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The adenosine receptor activity of EMD 28422, a purine derivative with reported actions on benzodiazepine receptors

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1 The effects of a novel purine derivative, N^6-[2-(4-chlorophenyl)-bicyclo-2.2.2.octyl-(3)]-adenosine (EMD 28422), that has been found to influence central benzodiazepine receptors, has been compared to those of other adenosine analogues such as L-phenylisopropyladenosine (L-PIA), cyclohexyladenosine (CHA) and adenosine-5'-N-ethyl-carboxamide (NECA).

2 EMD 28422 was about 30 times less potent than CHA and 4 times less potent than NECA in displacing bound [^{3}H]-L-PIA from specific binding sites in the rat brain, presumably reflecting adenosine A_1-receptors. A similar relative potency was found using depression of field e.p.s.p. in the hippocampal slice *in vitro*.

3 In isolated fat cells EMD 28422 was antilipolytic, but some 1000 times less potent than L-PIA.

4 In rat isolated hippocampal slices, which have adenosine A_2-receptors, EMD 28422 was more than 300 times less potent than NECA and in guinea-pig thymocytes, which similarly have A_2-receptors, EMD 28422 was about 60 times less potent.

5 The results are compatible with the opinion that EMD 28422 is a rather weak agonist at adenosine receptors, with limited selectivity for A_1- or A_2-receptors. The compound is highly lipophilic, which plays a role in determining its potency in a given biological system. The results are discussed in relation to reported adenosine modulation of benzodiazepine receptors.

Introduction

As originally shown by Drury & Szent-Györgyi (1929) adenosine has a number of biological effects. The actions of adenosine and adenosine analogues are probably mediated via specific adenosine receptors located on the cell surface (see Burnstock, 1981). These adenosine receptors are of two types: A_1 and A_2 (Van Calker *et al.*, 1979; see Fredholm, 1982). At A_1-receptors two N^6-substituted adenosine analogues, L-phenylisopropyladenosine (L-PIA) and cyclohexyladenosine (CHA), are more potent than adenosine-5'-ethyl-carboxamide (NECA). Specific binding sites exhibiting many of the characteristics of A_1-type adenosine receptors have been found in the brain of many species (Bruns *et al.*, 1980; Williams & Risley, 1980; Schwabe & Trost, 1980). A_2-type adenosine receptors can be studied by examining cyclic adenosine 3',5'-monophosphate (cyclic AMP) accumulation in different preparations and are characterized by having a higher affinity for NECA than for PIA and CHA.

The possibility exists that adenosine and other purines may be related to benzodiazepine receptors and benzodiazepine effects (Skolnick & Paul, 1981; Phillis & Wu, 1981). For example, Skolnick & coworkers (1980a) found that a novel purine derivative, EMD 28422 (N^6-[2-(4-chlorophenyl)-bicyclo-2.2.2.octyl-(3)-]-adenosine), increased the number of benzodiazepine binding sites in the brain. The authors also showed that EMD 28422 was able to increase the number of binding sites for [^{3}H]-diazepam *in vitro*, without an accompanying increase in the affinity. Because such effects on benzodiazepine receptors have not been observed with other adenosine analogues we have tried to characterize EMD 28422 as an adenosine receptor agonist in several model systems to determine if this compound has a particularly high affinity or selectivity for an adenosine receptor sub-type. The results indicate that EMD 28422 is a relatively weak adenosine receptor agonist with limited selectivity towards A_1- or A_2-receptors.

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Methods

Adult male Sprague-Dawley rats weighing 180–250 g were obtained from Anticimex, Sweden, (for the biochemical experiments) or from Charles River Laboratories (for the electrophysical experiments). Guinea-pigs weighing 400–600 g were used to prepare thymocytes. Cats were obtained from a breeding colony at the department.

Binding studies

The effect of EMD 28422 and other adenosine analogues as displacers of [^{3}H]-L PIA binding was studied using membranes from cat cortex, essentially as described elsewhere (Fredholm *et al.*, 1982; Dunwiddie & Fredholm, 1984). The drugs were added to the concentration indicated together with 10 nM[^{3}H]-L-PIA and the binding was allowed to proceed for 120 min at 30°C. Non-specific binding was defined as the amount of binding occurring in the presence of 10 μM 2-chloroadenosine (Dunwiddie & Fredholm, 1984).

