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Adenosine receptor-induced second messenger production in adult guinea-pig cerebellum

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1 The effects of adenosine receptor agonists on cyclic nucleotides accumulation were investigated in adult guinea-pig cerebellar slices by use of radioactive precursors.

2 Adenosine elicited a rapid and maintained increase in cyclic AMP, that was fully reversed upon addition of adenosine deaminase. Adenosine analogues stimulated cyclic AMP generation up to 40 fold with the rank order of potency: 5'-N-ethylcarboxamidoadenosine (0.6 μM) > 2-chloroadenosine (6 μM) > adenosine (13 μM). CGS 21680 (10 μM) elicited only a small stimulation (1.2 fold).

3 The cyclic AMP response to NECA was reversed by the 1,3-dipropylxanthine-based adenosine receptor antagonists 8-[4-[[[[(2-aminoethyl)amino]carbonyl]methyl]oxy]-phenyl]-1,3-dipropylxanthine (XAC), 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) and N-[2-(dimethylamino)ethyl]N-methyl-4-(1,3-dipropylxanthine)benzene sulphonamide (PD 115,199) with estimated apparent inhibition constants of 15, 81 and 117 nM, respectively.

4 Pretreatment with adenosine also potentiated the cyclic GMP response to sodium nitroprusside, abolishing the decline in [^{3}H]-cyclic GMP observed with sodium nitroprusside alone, and allowing [^{3}H]-cyclic GMP levels to be maintained for at least an additional 10 min. This potentiation was fully reversed by adenosine deaminase.

5 Adenosine analogues potentiated the sodium nitroprusside-elicited cyclic GMP generation with the rank order of potency: 5'-N-ethylcarboxamidoadenosine (0.7 μM) > 2-chloroadenosine (6 μM) > adenosine (42 μM).

6 NECA potentiation of cyclic GMP formation was reversed by the antagonists XAC, DPCPX and PD 115,199 with apparent inhibition constants of 17, 102 and 242 nM, respectively.

7 The similar potencies of adenosine analogues and xanthine antagonists for stimulation of cyclic AMP and potentiation of cyclic GMP lead to the suggestion that these phenomena are mediated through the same adenosine receptor, the A_{2b} receptor. Furthermore, we suggest that potentiation of the sodium nitroprusside-induced cyclic GMP response may be mediated at the level of phosphodiesterase hydrolysis of the cyclic nucleotides.

Keywords: Cyclic GMP; cyclic AMP; A_{2b} adenosine receptor; guinea-pig cerebellum

Introduction

Adenosine appears to act as a general inhibitory modulator of neurotransmission throughout the CNS (Jacobson *et al.*, 1992; van Galen *et al.*, 1992). Although the physiological roles for adenosine in the cerebellum have yet to be fully clarified, a role for adenosine has been proposed in the regulation of long-term potentiation in hippocampus (de Mendonça & Ribeiro, 1990), a phenomenon similar to long-term depression in cerebellum, a proposed cellular mechanism for cerebellar motor learning. A neuromodulatory role for adenosine in the cerebellum has also been proposed (Ross *et al.*, 1990), whereby liberated adenosine (present at high levels in Purkinje cells, Braas *et al.*, 1986) might influence glutamate release from adjacent parallel fibres.

Adenosine receptors have been most closely studied in tissues of CNS origin, in which at least three classes have been described (Daly *et al.*, 1983). Thus, the A_1 class is linked to inhibition of adenylyl cyclase activity, and also appears coupled to a variety of other signal transduction mechanisms (for reviews see Jacobson *et al.*, 1992; van Galen *et al.*, 1992). In contrast, the A_2 class appears to be coupled exclusively to stimulation of adenylyl cyclase and adenosine 3':5'-cyclic monophosphate (cyclic AMP) generation. Evidence for two subtypes of A_2 receptor was originally derived from the finding that although adenosine and its analogues could stimulate cyclic AMP generation in tissue slices from most brain regions (Daly, 1977) stimulation of adenylyl cyclase in particulate preparations from the CNS was limited to neostriatum, nucleus accumbens and olfactory tubercle (Fredholm, 1977). These subtypes were initially termed low and high affinity A_2 receptors based on the relative potency of adenosine analogues, and were later renamed A_{2b} and A_{2a} receptors, respectively (Bruns *et al.*, 1986). Further evidence for distinguishing these two subtypes has been gained through the A_{2a}-selective nature of the agonist CGS 21680 (Lupica *et al.*, 1990) and antagonist PD 115,199 (Bruns *et al.*, 1987b). Autoradiographic analysis of the binding of these two compounds labelled with ^{3}H underlines the discrete regional distribution of A_{2a} receptors (Bruns *et al.*, 1987b; Jarvis *et al.*, 1989). As yet, no selective agonist or antagonist, or radioligand binding assay is available for the A_{2b} receptor in the CNS.

High densities of A_1 receptors have been identified in the molecular layer of the cerebellum (Lewis *et al.*, 1981), the receptor class associated with inhibition of glutamate release in other brain regions (Fredholm & Dunwiddie, 1988). The use of mice with neurological deficits (Goodman *et al.*, 1983), as well as selective brain lesions (Wojcik & Neff, 1983), have implicated an association of A_1 receptors with cerebellar granule cells. These A_1 receptors inhibit adenylyl cyclase in *in vitro* assays (Wojcik & Neff, 1983). A_1 receptor radioligand

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binding has also been observed in cerebellum from rat, rabbit, guinea-pig and man (Murphy & Snyder, 1982). In the rodent neostriatum, the adenosine receptor agonists N^6-phenylisopropyladenosine exhibits a biphasic action on particulate adenylyl cyclase activity, with an inhibition at low concentrations (mediated through an A_1 receptor) and a stimulation at higher concentrations (via the A_{2a} receptor). In contrast, high concentrations of N^6-phenylisopropyladenosine failed to activate the particulate adenylyl cyclase in membranes from cerebellum. It was proposed, therefore, that A_2 receptors do not exist in mouse cerebellum (Wojcik & Neff, 1983). In cerebellar slices from the guinea-pig, adenosine had been shown to increase the generation of cyclic AMP, and also to increase levels of guanosine 3':5'-cyclic monophosphate (cyclic GMP) in a Ca^{2+}-dependent manner (Ohga & Daly, 1977; Saito, 1977). In mouse cerebellum, there are contradictory reports of both increases and decreases in cyclic GMP formation (Ferrendelli *et al.*, 1973; Saito, 1977).

