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Characterization of adenosine receptors in isolated cerebral arteries of cat

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1 The effect of some adenosine analogues and xanthine derivatives were studied on isolated cerebral arteries from cats.

2 The adenosine analogues caused an almost complete relaxation of cerebral arteries contracted by prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$, 30 μM). The order of potency was: 5-N-ethylcarboxamide adenosine (NECA) > 2-chloroadenosine > adenosine > L-N^6-phenylisopropyl adenosine (L-PIA). The analogue D-PIA was very weak and its maximum effect was small.

3 NECA and L-PIA enhanced [^{3}H]-cyclic AMP accumulation in [^{3}H]-adenine labelled feline pial vessels with similar absolute and relative potency to their relaxant effects.

4 The relaxant effects of adenosine and of NECA were competitively antagonized by 8-phenyl-theophylline ($pA_2 = 6.5$). The effect of theophylline and enprofylline could not be tested in higher concentrations than 30 or 10 μM because they affected the vessels directly. At these concentrations they were essentially inactive as adenosine antagonists. The non-xanthine phosphodiesterase inhibitor rolipram (0.1 and 100 μM) caused a slight but non-significant potentiation of the relaxant effect of adenosine.

5 The results are compatible with the opinion that adenosine relaxes cerebral vessels by an action on adenosine A_2-receptors. The effect may be linked to adenylate cyclase and can be antagonized by 8-phenyl-theophylline.

Introduction

Adenosine has been proposed as a mediator in the dilatation of cardiac, skeletal (Berne, Rubio, Dobson & Curnish, 1971), adipose tissue (Sollevi & Fredholm, 1981) and cerebral blood vessels (Winn, Rubio & Berne 1981). In brain, it has been suggested that adenosine acts as a link between flow and metabolism since levels of brain tissue adenosine were found to be increased in conditions such as ischaemia, hypotension, hypoxia and during bicuculline-induced seizures (Berne, Winn & Rubio, 1981; Winn *et al.*, 1981). Adenosine dilates cerebral arteries both *in vitro* (Hardebo & Edvinsson, 1979; Muramatsu, Fujiwara, Miura & Shibata, 1980) and *in situ* (Berne, Rubio & Curnish, 1974; Wahl & Kuschinsky, 1976; Gregory, Boisvert & Harper, 1980). Furthermore, adenosine and some of its analogues are able to increase cerebral blood flow (Forrester, Harper, MacKenzie & Thompson, 1979; Heistad, Marcus, Gourley & Busija, 1981; Winn, Rubio, Curnish & Berne, 1981). The adenosine uptake inhibitor, dipyridamole, has a similar action (Heistad *et al.*, 1981).

The cerebrovascular effects of adenosine are blocked by methylxanthines such as theophylline (see Winn *et al.*, 1981). Theophylline does not influence cerebral blood flow during normoxia, but causes a marked reduction during hypoxia in the dog (Emerson & Raymond, 1981). In an earlier study Oberdörster and colleagues (Oberdörster, Lang & Zimmer, 1975) found that theophylline actually reduced cerebral vascular resistance at normal perfusion pressure, but increased it at decreased perfusion pressure or during adenosine infusion. This suggests that adenosine is particularly important in cerebral blood flow regulation during hypoxia or in hypotension.

Adenosine is known to act on specific receptors located on cell surfaces (Schwabe, 1981; Fredholm, 1982). These receptors can be subdivided into two types: A_1 and A_2. The former type shows a preference for N^6-substituted adenosine analogues over 5-N-ethylcarboxamide adenosine (NECA) and activation of the receptor is often associated with an inhibition of adenylate cyclase (Londos, Wolff & Cooper, 1981). By contrast, the A_2 receptor shows a

preference for NECA over N^6-substituted adenosine analogues and is frequently associated with an enhanced cyclic AMP accumulation.

In the present study we have attempted to characterize the adenosine receptors responsible for the relaxation of cerebral arteries of cats by the use of adenosine analogues with different selectivities for A_1- and A_2-receptors. The effects of some xanthine derivatives as adenosine antagonists were also examined.