Cyclic AMP accumulation in rat hippocampus

The ability of EMD 28422 and other adenosine analogues to increase the accumulation of [^{3}H]-cyclic AMP in [^{3}H]-adenine labelled slices of rat hippocampus was determined as described in detail elsewhere (Fredholm *et al.*, 1982). Briefly slices of rat hippocampus were cut to a thickness of 400 μm and incubated in Krebs Ringer bicarbonate buffer for 15 min with the drug after labelling. The conversion of radioactivity to cyclic AMP was determined by a combined alumina and Dowex chromatography. No correction was made for incomplete recovery of cyclic AMP (60 ± 5%).

Lipolysis in fat cells

The effect of EMD 28422 and other adenosine analogues as inhibitors of lipolysis in rat fat cells was studied as described elsewhere (Fredholm & Lindgren, 1984). After preparation of the fat cells they were incubated in the presence of adenosine deaminase (1 μg ml^{-1}), noradrenaline (10^{-8} M) and the adenosine analogues to be tested. Lipolysis was measured as the release of glycerol into the medium during a 1 h incubation.

Electrophysiological experiments

Slices of rat hippocampus were prepared and incubated as has been described previously (Dunwiddie & Lynch, 1978; Dunwiddie & Hoffer, 1980). Slices were continuously superfused with control medium (modified Krebs Ringer bicarbonate) and synaptic

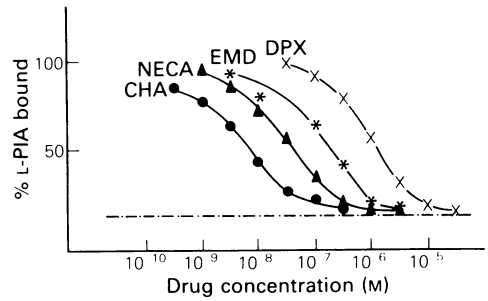

Figure 1 The displacement of [^{3}H]-L-phenylisopropyladenosine (L-PIA) from membranes of rat parietal cortex by cyclohexyladenosine (CHA), adenosine-5′-ethylcarboxamide (NECA), EMD 28422 and 1,3-diethyl-8-phenylxanthine (DPX). L-PIA was present in a concentration of 5 nM. The incubation was performed at 22°C for 120 min. Mean of duplicate determinations from two separate experiments. (—.—.—) Non-specific binding.

responses evoked from the Schaffer collateral-commissural pathway to the CA1 region at 1 min intervals. This response is typically inhibited by 90–95% by adenosine and other A_1-receptor agonists (Dunwiddie & Hoffer, 1980; Dunwiddie & Fredholm, 1984).

EMD 28422 (100 μM in Krebs Ringer) was added to the flow of perfusion fluid via a calibrated syringe pump during continuous testing of the response to give a final concentration of 0.25–5 μM in the medium. Following 60–90 min of drug perfusion, the slices were washed for 10 min with drug-free medium, and 8-phenyl-theophylline (8PT; 10 μM) was added to the medium to reverse the effects of residual drug. In all cases, responses were quantified as the % depression relative to the post-8PT response; the EC_{50} value was then determined from a Hill plot of the resulting data. This way of quantifying drug responses was used because of the extremely slow onset of action of EMD 28422, the fact that responses to this drug did not recover with washing within at least 1 h, and the rapidity with which recovery set in after administration of 8PT.

Cyclic AMP accumulation in guinea-pig thymocytes

These experiments were carried out exactly as described elsewhere (Fredholm & Sandberg, 1983). The effect of an increase in concentrations of EMD 28422 and other adenosine analogues on the accumulation of cyclic AMP was studied in suspensions of guinea-pig thymocytes (5×10^6 cells ml^{-1}). The medium contained adenosine deaminase (0.5 units ml^{-1}) and the phosphodiesterase inhibitor rolipram (ZK 62,711 obtained from Schering AG) final concentration of

30 μM. At the end of a 15 min incubation at 37°C the reaction was stopped by the addition of 0.4 M perchloric acid. Cyclic AMP was measured in the non-purified extracts following removal of proteins and neutralization.

Effects on rectal temperature

Non-anaesthetized rats weighing 240 g were provided with a rectal temperature probe. Increasing doses of EMD 28422 were injected intraperitoneally and the temperature recorded until a steady level was obtained.

High pressure liquid chromatography of adenosine analogues

Adenosine analogues were chromatographed on reversed phase h.p.l.c. (μBondapak C18, Waters) using increasingly non-polar solvents, the most non-polar being 10 mM $NH_4H_2PO_4$ (pH 8.0): MeOH: acetonitrile, 50:20:30. On this system the retention times (in min) were: NECA (3), CHA (4.5), PIA (5), EMD 28422 (27).