In the present study we have evaluated the role that different adenosine receptors play in the control of cyclic AMP levels in adult guinea-pig cerebellar slices using a number of adenosine analogues and antagonists. Furthermore, since we have recently observed a potentiation of sodium nitroprusside-stimulated cyclic GMP levels by forskolin (Hernández, Alexander & Kendall, unpublished observations), we also tested whether adenosine regulates cyclic GMP levels in adult guinea-pig cerebellar slices.

Methods

Tissue preparation and second messenger accumulation

Preparation and incubation of slices were essentially the same as described by Donaldson *et al.* (1990). Cross-chopped cerebellar slices (350 × 350 μm) were prepared from guinea-pigs (Dunkin-Hartley, either sex, weighing 200–300 g) with a McIlwain tissue chopper. They were then incubated in a shaking water bath for 60 min at 37°C in several changes of Krebs-bicarbonate buffer which contained (mM): NaCl 118, KCl 4.7, $MgSO_4$ 1.2, KH_2PO_4 1.2, glucose 11.7, $NaHCO_3$ 25, $CaCl_2$ 1.2m equilibrated with 95% O_2:5% CO_2. The slices were next suspended in fresh Krebs buffer and [^{3}H]-adenine or [^{3}H]-guanine was added to a final concentration of 74 kBq ml^{-1}. After an additional 60 min of incubation, the slices were washed and aliquots (25 μl) transferred into flat-bottomed plastic vials containing Krebs buffer (to a final volume of 300 μl). Slices were allowed to equilibrate for 15 min prior to addition of agents. The tubes were resealed under 95% O_2:5%CO_2 after each addition. Incubations were terminated by the addition of 200 μl of HCl (1 M), followed by 750 μl of ice-cold water. [^{3}H]-cyclic GMP and [^{3}H]-cyclic AMP were subsequently resolved by the double-column method of Salomon *et al.* (1974) using [^{14}C]-cyclic AMP and [^{14}C]-GMP as recovery markers. Typical basal levels of [^{3}H]-cyclic AMP and [^{3}H]-cyclic GMP were 2452 ± 108 and 1863 ± 103 d.p.m., respectively.

Slices used for determination of phosphoinositide turnover were pre-equilibrated with Krebs-bicarbonate buffer for 60 min and distributed as 25 μl aliquots into flat-bottomed vials in the presence of [^{3}H]-inositol (c. 40 kBq ml^{-1}) and LiCl (5 mM) to give a final volume of 300 μl as previously described (Alexander *et al.*, 1989). After 40 min, adenosine was added, followed by histamine. Following an incubation period of 45 min, the reaction was halted by the addition of 7.5% perchloric acid. After neutralization, [^{3}H]-inositol phosphates were then resolved by chromatography on Dowex-1 (chloride form) columns.

Binding of cyclopentyl-[^{3}H]-1,3-dipropylxanthine

Binding of the A_1-selective antagonist 8-cyclopentyl-[^{3}H]-1,3-dipropylxanthine ([^{3}H]-DPCPX) was carried out on a 20,000 *g* particulate preparation from guinea-pig cerebellum at 22°C for 90 min. The medium for radioligand binding was composed of 50 mM Tris, pH 7.4 containing 1 mM EDTA, 0.01% Triton X-100 and 1.25 u ml^{-1} adenosine deaminase. Saturation isotherms were constructed with six concentrations of [^{3}H]-DPCPX over the range 0.3–13 nM, defining non-displaceable binding in the presence of 2.5 mM theophylline. Incubation was halted by rapid filtration through Whatman GF/B filters using a cell harvester (Brandel Scientific/SEMAT, Herts, U.K.), washing the filters with 3 × 3 ml ice-cold 50 mM Tris, pH 7.4 containing 1 mM EDTA.

Calculations and statistical analysis

The computer programme GraphPad (GraphPad, California, U.S.A.) was used to generate parameters from radioligand binding data, and agonist and antagonist concentration-response curves. Antagonist dissociation constants (K_I) were estimated by a modification of the null method described by Lazareno & Roberts (1987). Briefly, a concentration-response curve to NECA was generated and a concentration (C, 10 μM) of NECA was chosen which exceeded the EC_{50} value. The concentration of antagonist (IC_{50}) required to reduce the response of this concentration (C) of NECA by 50% was then determined. The NECA concentration-response curve was fitted to a logistic equation as described above and a concentration of NECA (C') identified which yielded a response equivalent to 50% of that produced by concentration C (in the absence of antagonist). The apparent K_I was then determined from the relationship:

$$C/C' = (IC_{50}/K_I) + 1$$

In the text, values represent mean ± s.e.mean (except where indicated) of *n* independent experiments conducted in triplicate. Statistical analysis was performed with Student's unpaired *t* test.