Methods

Specimens were obtained from 17 adult cats of either sex, weighing 2–3.5 kg. The animals were exsanguinated under sodium pentobarbitone anaesthesia (Nembutal, 30 mg kg^{-1} i.p.), the brain removed, the middle cerebral arteries excised and placed in aerated, buffered Krebs solution (for composition, see below). The individual arteries were dissected free under an operation microscope and usually examined immediately or occasionally stored in cold (+4 °C) Krebs solution for up to 24 h.

Recording of vasomotor activity

Small circular segments of the brain arteries (approximately 400–500 μm in diameter and 3 mm long) were suspended between two L-shaped metal prongs in tissue baths containing 5 ml of the buffered Krebs solution. The Krebs solution was continuously gassed with 5% CO_2 in oxygen resulting in a pH of 7.4 in both the stock and the organ bath solutions. The solutions were kept at 37 °C.

Isometric contractions of the circular muscle were recorded through FT 03 C Grass transducers and displayed on a Grass 7 B polygraph. The vessels were given a passive load of 4 mN and allowed to stabilize at this level of tension for 1.5 h. For further methodological details, see Edvinsson, Nielsen & Owman (1974) and Högestätt, Andersson & Edvinsson (1983).

Analysis of data

After two successive stable and reproducible contractions of the vessels which had been evoked by exposure to a potassium rich (124 mM) solution (15.3 ± 3.2 mN), concentration-response curves to drugs were obtained by their cumulative application to the tissue bath. Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) evoked an increase in tension (10.7 ± 3.2 mN) which was stable and during which relaxation responses could be studied (Edvinsson, Hardebo & Owman, 1978). Results are presented as maximum relaxations of $PGF_{2\alpha}$-contraction (E_{max}) and as concentrations of agonists eliciting half maximum relaxation (EC_{50}) or as $-\log EC_{50}$ (pD_2). Antagonists were administered 15–20 min beforehand and were present during the test with the agonists. The results are given as mean values ± s.e.mean and with 95% confidence limits. The agonist results, obtained from 5–14 individual vessel preparations tested with each drug or drug combination, were transformed according to Hill and linear regression analysis, performed as described by Tallarida, Cowan & Adler (1979).

The antagonists were added 20 min before cumulative administration of the agonists adenosine or NECA. Additional vessel segments, in some instances, served as controls to correct for any spontaneous shift of the concentration-response curve.

The dissociation constant (K_B) of 8-phenyltheophylline was calculated from the relationship

$$K_B = \frac{B}{\text{dose-ratio} - 1}$$

where B is the concentration of the antagonist, and the dose-ratio is the ratio of equieffective concentrations of the agonist in the presence and absence of the antagonist (Furchgott, 1972). The dose-ratio used in the calculation of K_B was an average figure derived from the mean curves of all experiments and obtained from Figure 3 a and b. Experiments on antagonists were also analysed using the Schild plot (Arunlakshana & Schild, 1959) and the pA_2 value obtained using conventional regression analysis (as in Figure 4).

[^3H]-cyclic AMP accumulation

Cerebral arteries were placed in Krebs solution gassed with 5% CO_2 in oxygen. After preincubation for 2 × 15 min the vessels were incubated with 50 μCi [^{3}H]-adenine (28 Ci $mmol^{-1}$, Radiochemical Centre, Amersham), which caused a labelling of the adenine nucleotide stores. The cerebral arteries from two cats (approximately 150 mg) accumulated 0.4 μCi during 30 min incubation after which the vessels from each cat were divided into 30 aliquots; each aliquot was incubated in 1 ml Krebs solution, pH 7.4, gassed with 5% CO_2 in oxygen, containing rolipram (Zk 62711, Schering AG) in a concentration of 30 μM to block the cyclic AMP phosphodiesterase. Drugs were added as described under results and the incubation (37 °C) proceeded for 15 min when the medium was discarded and the pial vessels homogenized in 0.4 M perchloric acid, after washing in fresh medium. After neutralization, an aliquot was taken for the determination of total radioactivity. The remainder of the homogenate was passed over alumina and Dowex 50 column to isolate [^{3}H]-cyclic AMP from other [^{3}H]-adenine nucleotides. The percentage of [^{3}H]-cyclic AMP of total [^{3}H]-radioactivity was calculated (for further details see Fredholm, Janzon, Lindgren & Lindström, 1982).