Drugs

EMD 28422 (N^6-[2-(4-chlorophenyl)-bicyclo-2.2.2.octyl-(3)]-adenosine, was from Merck, Darmstadt, BRD. It was dissolved to a concentration of 1 mM in 50% propylene glycol, 10% ethanol in water. From this stock solution dilutions were made in saline. L-phenylisopropyladenosine (L-PIA) was obtained from Boehringer, Mannheim, which was also the source of adenosine deaminase. 2-Chloroadenosine, adenosine and noradrenaline were obtained from Sigma. Adenosine 5'-ethylcarboxamide was a kind gift from Byk Gulden, Konstanz, BRD. N^6-cyclohexyladenosine was obtained from Calbiochem, Behring, San Diego, California. 1,3-diethyl-8-phenylxanthine and 8-phenyl-theophylline were obtained from Research Biochemicals Inc., Wayland, MA, U.S.A. [^{3}H]-L-PIA was obtained from the Radiochemical Centre, Amersham.

Results

As seen in Figure 1 EMD 28422 was able to displace [^{3}H]-L-PIA from binding sites in the rat parietal cortex. It was approximately 30 times less potent than CHA, 4 times less potent than NECA and about 7 times more potent than the xanthine derivative, 1,3-diethyl-8-phenylxanthine (DPX). The K_D value for EMD 28422 is close to 0.1 μM.

Inhibition of lipolysis in rat fat cells is an archetypal A_1-receptor effect of adenosine and adenosine

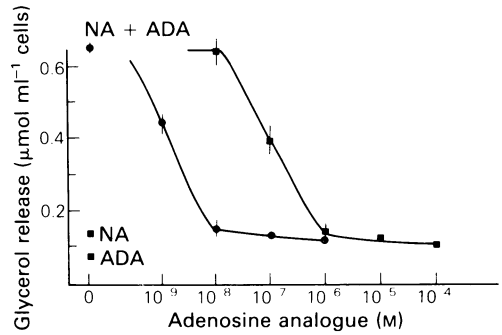

Figure 2 Inhibition of lipolysis (glycerol release) by 2-chloroadenosine (●) and EMD 28422 (■) in rat isolated fat cells. Mean ± s.e.mean of 4 determinations. Noradrenaline (NA) was present at 10^{-8} M and adenosine deaminase (ADA) at 1 μg ml^{-1}.

analogues. The results shown in Figure 2 demonstrate that EMD 28422 is about 80 times less potent than 2-chloroadenosine as an inhibitor of lipolysis which, based on previous work (Fredholm & Lindgren, 1984), would make it almost 1000 times less potent than L-PIA, and close to 200 times less potent than NECA. EMD 28422 did not stimulate lipolysis *per se* and did not antagonize the effect of 2-chloroadenosine, indicating that it is not an antagonist or partial agonist.

We have previously shown (Dunwiddie & Fredholm, 1984) that the inhibition of activity in rat hippocampal slices measured electrophysiologically is also mediated by A_1-adenosine receptors. EMD 28422 produced results which were qualitatively similar to those seen with adenosine in this system, and which could be antagonized or reversed by treatment with adenosine antagonists such as theophylline. However, these responses were extremely slow in onset (compared with adenosine), in that it took more than 60 min to reach a maximal response (see Figure 3). Similar slow time courses have been observed for other lipid soluble adenosine analogues (L-PIA and CHA), and appear to be related to the amount of time required for such compounds to diffuse into the brain slice (Dunwiddie & Fredholm, 1984; Dunwiddie *et al.*, 1984). For this reason, it was somewhat difficult to ascertain the absolute potency of this drug, because responses from these slices typically are not stable over the amount of time required to elicit a maximal response. However, by measuring the increase in response amplitude following addition of the adenosine antagonist 8PT (this response was usually maximal within 3–4 min), we could determine the amount of depression of the response which had occurred independently of long-term changes in the baseline. Addition of 8PT alone to the slices produced less than 5% change in the amplitude of the synaptic response. Using this technique, we calculated an EC_{50} of 0.38 μM

