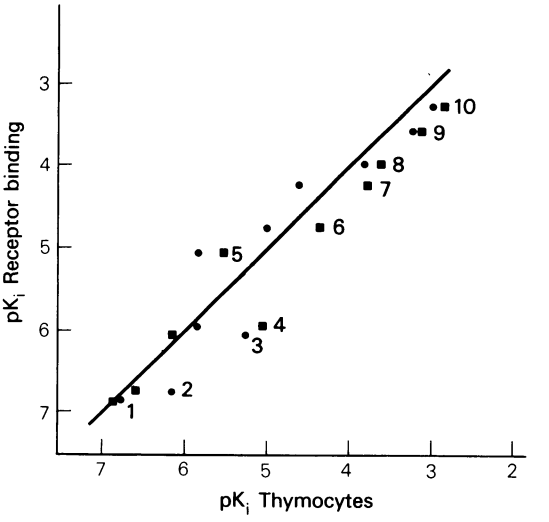

Figure 4 Relationship between the potency of xanthine derivatives as inhibitors of adenosine 5'-ethylcarboxamide (NECA) responses in rat (●, Table 2) and guinea-pig thymocytes (■, Table 3) and the potency as displacers of phenylisopropyladenosine L-PIA) binding to rat cortical membranes (data from Fredholm & Persson, 1982). The line indicates the line of identity. The numbers represent the xanthine derivatives used: (1) 1,3-diethyl-8-phenylxanthine; (2) 8-phenyl-theophylline; (3) 8-*p*-sulplophenyl-theophylline; (4) verrophylline; (5) 1-isobutyl-3-methylxanthine; (6) theophylline; (7) caffeine; (8) enprofylline; (9) theobromine; (10) pentoxifylline.

L-PIA from membranes prepared from the rat cerebral cortex (Fredholm & Persson, 1982). As seen in Figure 4, both the absolute and relative potency of the xanthines in the thymocyte system is very similar to that found in the binding assay. This indicates that the xanthines tested here do not exhibit any important selectivity for A1 or A2 receptors. In particular the new bronchodilator enprofylline (Persson & Erjefält, 1982) had the potency as an adenosine antagonist in the thymocyte system that would be expected from its ability to displace L-PIA from its binding sites in the CNS. We previously showed that enprofylline was more potent as an atagonist of the NECA-induced cyclic AMP accumulation in rat hippocampal slices (another A2-receptor mediated effect) than could be anticipated from its activity on A1 adenosine receptors (Fredholm & Persson, 1982). The present finding suggests that this aberrant behaviour is unlikely to be due to the fact that the compound is a selective antagonist of all A2 adenosine receptors.

Adenosine is known to inhibit many types of lymphocyte function. This may be of more than pharmacological interest since adenosine is active already in low μmolar concentrations (Wolberg *et al.*, 1978 and present results). There are several possible mechanisms of action of which receptor-mediated increase in cyclic AMP accumulation is only one (see Fox, 1981). If adenosine receptor-mediated changes in cyclic AMP are of biological significance it may be assumed that theophylline and other xanthine derivatives could exert some action on the immune system via an interaction with such receptors. A study of the immunological consequences of long term administration of one of the more potent xanthine derivatives, such as 8-phenyl-theophylline, could provide valuable insights into the biological importance of adenosine receptors on lymphocytes.

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