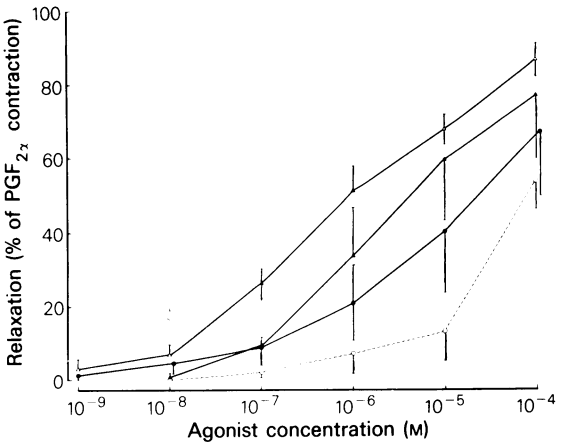

Figure 1 Concentration-dependent relaxation of feline middle cerebral arteries, contracted beforehand by 3×10^{-6} M $PGF_{2\alpha}$. The four adenosine agonists 2-chloroadenosine (△), adenosine (▲), L-N^6-phenylisopropyl adenosine L-PIA (●) and D-PIA (○) relaxed the vessels in the mentioned order of potency. The raw data and statistics are given in Tables 1 and 2.

Solutions and drugs

The standard buffered Krebs solution used was of the following composition (mM: NaCl 119, KCl 4.6, $CaCl_2$ 1.5, $MgCl_2$ 1.2, $NaHCO_3$ 15, NaH_2PO_4 1.2 and glucose 11.

The following agents were used in the experiments: adenosine, 2-chloroadenosine (Sigma, St. Louis, USA); 5-N-ethylcarboxamide adenosine (NECA) (a gift from Dr H.C. Erbler, Byk Gulden, Constanz, FRG); theophylline (ACO, Sweden); prostaglandin $F_{2\alpha}$ (Amoglandin, Astra, Sweden); D- and L-N^6-phenylisopropyl adenosine (PIA) (a gift from Boehringer, Ingelheim); enprofylline (3,7-dihydro-propyl-1H-purin-2,6-dione) (a gift from Dr C.G.A. Persson, Draco, Lund); and rolipram, Zk 62711 (a gift from Dr W. Kehr, Schering AG, Berlin, FRG).

8-Phenyl-theophylline (Calbiochem, Calif., USA) was made up into a 10 mM stock solution in 1 mM tetraphenylboronate in water. All substances were diluted in 0.9% w/v NaCl solution (saline), containing 0.2% asorbic acid to minimize oxidation when studying labile compounds. The drugs were added directly to the tissue baths in volumes of 50 μl and the concentrations given below are the final concentrations in the baths in mol l^{-1} (M).

Table 2 Analysis of the dose-response curves to adenosine and its analogues

	pD_2	*Slope*
NECA	6.03 ± 0.87	0.60 ± 0.07
2-chloroadenosine	5.85 ± 0.18	0.46 ± 0.08
Adenosine	5.32 ± 0.13	0.65 ± 0.07
L-PIA	4.99 ± 0.31	0.43 ± 0.10
D-PIA	3.56 ± 0.38	0.39 ± 0.13

The results given are the pD_2 values and the slopes of Hill plots (mean ± s.e.mean).

Results

Effects of adenosine agonists

Adenosine caused a concentration-dependent relaxation of feline (Figure 1) cerebral arteries that had been contracted by $PGF_{2\alpha}$ (3×10^{-6}–10^{-5} M), but had no effect on arteries without tone. The relaxation in $PGF_{2\alpha}$-contracted feline arteries amounted to 88 ± 10% of the induced tone and the EC_{50} of adenosine was $2.5 \pm 1.6 \times 10^{-6}$ M.