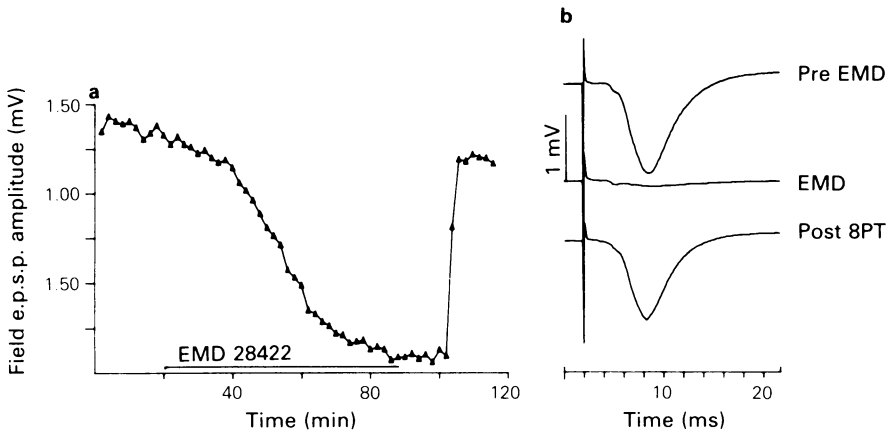

Figure 3 Typical responses of field excitatory postynaptic potential (e.p.s.p.) responses in the rat hippocampal CA1 region to EMD 28422. (a) Time course of inhibition following addition of EMD 28422 2 μM. After about 100 min 8-phenyl-theophylline (8PT) was added. (b) Actual records of e.p.s.ps before the drug, after 60 min exposure to EMD 28422 (2 μM), and after addition of 8PT.

(95% confidence limits 0.14–10.2 μM, calculated from 10 separate perfusion experiments) for EMD 28422; this makes this compound slightly less potent than NECA, and about 20 times less potent than analogues such as L-PIA and CHA in this system.

The effect of some adenosine analogues on the accumulation of cyclic AMP in rat hippocampus slices is shown in Figure 4. In agreement with our previous results NECA was found to be more potent than CHA indicating that this response can be characterized as

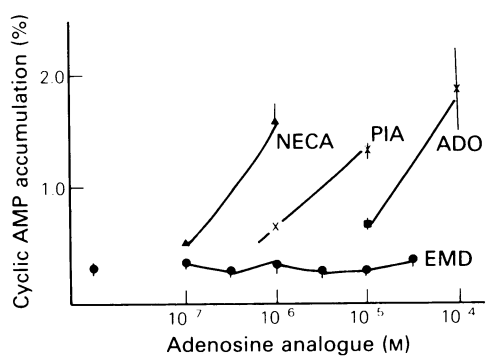

Figure 4 The effect of adenosine (ADO) and the three adenosine analogues adenosine-5'-ethylcarboxamide (NECA), L-phenylisopropyladenosine (PIA) and EMD 28422 on the accumulation of [^{3}H]-cyclic AMP in rat hippocampal slices incubated for 15 min in the presence of 30 μM rolipram. Mean of 3–9 determinations in four separate experiments is shown and vertical lines represent s.e.means.

being mediated by A_2-receptors (Fredholm *et al.*, 1982). It may be seen that EMD 28422 is considerably less potent than either of those two adenosine analogues. In fact, no significant effect was obtained in these experiments even at concentrations of 3×10^{-5} M. Since the electrophysiological experiments indicated that the effect of EMD 28422 may take some time to develop we also carried out experiments where the effect of EMD 28422 on cyclic AMP accumulation was studied for longer periods of time. When the incubation with 30 μM EMD 28422 was prolonged to 45 min there was a statistically significant increase in the accumulation of cyclic AMP from 0.4 ± 0.1 to 0.74 ± 0.13% cyclic AMP. Lower concentrations of EMD 28422 showed no significant effect even after preincubation. Thus, EMD 28422 is at least 300 times less potent than NECA and at least 30 times less potent than L-PIA in this A_2-receptor system. EMD 28422 did not antagonize the actions of NECA.

The accumulation of cyclic AMP in guinea-pig thymocytes is mediated by A_2-receptors. EMD 28422 caused a doubling of the cyclic AMP content in these cells at about 3 μM (Figure 5).

The effect of EMD 28422 on rat rectal temperature was studied in three animals. The control rectal temperature was 38.2 ± 0.1°C. After EMD 28422 3 mg kg^{-1} i.p. a decrease of 1°C was obtained and after 10 mg kg^{-1} EMD 28422 the rectal temperature decreased to 33.5 ± 0.6°C. These effects are quite weak relative to the potency of L-PIA and NECA which are 100–1000 times more potent.

The effect of EMD 28422 in the different *in vitro*

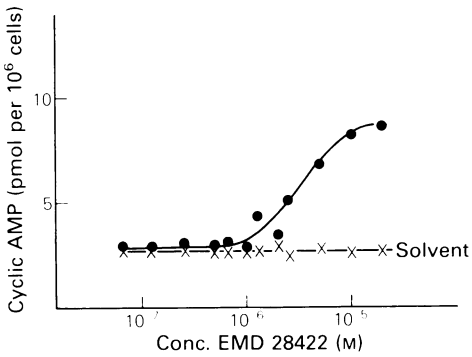

Figure 5 Stimulation of cyclic AMP accumulation in guinea-pig thymocytes by EMD 28422 (●). Mean of duplicate determinations from two separate experiments.