Chemicals

[8-^{3}H]-guanine (324 GBq mmol^{-1}) and [8-^{14}C]-guanosine 3',5'-cyclic monophosphate (1.9 GBq mmol^{-1}) were purchased from Rotem Industries Ltd, Beer-Sheva, Israel and Moravek Biochemicals, California, U.S.A., respectively. [8-^{3}H]-adenine (962 GBq mmol^{-1}) was from Amersham International, Bucks, U.K. [Adenine-U-^{14}C]-adenosine 3',5'-cyclic monophosphate (11.4 GBq mmol^{-1}), [^{3}H]-inositol (455.1 GBq mmol^{-1} and [^{3}H]-DPCPX (4007.1 GBq mmol^{-1}) were purchased from DuPont NEN, U.K., respectively. Adenosine, 2-chloroadenosine, sodium nitroprusside and N^G-nitro-L-arginine were from Sigma Chemicals, Poole, Dorset, U.K. Adenosine deaminase was obtained from Boehringer Mannheim. 5'-N-ethylcarboxamidoadenosine (NECA), N^6-cyclopentyladenosine (CPA), CGS 21680, 8-[4-[[[[(2-aminoethyl)amino]carbonyl]methyl]oxy]-phenyl]-1,3-dipropylxanthine (XAC), 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) were obtained from RBI SEMAT, Herts, U.K. PD 115,199 (N-[2-(dimethylamino) ethyl] N-methyl-4-(1,3-dipropylxanthine) benzene sulphonamide) was a generous gift from Warner Lambert, Ann Arbor, U.S.A. All other compounds were from standard commercial sources.

Results

Effects of adenosine on cyclic AMP accumulation

Incubation of [^{3}H]-adenine-prelabelled adult guinea-pig cerebellar slices in the presence of adenosine (1 mM) led to an increase in [^{3}H]-cyclic AMP levels. The response to adenosine was maximal by about 10 min (15 ± 1.7 fold over basal levels, $n = 6$) and stable for up to 30 min. This activation was fully reversible: addition of adenosine deaminase (1 u ml^{-1})

led to a rapid return of [^{3}H]-cyclic AMP to basal levels within a few minutes (Figure 1a).

The adenosine-induced increase of [^{3}H]-cyclic AMP levels was further characterized with a number of adenosine receptor agonists. Concentration-response curves for 5'-N-ethylcarboxamidoadenosine (NECA, EC_{50} value 0.62 ± 0.15 μM, $n = 3$), 2-chloroadenosine (2CA, 6 ± 1.3 μM, $n = 3$) and adenosine (12.8 ± 0.9 μM, $n = 3$) are shown in Figure 2a. Adenosine exhibited a reduced maximal response compared to NECA and 2CA (1 mM adenosine gave a response 56% of the response to 10 μM NECA, $P < 0.001$, $n = 6$). The class of adenosine receptor involved was further examined by the use of adenosine receptor antagonists. Increasing concentrations of antagonists were pre-incubated with slices for 10 min prior to a 10 min incubation period in the presence of a constant NECA concentration (10 μM). The inhibition curves for XAC (IC_{50} value 0.24 ± 0.1 μM, $n = 4$), DPCPX (1.27 ± 0.05 μM, $n = 3$) and PD 115,199 (1.83 ± 0.21 μM, $n = 3$) are shown in Figure 3a. The apparent inhibition constants were calculated (see Methods) and were 15, 81 and 117 nM for XAC, DPCPX and PD 115,199, respectively.

The potential role of the A_1 receptor subtype was further analysed with forskolin as stimulus of cyclic AMP generation and CPA as a selective agonist of this class of receptor. As can be seen in Figure 4a, forskolin (1 μM) increased [^{3}H]-cyclic AMP in [^{3}H]-adenine-prelabelled adult guinea-pig cerebellar slices. However, a 10 min preincubation with CPA failed to inhibit the forskolin response in the range of concentrations effective in cerebral cortex (10 nM–1 μM, Alexander *et al.*, 1992). A similar pattern was observed with 10 μM forskolin (data not shown). CPA increased [^{3}H]-cyclic AMP at concentrations of 300 nM or greater, suggesting a

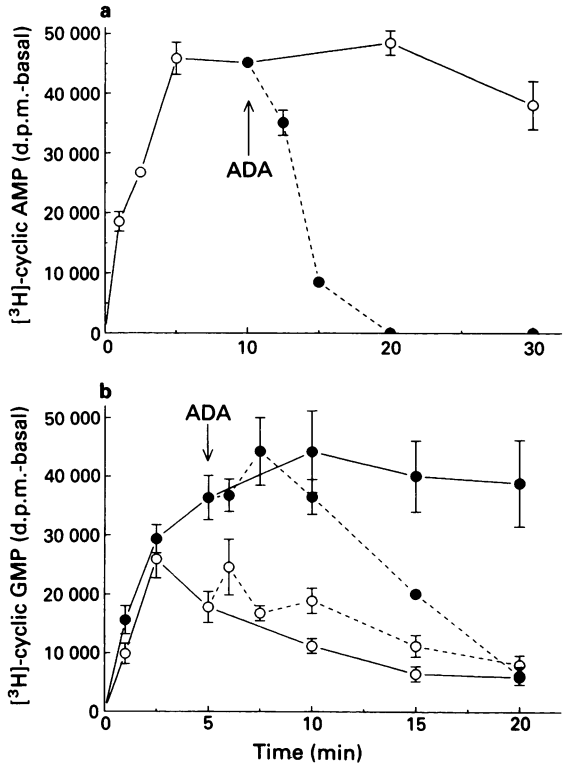

Figure 1 Time course profiles for adenosine-stimulated (a) [^{3}H]-cyclic AMP or (b) [^{3}H]-cyclic GMP accumulations. In (b), slices were incubated in the absence (○) or presence (●) of 1 mM adenosine for 10 min prior to addition of sodium nitroprusside. At the timepoint indicated by the arrow, 1 u ml^{-1} adenosine deaminase was added to some slices (dashed lines), which were then incubated further. Data are from a single experiment repeated on two additional occasions with essentially similar results. Bars show s.e.mean of triplicate determinations.