Feline arteries were relaxed in a concentration-dependent manner by NECA, 2-chloroadenosine, adenosine, L-PIA and D-PIA (Figure 1). In order to obtain the relative potency of the agonists, two modes of calculation were used: a direct analysis of individual dose-response curves (Table 1) or analysis of data according to Hill (Table 2). The Hill-coefficients were all lower than 1, probably because some preparations which were essentially unrespon-

Table 1 Dilatory effect of adenosine receptor agonists on the feline middle cerebral artery *in vitro*

	n	E_{max}	pD_2
NECA	13	71.5 ± 10.3 (49.1 – 93.9)	6.5 ± 0.2 (6.2 – 6.8)
Adenosine	12	87.7 ± 9.8 (66.1 – 109.3)	5.6 ± 0.2 (5.2 – 6.0)
2-Chloroadenosine	7	91.6 ± 3.1 (84.0 – 99.2)	6.0 ± 0.2 (5.6 – 6.4)
L-PIA	6	73.6 ± 11.2 (44.8 – 102.4)	5.1 ± 0.3 (4.3 – 5.8)
D-PIA	7	5.5 ± 4.8* (43.7 – 67.3)	

The data are given as mean pD_2; or as percentage relaxation of $PGF_{2\alpha}$ contraction (E_{max}) ± s.e.mean, with the 95% confidence intervals within parentheses.
*Since D-PIA did not reach the maximum dilatation ($P < 0.05$) the values could not be used for an accurate calculation of the pD_2.

sive were included in the calculations. Their inclusion also tended to reduce our estimates of the absolute potency.

Effects of enprofylline and theophylline

These xanthine derivatives affected the vessels directly. Enprofylline (10^{-5} M) relaxed untreated pial arteries by between 0–0.8 mN and $PGF_{2\alpha}$-contracted arteries by between 0.6–1.4 mN. The corresponding values for theophylline (3×10^{-5} M) which acted similarly were 0–0.7 and 0.4–1.1 mN respectively. In some cases the relaxation by theophylline was transient and followed by a small contraction (Figure 2). Higher concentrations of enprofylline (3×10^{-5} M) and theophylline (6×10^{-5} M) elicited complex responses such as are illustrated in Figure 2. Since this might be due to interference with membrane and/or intracellular bound Ca^{2+} stores (Manzini, Maggi & Meli, 1982), we only examined the response of adenosine in the presence of 10^{-5} M enprofylline and of 3×10^{-5} M theophylline neither of which had any significant effect on the adenosine induced relaxations.

Effects of 8-phenyl-theophylline (8-theo)

In non-contracted vessels, a minimum concentration of 10^{-4} M 8-theo was needed to induce significant relaxation whereas concentration-related relaxations occurred with lower concentrations during $PGF_{2\alpha}$-induced contractions (Figure 2). Therefore the antagonistic effect of 8-theo against adenosine and

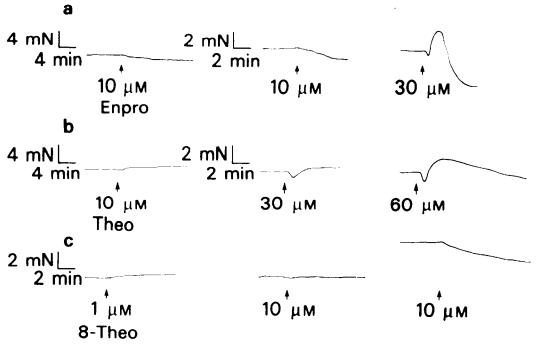

Figure 2 Typical examples of the effect of the adenosine antagonists enprofylline (a), theophylline (b) and 8-phenyl-theophylline (c) on relaxed arteries to the left or arteries contracted by prostaglandin $F_{2\alpha}$. (a) Enprofylline 10 μM caused a somewhat larger effect in contracted than in relaxed arteries; at 30 μM it produced a biphasic response in $PGF_{2\alpha}$-contracted arteries. (b) Theophylline, in increasing concentrations induced biphasic responses in relaxed arteries. (c) 8-theo at 1 and 10 μM had no effect *per se* in relaxed vessels, whereas 10 μM caused a relaxation of $PGF_{2\alpha}$-contracted arteries.