Table 1 Comparison of the potency of EMD 28422 with that of other adenosine analogues in different test systems

	EMD 28422	*NECA*	*2-CA*	*L-PIA*
A_1-receptors	*Absolute potency* (nM)			
Displacement of [^{3}H]-L-PIA[1]	150	40	10	4
Inhibition of lipolysis[2]	120	9	4	0.4
Inhibition of field e.p.s.p.[3]	380	200	400	18
A_2-receptors	*Absolute potency* (μM)			
Cyclic AMP in thymocytes[4]	3.0	0.04	0.11	0.41
Cyclic AMP in hippocampus[5]	>30	0.1	1.7	1.5
A_2/A_1[6]	20–30	1–4	10–25	100–1000

NECA: adenosine-5′-ethylcarboxamide; 2-CA: 2-chloroadenosine; L-PIA: L-phenylisopropyladenosine.

[1] Apparent K_D determined by Eadie-Hofstee plot. Data from Figure 1.

[2] Apparent K_i determined as described in Fredholm & Lindgren (1984). Data from that study and Figure 2.

[3] IC_{50} for inhibition of field e.p.s.p. Data from Dunwiddie & Fredholm (1984) and present study.

[4] Apparent K_D determined as described by Fredholm & Sandberg (1983). Data from that study and from Figure 5.

[5] Ability of adenosine analogue to increase the cyclic AMP accumulation by 100%. Data from Figure 4.

[6] Relative potency for K_D-ratio A_1 and A_2 sites. ([1] and [2] vs, [4] above).

systems studied is shown in Table 1. For comparison the potency of L-PIA, NECA and 2-chloroadenosine from the present or earlier studies is given.

Discussion

The present results show that the N^6-substituted adenosine derivative EMD 28422 is a rather weak agonist at adenosine receptors. It seems no more selective at the A_1-receptor than 2-chloroadenosine, which is generally considered to be a non-selective agonist. In terms of its absolute potency it is comparable to adenosine itself. Thus, in terms of its potency and selectivity it appears quite unremarkable.

However, it is remarkably lipophilic and difficult to maintain in aqueous solution. Furthermore, the compound had an extremely slow onset of action in the electrophysiological experiments and the effect once established was also difficult or impossible to wash out. We have previously found that the time-course of action in this system is related to the time it takes for the compound to equilibrate with the slice and that this appears to be related to its lipophilicity (Dunwiddie & Fredholm, 1984; Dunwiddie *et al.*, 1984). The present results enforce this interpretation since EMD 28422 is the compound with the highest lipophilicity and the slowest time course. It is also interesting to note that the lowest potency of EMD 28422 relative to the other agonists was observed in the fat cells, which contain large amounts of fat capable of accumulating a lipophilic drug in a biologically inactive compartment.

The interest in EMD 28422 derives from the finding that it is able to stimulate binding of benzodiazepines, both when administered *in vivo* (Skolnick *et al.*, 1980a) and *in vitro* (Skolnick *et al.*, 1980b; Fehske *et al.*, 1982). This effect has a dose-response relationship that is different from that found in the present study. Thus a 50% enhancement of benzodiazepine binding was observed at approximately 40 μM (Skolnick *et al.*, 1980b), i.e. considerably higher than the potency we found in the adenosine systems, with the possible exception of A_2-receptor mediated cyclic AMP accumulation in hippocampal slices.

Thus, EMD 28422 appears to be a relatively weak adenosine receptor agonist with limited selectivity twards A_1- and A_2-receptors. A high potency or a high selectivity towards a subtype of adenosine receptor therefore cannot explain why this particular adenosine analogue has the ability to influence the number of benzodiazepine binding sites. Hence, the latter effect may be fortuitous and the significance of the data with EMD 28422 for the concept that there is a close relationship between adenosine and benzodiazepine receptors may be limited. Skolnick & coworkers

(1980b) found that the enhancement of diazepam binding produced by EMD 28422 could be inhibited by bicuculline in a stereospecific manner. This could indicate that it acts via a GABA receptor. However, it was later found that the binding of β-carboline-3-carboxylate, which is much less susceptible to α-aminobutyric acid (GABA)-ergic modulation, was also enhanced by EMD 28422 and that this was not antagonized by bicuculline (Fehske *et al.*, 1982). This indicates that the role of GABA receptors in mediating the effect of EMD 28422 is similarly unclear.

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