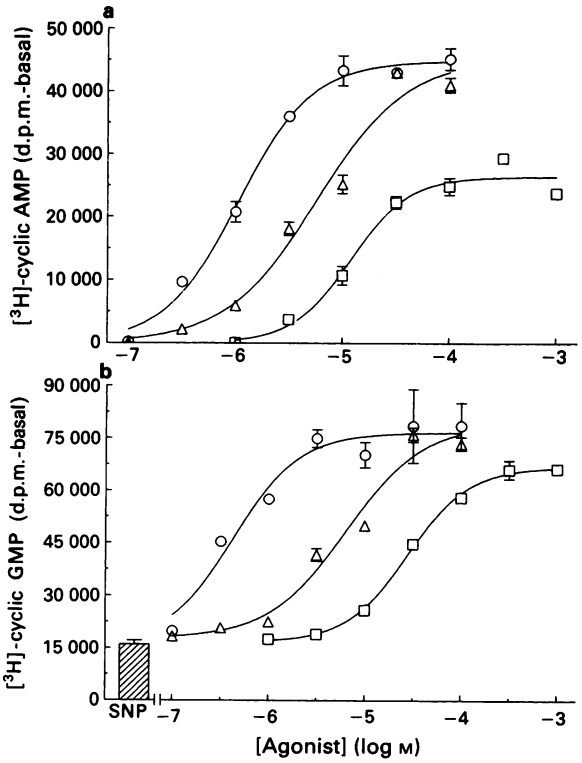

Figure 2 Concentration-response curves for the stimulation of (a) [^{3}H]-cyclic AMP or (b) sodium nitroprusside (SNP)-induced [^{3}H]-cyclic GMP accumulation in the presence of NECA (○), 2CA (△) or adenosine (□). The responses to NECA and 2CA were carried out, in both (a) and (b), after 10 min preincubation with 1 u ml^{-1} adenosine deaminase. Data are from single experiments representative of 3/4 separate experiments. Bars show s.e.mean of triplicate determinations. For abbreviations, see text.

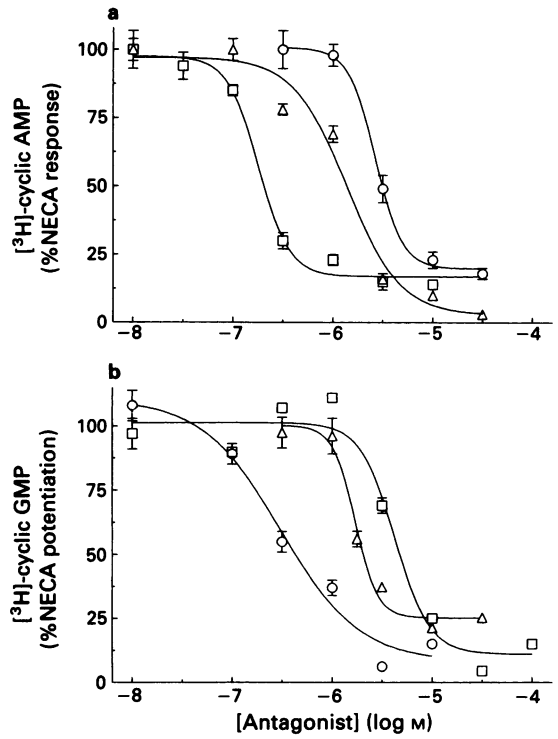

Figure 3 Inhibition of (a) NECA-stimulated [^{3}H]-cyclic AMP or (b) NECA-augmented [^{3}H]-cyclic GMP. Antagonists (a) XAC (□), DPCPX (△) or PD 115,199 (○) and (b) XAC (○), DPCPX (△) or PD 115,199 (□) were added 10 min prior to addition of 10 μM NECA. Data are from single experiments representative of 3/5 separate experiments. Bars show s.e.mean of triplicate determinations. For abbreviations, see text.

stimulation of A_{2b} receptors at such concentrations. Interestingly, at such concentrations CPA also enhanced the response to forskolin, suggesting that forskolin may potentiate receptor-mediated increases in cerebellar cyclic AMP. This hypothesis was further analysed by studying the response to forskolin in the presence and absence of adenosine deaminase (1 u ml^{-1}). As can be seen in Figure 5, adenosine deaminase shifted the concentration-response curve to forskolin to the right, suggesting a role for endogenous adenosine in the stimulation of cyclic AMP generation by low concentrations of forskolin in cerebellum.