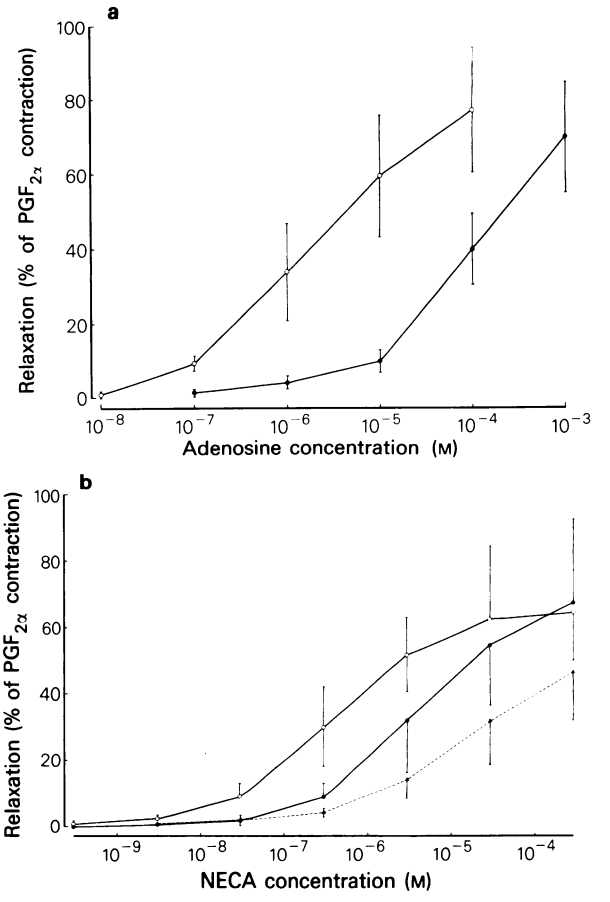

Figure 3 Effect of adenosine (a) and 5′-N-ethylcarboxamide adenosine (NECA) (b) on feline middle cerebral arteries contracted with $PGF_{2\alpha}$. The relaxant effect of the two adenosine agonists were shifted towards higher concentrations in the presence of 8-phenyl-theophylline (8-theo). In (a) adenosine control (○); adenosine plus 10^{-5} M 8-theo (●). In (b) NECA control (○); NECA plus 10^{-6} M 8-theo (●); NECA plus 10^{-5} M 8-theo (▲).

NECA was only tested using 10^{-6} M and 10^{-5} M solutions.

As can be seen in Figure 3a, 8-theo (10^{-5} M) displaced the concentration-response curve of adenosine to the right and similarly shifted the curve of NECA (Figure 3b). The $-\log K_B$ of 8-theo was calculated from these results using the concentrations eliciting 50% relaxation of the $PGF_{2\alpha}$-induced contraction. These values were found to be 6.8 with adenosine as agonist and 6.9 with NECA as agonist. Schild regression analysis of the NECA data (in Figure 3b) revealed a straight line which did not differ from unity (Student's *t* test), suggestive of an

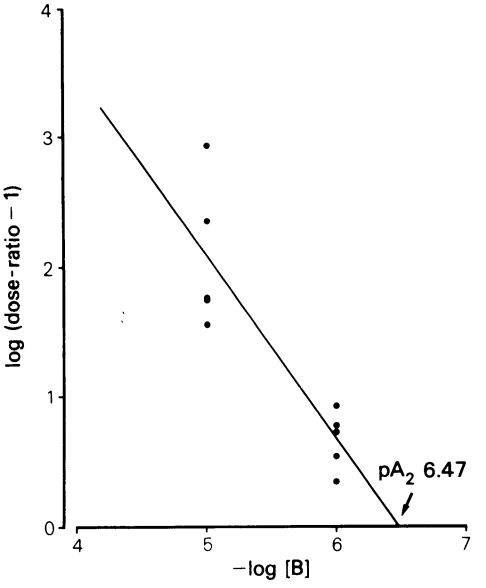

Figure 4 A Schild plot of the antagonistic effect of 8-phenyl-theophylline on 5'-N-ethylcarboxamide adenosine (NECA)-induced relaxation. The mean concentration-response curves are shown in Figure 3b. Regression analysis resulted in a slope that was not different from unity. The pA_2 value was obtained from the intercept with the x-axis of the regression line ($y = 9.12 - 1.41x$). B is the concentration of 8-phenyl-theophylline (M).

action on one receptor site only. The graphically determined pA_2 was found to be 6.5 (Figure 4).

Accumulation of [^{3}H]-cyclic AMP

The effects of NECA and L-PIA on the accumulation of [^{3}H]-cyclic AMP in feline vessel preparations are shown in Figure 5. For comparison the effect of these analogues on vessel relaxation is also given. In this graph only those preparations that showed a good responsiveness to NECA or L-PIA were included, i.e. those that responded to 0.3 μM NECA. The similarity of the two concentration-response curves indicates that cyclic AMP could mediate the relaxant effects of adenosine analogues since the dilatation and the cyclic AMP accumulation occur at the same concentrations. Consequently, we examined the effect of a phosphodiesterase inhibitor, rolipram, on the adenosine-induced relaxation in feline cerebral arteries. As can be seen in Figure 6, rolipram (10^{-7} M or 10^{-4} M) caused a slight but not statistically significant potentiation of the adenosine-induced response. It must be noted that $PGF_{2\alpha}$ induced a contraction that was 59 ± 21% of the maximum potassium contraction both in the absence and in the presence of

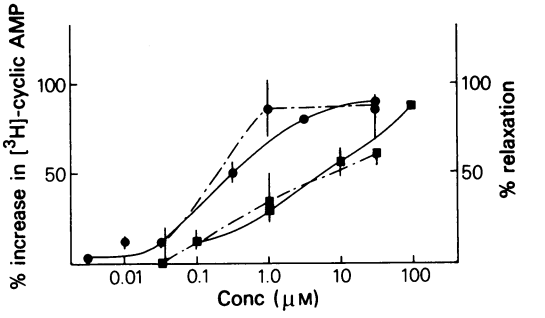

Figure 5 Concentration-dependent increase in [^{3}H]-cyclic AMP accumulation (broken line) and relaxation of pial vessels (unbroken lines) by 5'-N-ethylcarboxamide adenosine (NECA) (●) and L-N^6-phenylisopropyl adenosine (L-PIA) (■). The [^{3}H]-cyclic AMP accumulation was determined in six incubations (three each from two cats). The relaxation was determined in 4 strips from 3 cats (L-PIA) or 8 strips from 5 cats (NECA). Note that in these experiments those vessel preparations showing less than 50% relaxation with 30 μM NECA were excluded. Results are shown as mean ± s.e.mean.

10^{-7} M rolipram. However, the $PGF_{2\alpha}$ contraction was only 10 ± 4% of its maximum (Student's *t* test, $P < 0.001$) in arteries pretreated with 10^{-4} M rolipram.

Discussion

We have in the present study confirmed the relaxant effect of adenosine on isolated cerebral arteries. A 50% relaxation was induced by approximately 1–5 μM adenosine, in agreement with previous studies (Hardebo & Edvinsson, 1979; Muramatsu *et*