Effects of adenosine on cyclic GMP accumulation

Pretreatment of cerebellar slices with adenosine (1 mM, 10 min) substantially changed the response to 1 mM sodium nitroprusside (SNP, Figure 1b). Adenosine pretreatment abolished the decline in [^{3}H]-cyclic GMP observed with SNP alone (10 min, 2.7 ± 0.2 fold over SNP alone, $n = 5$). The same pattern was obtained with 10 μM NECA (10 min, 3.36 ± 0.8 fold over SNP alone, $n = 10$). This effect of adenosine was fully reversible: addition of adenosine deaminase (1 u ml^{-1}) returned [^{3}H]-cyclic GMP content to basal levels within a few minutes (Figure 1b); 10 min treatment with 1 mM adenosine alone increased the basal levels of [^{3}H]-cyclic GMP (3.2 ± 0.3 fold, mean ± range, $P < 0.05$, $n = 2$). The same effect was observed with 10 μM NECA (10 min, 2.07 ± 0.25 fold over basal levels, $P < 0.001$, $n = 6$). In the presence of N^G-nitro-L-arginine (100 μM, 10 min preincubation), the response to 10 μM NECA alone was 63 ± 11% ($P < 0.01$, $n = 3$) of the response obtained in the absence of inhibitor. The potentiation of SNP-induced [^{3}H]-cyclic GMP levels produced by adenosine was analysed further with a number of adenosine receptor agonists. Concentration-response curves for NECA (EC_{50} value 0.67 ± 0.21 μM, $n = 3$), 2CA (5.85 ± 1.1 μM, $n = 4$) and adenosine (42 ± 16 μM, $n = 3$) are shown in Figure 2b. In this case, adenosine showed a similar maximal response compared to NECA (1 mM adenosine gave 81% of the response observed with 10 μM NECA, $P < 0.2$). The class of adenosine receptor involved was further examined by the use of adenosine receptor antagonists. Increasing concentrations of antagonists were pre-incubated with slices for 10 min prior to a 10 min incubation period in the presence of a constant NECA concentration (10 μM). The inhibition curves for XAC (IC_{50} value 0.25 ± 0.02 μM, $n = 4$), DPCPX (1.44 ± 0.1 μM, $n = 5$) and PD 115,199 (3.42 ± 0.36 μM, $n = 3$) are shown in Figure 3b, permitting calculation of the apparent inhibition constants as before (17, 102 and 242 nM for XAC, DPCPX and PD 115,199 respectively).

The role of A_{2a} and A_1 receptors in enhancing cyclic GMP levels was analysed with the specific agonists CGS 21680 and CPA, 10 min preincubation with 10 μM CGS 21680 potentiated only slightly the levels of [^{3}H]-cyclic GMP induced by 1 mM SNP (10 min, 1.2 ± 0.06 fold, $P < 0.1$, $n = 3$). Using concentrations in the range of A_1 receptor selectivity (up to 1 μM), CPA did not change either the response to SNP or basal levels of [^{3}H]-cyclic GMP (Figure 4b). However, in concentrations greater than 1 μM, the response to 1 mM SNP was potentiated, presumably through activation of A_{2b} receptors at these concentrations.

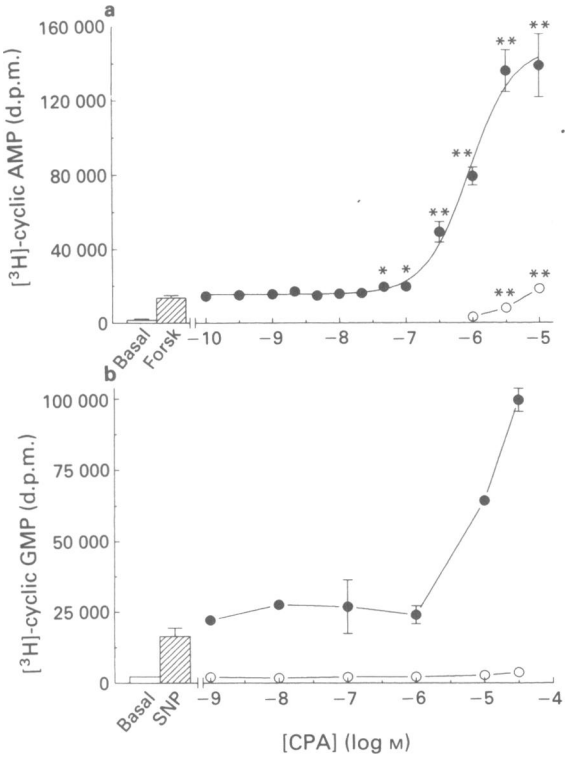

Figure 4 Concentration-response curves for CPA effects on (a) [^{3}H]-cyclic AMP or (b) [^{3}H]-cyclic GMP in the absence (○) or presence (●) of (a) forskolin (Forsk) or (b) sodium nitroprusside (SNP). Data are from single experiments representative of 3/4 separate experiments. Bars show s.e.mean of triplicate determinations. $*P < 0.05$, $**P < 0.01$ absence vs. presence of CPA. For abbreviations, see text.

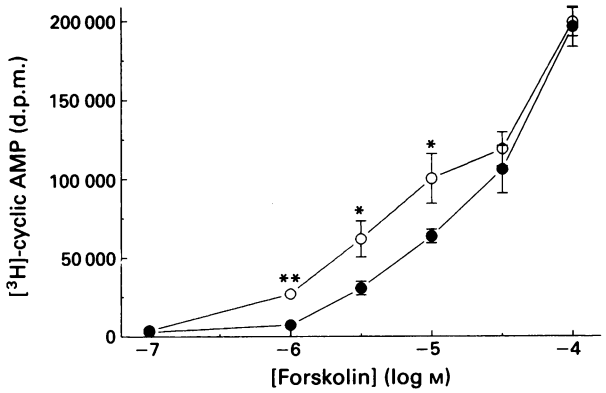

Figure 5 Concentration-response curves for forskolin-stimulated [^{3}H]-cyclic AMP accumulation in the absence (○) or presence (●) of of 1 u ml^{-1} adenosine deaminase. Data are from a single experiment repeated on a further occasion with essentially identical results. Bars show s.e.mean of triplicate determinations. $*P < 0.05$, $**P < 0.01$ absence vs. presence of adenosine deaminase.

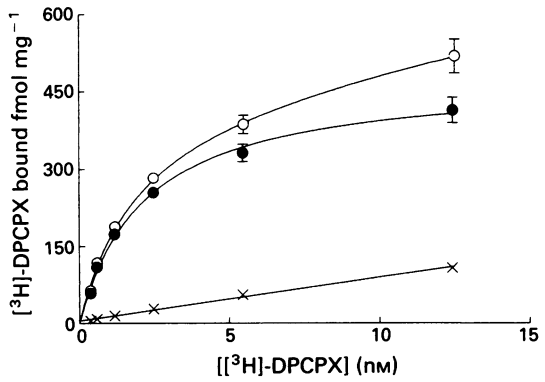

Figure 6 [^{3}H]-DPCPX binding to guinea-pig cerebellar membranes at the indicated concentrations in the absence (○) or presence of 5 mM theophylline (×). Displaceable [^{3}H]-DPCPX binding is indicated by (●). Data are means ± s.e.mean of triplicate determinations from three separate experiments.

Adenosine effects on phosphoinositide turnover in the guinea-pig cerebellum

Since adenosine has been observed to potentiate specifically H_1 histamine receptor-stimulated phosphoinositide turnover in guinea-pig cerebral cortical slices via an A_1-type adenosine receptor (Hill & Kendall, 1987), we investigated whether this phenomenon was apparent in the cerebellum. Basal accumulations of [^{3}H]-inositol phosphates (3004 ± 494 d.p.m., $n = 3$) were elevated in the presence of 100 μM histamine ($470 \pm 85\%$ basal, $n = 3$). However, the presence of 100 μM adenosine failed to alter significantly the response to histamine ($91 \pm 15\%$ histamine response, $n = 3$).

Binding of [^{3}H]-DPCPX to guinea-pig cerebellar membranes

Since we were unable to provide evidence from studies of second messenger responses for the presence of A_1 adenosine receptors in guinea-pig cerebellum, we investigated whether radioligand binding could demonstrate A_1 receptors in this tissue. Using a total particulate preparation from guinea-pig cerebellum, we observed saturable binding of the A_1-selective antagonist radioligand [^{3}H]-DPCPX, with a calculated K_D value of 2.1 ± 0.1 nM and a maximal binding capacity of 474 ± 32 fmol mg^{-1} protein ($n = 3$) (Figure 6).

Discussion

Adenosine receptor stimulation of cyclic AMP levels in guinea-pig cerebellum

Stimulation of cyclic AMP generation in guinea-pig cerebellum was enhanced by adenosine and its analogues with the rank order of potency; NECA > 2CA > adenosine. This rank order of potency is identical to that at the A_{2b} adenosine receptors of guinea-pig (Losinski *et al.*, 1993) and rat cerebral cortex (Bazil & Minneman, 1986), although the absolute potencies of the adenosine analogues appeared highest in guinea-pig cerebellum. The antagonists investigated showed an identical rank order of potency (XAC > DPCPX > PD 115,199) when compared with guinea-pig cerebral cortex (Losinski *et al.*, 1993), with, again, a slightly-enhanced affinity. DPCPX has been characterized as an A_1-selective antagonist (Lee & Reddington, 1986; Bruns *et al.*, 1987a; Lohse *et al.*, 1987), with low affinity at A_{2a} receptors. In contrast, PD 115,199 exhibits similar affinity at A_1 and A_{2a} receptors with low affinity at A_{2b} receptors (Bruns *et al.*, 1987b). XAC has been defined as an antagonist which exhibits high affinity at A_1 and A_2 receptors (Jacobson *et al.*, 1985; 1987). The rank order of potencies of both agonists and antagonists, therefore, identifies the adenosine receptor as an A_{2b} adenosine receptor.

Adenosine receptor stimulation of cyclic GMP levels in guinea-pig cerebellum

In the presence of 1 mM SNP, a transient elevation of [^{3}H]-cyclic GMP levels was observed which was enhanced and prolonged in the presence of adenosine or its analogues (Figure 1b). The rank order of potency of adenosine analogues for eliciting a potentiation of the SNP-induced [^{3}H]-cyclic GMP response was identical when compared to that observed for stimulation of cyclic AMP generation. Similarly, the antagonists elicited inhibition of NECA-enhanced [^{3}H]-cyclic GMP accumulation with the same rank order of potency as observed for NECA-stimulated [^{3}H]-cyclic AMP accumulation. These data strongly implicate A_{2b} adenosine receptors in both phenomena. This raises the possibility of a causative linkage between the two events, a suggestion which is further strengthened by our recent findings that forskolin and the phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine, also exhibit a similar profile of enhancement of SNP-induced [^{3}H]-cyclic GMP accumulation in this tissue (Hernández, Alexander & Kendall, unpublished observations). Our present hypothesis to explain the enhancement of [^{3}H]-cyclic GMP levels is that competition at the phosphodiesterase level by either cyclic AMP (generated by forskolin or A_{2b} receptor stimulation) or 3-isobutyl-1-methylxanthine leads to an enhanced accumulation of [^{3}H]-cyclic GMP. We can provide a potential explanation for the increased maximal response to adenosine relative to NECA for augmenting cyclic GMP accumulation compared to the generation of cyclic AMP by suggesting that the phosphodiesterase responsible for degradation of [^{3}H]-cyclic GMP may become saturated by the cyclic AMP generated at ca. 70% of the maximal NECA response. Thus, since adenosine generates 56% of the cyclic AMP response to a maximally-active concentration of NECA, this will approach 80% of the cyclic GMP response.