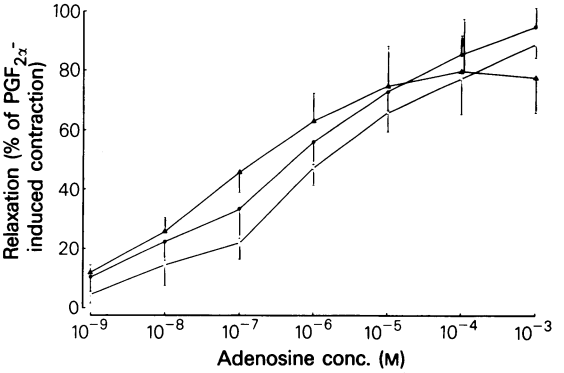

Figure 6 Effect of rolipram 10^{-7} M (●) and 10^{-4} M (▲) on the relaxation induced by adenosine alone (○) in $PGF_{2\alpha}$-contracted arteries. There was no statistically significant difference between arteries pretreated with rolipram or not.

al., 1980; Toda, Okunishi, Taniyama & Miyazak, 1982). NECA and 2-chloroadenosine were more potent as dilators than adenosine, whereas L-PIA was less potent. This order of potency defines the receptor site involved as being of the A_2-subtype. The D-isomer of PIA was approximately 30 times less active than the L-isomer, showing the pronounced stereo-selectivity of these agonists on A_2-receptors (Fredholm *et al.*, 1982).

Adenosine receptors of the A_2-type are commonly found in systems where adenosine may cause an increase in the cyclic AMP production (see Londos *et al.*, 1981; Schwabe, 1981; Fredholm, 1982). Indeed, stimulation, as opposed to inhibition of adenylate cyclase was the first criterion used to subdivide adenosine receptors. We have shown that NECA and L-PIA stimulated cyclic AMP accumulation in cerebral blood vessels with the same relative and, approximately, the same absolute potency as they relax the arteries (Figure 5). Brain microvessels from the rat have recently been found to contain an adenylate cyclase system which is functionally linked with an adenosine receptor, as demonstrated by binding of [^{3}H]-NECA (Palmer & Ghai, 1982; Schütz, Steurer & Tuisl, 1982). This might indicate that stimulation of cyclic AMP accumulation is the mechanism behind the adenosine-induced relaxation in cerebral vessels. There is evidence from coronary vessels that cyclic AMP may be the mediator of adenosine-induced vascular relaxation (Kukovetz, Pöch, Holzmann, Wurm & Riuner, 1978) but there is contradictory evidence regarding relaxation of other vascular smooth muscle preparations (Verhaege, 1977). Since the cerebrovascular preparations used by us contain many cell types, besides smooth muscle cells, it is possible that the cyclic AMP accumulation had occurred in non-muscle cells and was, therefore, unrelated to smooth muscle function. Experiments with the phosphodiesterase inhibitor rolipram demonstrated a slight potentiation (but not statistically significant) of the adenosine-induced relaxation. This may indicate a causal relationship between the dilatation and the cyclic AMP accumulation. However, since results with 10^{-4} M rolipram were unreliable because of the extent to which the drug itself suppressed $PGF_{2\alpha}$ contractions, a firm conclusion will await a more detailed analysis with phosphodiesterase inhibitors.

Whereas the present results have defined the receptors mediating relaxation of feline cerebral vessels as being of the A_2-subtype, the receptors that mediate inhibition of transmitter release and inhibition of evoked potentials in the hippocampus are of the A_1-subtype (Dunwiddie & Fredholm, 1982). Thus, the possibility exists that one may enhance cerebral blood flow without affecting neuronal function and vice versa by using selective adenosine analogues. The natural agonist, adenosine, caused a 50% relaxation of cerebral blood vessels at about 5 μM (present results). Hence, there is no major difference in the potency of adenosine in causing responses at these two types of adenosine receptors. The concentration of free diffusible adenosine in the rat brain was recently found to be 1 μM under resting conditions (Zetterström, Vernet, Ungerstedt, Tosimon, Jonzon & Fredholm, 1982), a concentration sufficient to cause some slight relaxation of pial blood vessels (Figure 1) and to depress neuronal firing (Dunwiddie & Fredholm, 1982). Following mild hypoxia the brain levels of adenosine increased to close to 10 μM (Zetterström *et al.*, 1982), at which concentration there are marked effects on both parameters. Thus, both inhibition of neuronal firing and dilatation of cerebral blood vessels may be physiologically relevant actions of adenosine.

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