In the absence of SNP, a small enhancement of [^{3}H]-cyclic GMP accumulation could be observed in the presence of adenosine. The reduction in basal [^{3}H]-cyclic GMP levels in the presence of oxyhaemoglobin and N^G-nitro-L-arginine suggests some endogenous production of nitric oxide in these slices (Hérnandez, Alexander & Kendall, unpublished observations), indicating the potential for adenosine receptor enhancement of endogenous nitric oxide-evoked cyclic GMP accumulation.

Other adenosine receptors in guinea-pig cerebellum

In guinea-pig cerebral cortical slices, it is possible to observe A_1 adenosine receptor-mediated inhibitions of cyclic AMP formation when forskolin is used as a stimulus (Alexander *et al.*, 1992). However, in the cerebellum, we were unable to provide evidence for inhibition of forskolin-stimulated cyclic AMP generation by concentrations of CPA active in the cortex. At relatively high concentrations of CPA, a direct stimulation of [^{3}H]-cyclic AMP generation was observed, together with an enhancement of the forskolin response, presumably through activation of A_{2b} receptors (DeLapp & Eckols, 1992). We were also unable to observe adenosine receptor potentiation of histamine-stimulated phosphoinositide turnover in the guinea-pig cerebellum, although this phenomenon is present in cerebral cortex (Hill & Kendall, 1987), hippocampus and neostriatum (R.M. Straw & D.A. Kendall, unpublished observation). Our investigations of [^{3}H]-DPCPX radioligand binding in particulate preparations from the guinea-pig cerebellum indicates relatively dense binding. This compares with our previously-reported investigation of [^{3}H]-DPCPX binding to guinea-pig cerebral cortical A_1 adenosine receptors (Alexander *et al.*, 1992) in which we observed a K_D value of 4.2 ± 0.4 nM with a maximal binding capacity of 1560 ± 278 fmol mg^{-1} protein. The signal transduction mechanism for the relatively abundant A_1 receptors in guinea-pig cerebellum therefore remains to be elucidated. The minor effect of CGS 21680 on [^{3}H]-cyclic GMP accumulation suggests an absence of A_{2a} adenosine receptors from guinea-pig cerebellum, a finding in agreement with radioligand binding studies in the rat (Bruns *et al.*, 1987b; Jarvis *et al.*, 1989) and mouse (Wojcik & Neff, 1983).

Conclusion

We are able to furnish evidence for signal transduction pathways for the A_{2b} adenosine receptor, but not A_1 or A_{2a} receptor classes, in adult guinea-pig cerebellum. The enhancement of cyclic GMP accumulation by A_{2b} adenosine receptor activation in guinea-pig cerebellum appears to be mediated through the stimulation of cyclic AMP generation. This phenomenon of cross-talk between second messengers could

well have important connotations for other systems where a stimulation of cyclic AMP is observed, the associated phenomena may be better explained as mediated through cyclic GMP.

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References

ALEXANDER, S.P.H., KENDALL, D.A. & HILL, S.J. (1989). Differences in the adenosine receptor modulating inositol phosphates and cyclic AMP accumulation in mammalian cerebral cortex. *Br. J. Pharmacol.*, **98**, 1241–1248.

ALEXANDER, S.P.H., CURTIS, A.R., KENDALL, D.A. & HILL, S.J. (1992). The affinity of 8-cyclopentyl-1,3-dipropylxanthine at A1 adenosine receptors of guinea-pig cerebral cortex. *Br. J. Pharmacol.*, **107**, 75P.

BAZIL, C.W. & MINNEMAN, K.P. (1986). An investiation of the low intrinsic activity of adenosine and its analogs at low affinity (A2) adenosine receptors in rat cerebral cortex. *J. Neurochem.*, **47**, 547–553.

BRAAS, K.M., NEWBY, A.C., WILSON, V.S. & SNYDER, S.H. (1986). Adenosine-containing neurons in the brain localized by immunocytochemistry. *J. Neurosci.*, **6**, 1952–1961.

BRUNS, R.F., FERGUS, J.H., BADGER, E.W., BRISTOL, J.A., SANTAY, L.A., HARTMAN, J.D., HAYS, S.J. & HUANG, C.C. (1987a). Binding of the A1-selective adenosine antagonist 8-cyclopentyl-1, 3-dipropylxanthine to rat membranes. *Naunyn-Schmied. Arch. Pharmacol.*, **335**, 59–63.

BRUNS, R.F., FERGUS, J.H., BADGER, E.W., BRISTOL, J.A., SANTAY, L.A. & HAYS, S.J. (1987b). PD 115,199: an antagonist ligand for adenosine A2 receptors. *Naunyn-Schmied. Arch. Pharmacol.*, **335**, 64–69.

BRUNS, R.F., LU, G.H. & PUGSLEY, T.A. (1986). Characterization of the A_2 adenosine receptor labeled by [^{3}H]NECA in rat striatal membranes. *Mol. Pharmacol.*, **29**, 331–346.

DALY, J.W. (1977). *Cyclic Nucleotides in the Nervous System.* New York: Plenum Press.

DALY, J.W., BUTTS-LAMB, P. & PADGETT, W. (1983). Subclasses of adenosine receptors in the central nervous system: interaction with caffeine and related methylxanthines. *Cell. Mol. Neurobiol.*, **3**, 69–80.

DELAPP, N.W. & ECKOLS, K. (1992). Forskolin stimulation of cyclic AMP accumulation in rat brain slices is markedly enhanced by endogenous adenosine. *J. Neurochem.*, **58**, 237–242.

DE MENDONÇA, A. & RIBEIRO, J.A. (1990). 2-Chloroadenosine decreases long-term potentiation in the hippocampal CA1 area of the rat. *Neurosci. Lett.*, **118**, 107–111.

DONALDSON, J., KENDALL, D.A. & HILL, S.J. (1990). Discriminatory effects of forskolin and EGTA on the indirect cyclic AMP responses to histamine, 5-hydroxytryptamine, and glutamate in guinea pig cerebral cortical slices. *J. Neurochem.*, **54**, 1484–1494.

FERRENDELLI, J.A., KINSCHERF, D.A. & CHANG, M.M. (1973). Regulation of levels of guanosine cyclic 3′,5′-monophosphate in the central nervous system: effects of depolarizing agents. *Mol. Pharmacol.*, **9**, 445–454.

FREDHOLM, B.B. (1977). Activation of adenylate cyclase in rat striatum and tuberculum olfactorium by adenosine. *Med. Biol.*, **55**, 262–267.

FREDHOLM, B.B. & DUNWIDDIE, T.V. (1988). How does adenosine inhibit transmitter release? *Trends Pharmacol. Sci.*, **9**, 130–134.

GOODMAN, R.R., KUHAR, M.J., HESTER, L. & SNYDER, S.H. (1983). Adenosine receptors: autoradiographic evidence for their location on axon terminals of excitatory neurons. *Science*, **220**, 967–969.

HILL, S.J. & KENDALL, D.A. (1987). Studies on the adenosine-receptor mediating the augmentation of histamine-induced inositol phospholipid hydrolysis in guinea-pig cerebral cortex. *Br. J. Pharmacol.*, **91**, 661–669.

JACOBSON, K.A., KIRK, K.L., PADGETT, W.L. & DALY, J.W. (1985). Functionalized congeners of 1,3-dialkylxanthines: preparation of analogues with high affinity for adenosine receptors. *J. Med. Chem.*, **28**, 1334–1340.

JACOBSON, K.A., UKENA, D., PADGETT, W., DALY, J.W. & KIRK, K.L. (1987). Xanthine functionalized congeners as potent ligands at A2-adenosine receptors. *J. Med. Chem.*, **30**, 211–214.

JACOBSON, K.A., VAN GALEN, P.J.M. & WILLIAMS, M. (1992). Adenosine receptors: pharmacology, structure-activity relationships, and therapeutic potential. *J. Med. Chem.*, **35**, 407–422.

JARVIS, M.F., SCHULZ, R., HUTCHISON, A.J., DO, U.H., SILLS, M.A. & WILLIAMS, M. (1989). [3H]CGS 21680, a selective A2 adenosine receptor agonist directly labels A2 receptors in rat brain. *J. Pharmacol. Exp. Ther.*, **251**, 888–893.

LAZARENO, S. & ROBERTS, F.F. (1987). Measuring muscarinic antagonist potency using stimulated phosphoinositide breakdown in rat cortical slices. *Br. J. Pharmacol.*, **92**, 677P.

LEE, K.S. & REDDINGTON, M. (1986). 1,3-Dipropyl-8-cyclopentylxanthine (DPCPX) inhibition of [3H]N-ethylcarboxamidoadenosine (NECA) binding allows the visualization of putative non-A1 adenosine receptors. *Brain Res.*, **368**, 394–398.

LEWIS, M.E., PATEL, J., EDLEY, S.M. & MARANGOS, P.J. (1981). Autoradiographic visualization of rat brain adenosine receptors using N6-cyclohexyl[3H]adenosine. *Eur. J. Pharmacol.*, **73**, 109–110.

LOHSE, M.J., KLOTZ, K.-N., LINDEBORN-FOTINOS, J., REDDINGTON, M., SCHWABE, U. & OLSSON, R.A. (1987). 8-Cyclopentyl-1,3-dipropylxanthine (DPCPX) – a selective high affinity antagonist radioligand for A1 adenosine receptors. *Naunyn-Schmied. Arch. Pharmacol.*, **336**, 204–210.

LOSINSKI, A., HILL, S.J. & ALEXANDER, S.P.H. (1993). A_2 adenosine receptor relaxation of guinea-pig aorta. *Br. J. Pharmacol.*, **108**, 147P.

LUPICA, C.R., CASS, W.A., ZAHNISER, N.R. & DUNWIDDIE, T.V. (1990). Effects of the selective adenosine A2 receptor agonist CGS 21680 on in vitro electrophysiology, cAMP formation and dopamine release in rat hippocampus and striatum. *J. Pharmacol. Exp. Ther.*, **252**, 1134–1141.

MURPHY, K.M.M. & SNYDER, S.H. (1982). Heterogeneity of adenosine A1 receptor binding in brain tissue. *Mol. Pharmacol.*, **22**, 250–257.

OHGA, Y. & DALY, J.W. (1977). The accumulation of cyclic AMP and cyclic GMP in guinea pig brain slices. Effect of calcium ions, norepinephrine and adenosine. *Biochim. Biophys. Acta*, **498**, 46–60.

ROSS, C.A., BREDT, D. & SNYDER, S.H. (1990). Messenger molecules in the cerebellum. *Trends Neurosci.*, **13**, 216–222.

SAITO, M. (1977). Elevation of guanosine 3′:5′-monophosphate level by adenosine in cerebellar slices of guinea pig. *Biochim. Biophys. Acta*, **498**, 316–324.

SALOMON, Y., LONDOS, C. & RODBELL, M. (1974). A highly sensitive adenylate cyclase assay. *Anal. Biochem.*, **58**, 541–548.

VAN GALEN, P.J.M., STILES, G.L., MICHAELS, G. & JACOBSON, K.A. (1992). Adenosine A_1 and A_2 receptors: structure-function relationships. *Med. Res. Rev.*, **12**, 423–471.

WOJCIK, W.J. & NEFF, N.H. (1983) Adenosine A1 receptors are associated with cerebellar granule cells. *J. Neurochem.*, **41**, 759–763.

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